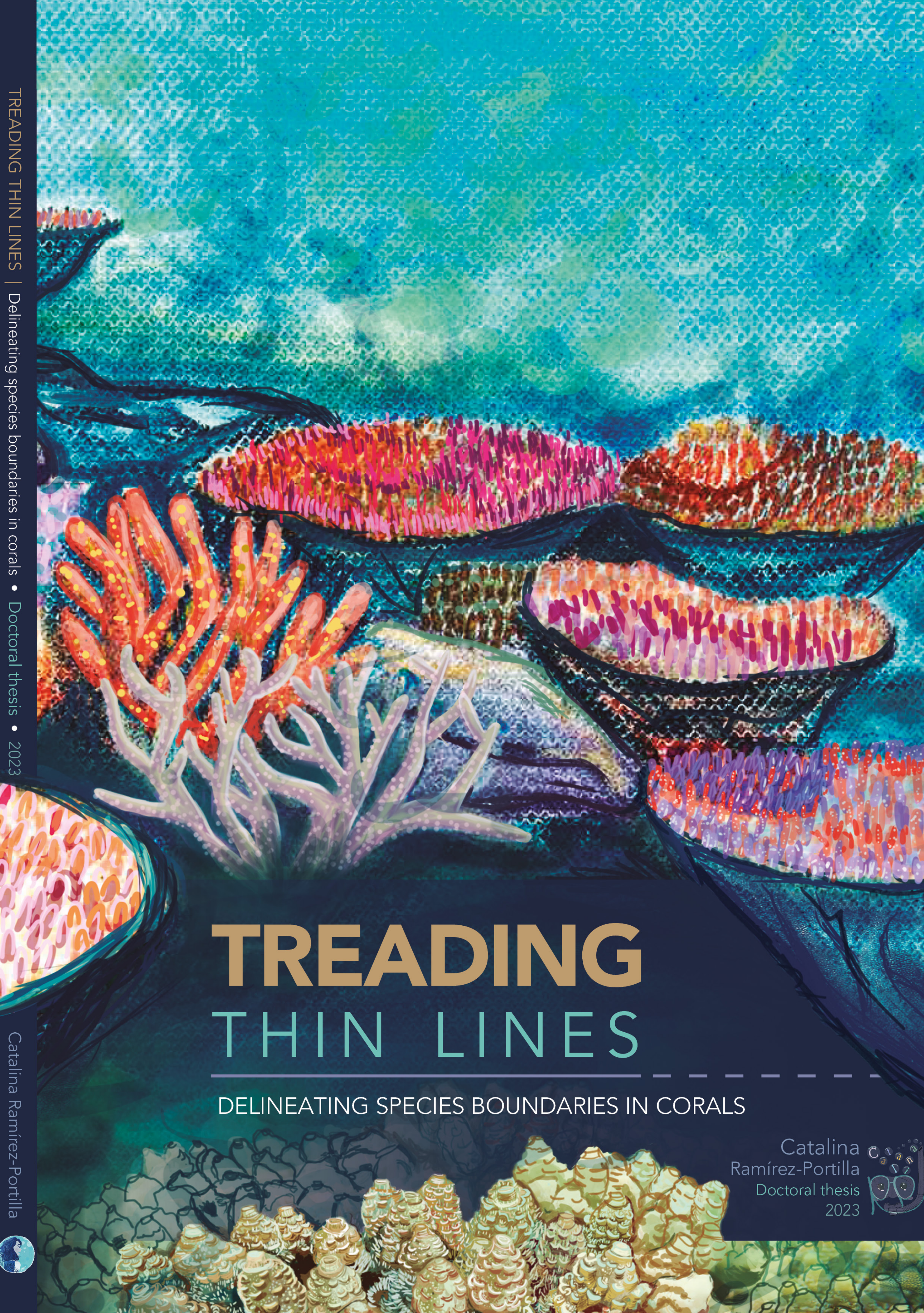
TREADING THIN LINES | Delineating species boundaries in corals • Doctoral thesis • 2023
Catalina Ramírez-Portilla
TREADING
THIN LINES
DELINEATING SPECIES BOUNDARIES IN CORALS
Catalina
Ramírez-Portilla
Doctoral thesis
2023

Cover illustration: *"Espuma y arrecife"*
Dry pastel and digital art by I. Portilla Portilla
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To my inner child who
trusted I would get past my
fears and get to explore the
"*island beneath the sea*"

"La isla bajo el mar"
Watercolor by C. Ramírez-Portilla

Thesis submitted by Catalina RAMÍREZ-PORTILLA

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DELINEATING SPECIES BOUNDARIES IN CORALS

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Sunset from the jetty at Sesoko Station
Motobu, Okinawa, Japan
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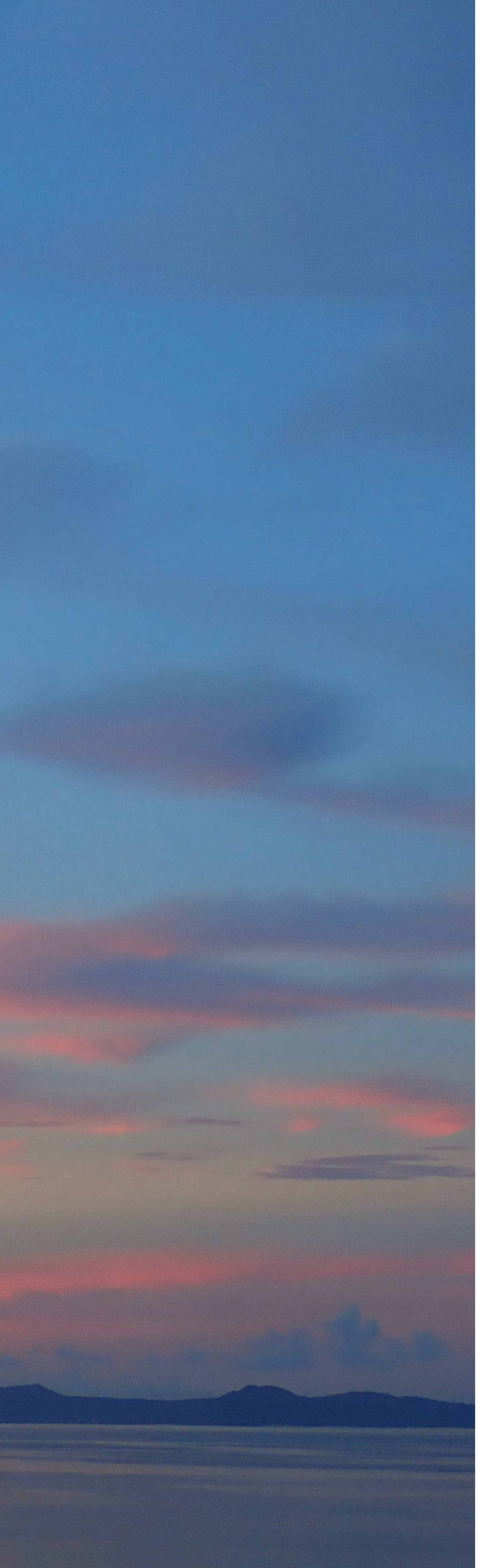

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Outer reef south of Sesoko Island
Motobu, Okinawa, Japan
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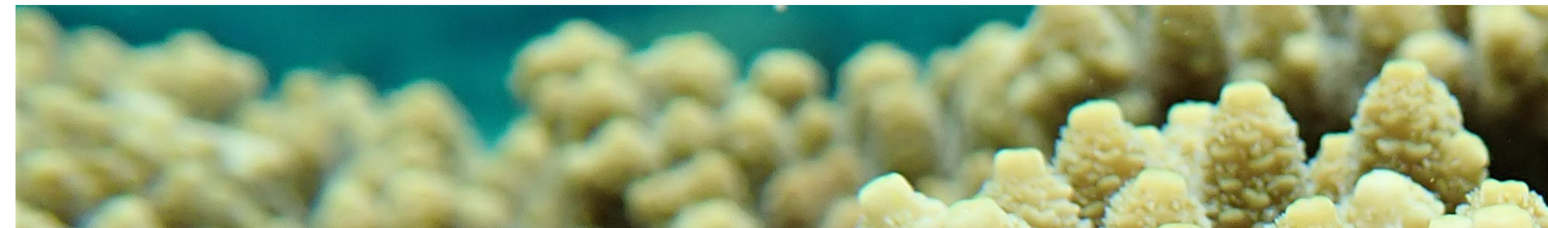
Oku beach point
Kunigami, Okinawa, Japan
Photo by C. Ramírez-Portilla

Abstract



Abstract (ENG)

KEYWORDS | coral taxonomy · species delimitation · integrative approaches · *Acropora*

Delimiting species is not only a central issue in evolutionary and systematic biology but also a prerequisite to physiological, ecological, and population genetic studies. However, species boundaries' delineation in highly diverse environments, such as shallow tropical coral reefs, remains challenging. Traditional hypotheses that separate lineages in corals have long been based on morphological traits that did not yield resolution at the species level. The plastic response of corals to environmental variations and reports of intermediate morphotypes in the field have also hindered this task, casting doubt on current species delimitations. Therefore, while anthropogenic disturbances threaten coral reef ecosystems, the taxonomy of vulnerable taxa that inhabits them remains obscure.

The difficulty to navigate this intricate taxonomic landscape is epitomized by the coral genus *Acropora*, which exhibits more than a hundred morphospecies and provides an excellent training ground to test and validate new practices. Species delimitation attempts in *Acropora* corals have so far been unsuccessful due to widespread genealogical incongruence among genetic markers and between morphological groupings, mating trials, and molecular clades obtained in phylogenetic analyses. Consequently, this thesis aimed to test the current species delimitation in scleractinian corals, outlining an integrative approach and establishing a methodology that paves the way for a taxonomic revision using the *Acropora* genus as a case study.

For this purpose, a historical overview highlighting the emergence of the main issues faced by coral taxonomy was compiled (**Chapter I**). Molecular approaches were then applied to delimitate sympatric species of ecologically important and closely related tabular *Acropora* species. The congruence of the resulting molecular species delineation with additional lines of evidence, such as mating trials and morphology, was then gauged to select the most robust taxonomic hypothesis (**Chapter II**). The potential of this integrative methodology was extended by assessing the discriminative power of novel 3D-based morphometrical approaches in *Acropora* species robustly delineated using other lines of evidence (**Chapter III**). Finally, the discussion, perspectives and conclusions of this thesis are presented (**Chapter IV**).

The results of this thesis support the feasibility of developing a robust coral taxonomy when combining approaches sensitive enough to detect species divergence amid the complexity of speciation scenarios. The agreement between *Acropora* species boundaries delineated using different lines of evidence challenged the widespread notion that coral morphospecies cannot be distinguished at the molecular level due to hybridization. Due to unwarranted expectations, allele sharing-based and coalescence-based multilocus approaches outperformed mainstream molecular approaches. Contrastingly, the discriminative power of 3D-based quantitative morphology was comparable to traditional morphometric analyses for differentiating closely related species. Ultimately, comparing multiple lines of evidence is crucial to address species delimitation and provide valuable support to conservation efforts of taxonomically confused and threatened keystone organisms like corals of the genus *Acropora*.

Résumé (FRA)

MOTS CLÉS | taxonomie des coraux · délimitation des espèces · approches intégratives · *Acropora*

La délimitation des espèces est non seulement une question centrale en biologie évolutive et en systématique, mais aussi une condition préalable aux études physiologiques, écologiques et de génétique des populations. Cependant, la délimitation des espèces dans des environnements très diversifiés, tels que les récifs coralliens tropicaux peu profonds, reste un défi. Les hypothèses traditionnelles de séparation des lignées coralliennes ont longtemps été basées sur des traits morphologiques qui n'ont pas permis une résolution au niveau des espèces. En effet, la réponse plastique des coraux aux variations environnementales et les observations de terrain de morphotypes intermédiaires ont également rendu cette délimitation difficile, remettant en question les délimitations actuelles des espèces. Par conséquent, alors que les perturbations anthropiques menacent les écosystèmes des récifs coralliens, la taxonomie des genres vulnérables qui les habitent reste obscure.

La difficulté de naviguer dans ce paysage taxonomique complexe est particulièrement bien illustrée par le genre de corail *Acropora*, qui compte plus de cent morpho-espèces et constitue un excellent terrain d'entraînement pour tester et valider de nouvelles pratiques. Les tentatives de délimitation des espèces dans le genre *Acropora* ont jusqu'à présent été infructueuses en raison de l'incongruité des généalogies obtenues sur base de marqueurs génétiques et entre les groupements obtenues sur des bases morphologiques, de tests de croisements et d'analyses phylogénétiques. Par conséquent, l'objectif de cette thèse était de tester la délimitation actuelle des espèces chez les coraux scléractiniaires, en esquissant une approche intégrative et en établissant une méthodologie qui ouvre la voie à une révision taxonomique en utilisant le genre *Acropora* comme étude de cas.

À cette fin, un descriptif historique soulignant l'émergence des principaux problèmes rencontrés par la taxonomie des coraux a été compilé (**Chapitre I**). Des approches moléculaires ont ensuite été utilisées pour délimiter les espèces sympatriques d'*Acropora* tabulaires, importantes sur le plan écologique et étroitement liées. La congruence de la délimitation moléculaire de ces espèces qui en résulte a ensuite été évaluée avec d'autres sources de données, telles que les expériences de croisements et la morphologie, afin de sélectionner l'hypothèse taxonomique la plus robuste (**Chapitre II**). Le potentiel de cette méthodologie intégrative a été étendu en évaluant le pouvoir discriminatoire des nouvelles méthodes morphométriques 3D sur des espèces d'*Acropora* précédemment délimitées en utilisant d'autres sources de données (**Chapitre III**). Enfin, la discussion, les perspectives et les conclusions de cette thèse sont présentées (**Chapitre IV**).

Les résultats de cette thèse soutiennent la faisabilité du développement d'une taxonomie corallienne robuste en combinant des approches suffisamment sensibles pour détecter la divergence des espèces dans la complexité des scénarios de spéciation. La concordance entre les limites des espèces d'*Acropora* délimitées à l'aide de différentes sources de données a remis en question l'idée répandue selon laquelle les morpho-espèces coralliennes ne peuvent être distinguées au niveau moléculaire en raison de l'hybridation. En raison d'attentes injustifiées, les approches multilocus basées sur le partage des allèles et sur la coalescence ont donné de meilleurs résultats que les approches moléculaires classiques. En revanche, le pouvoir discriminant de la morphologie quantitative basée sur la 3D était comparable à celui des analyses morphométriques traditionnelles pour différencier des espèces étroitement apparentées. En fin, la comparaison de plusieurs sources de données est cruciale pour aborder la délimitation des espèces et apporter un soutien précieux aux efforts de conservation d'organismes clés menacés et confus sur le plan taxonomique, comme les coraux du genre *Acropora*.

Zusammenfassung (DEU)

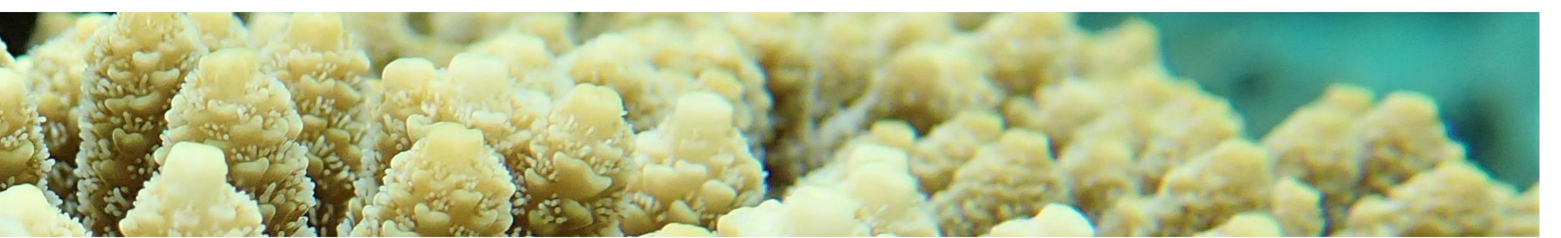
SCHLÜSSELWÖRTER | Korallentaxonomie · Abgrenzung der Arten · integrative Ansätze · *Acropora*

Die Abgrenzung von Arten ist nicht nur ein zentrales Thema in der Evolutionsbiologie und der systematischen Biologie, sondern auch eine Voraussetzung für physiologische, ökologische und populationsgenetische Studien. Die Abgrenzung von Arten in sehr unterschiedlichen Umgebungen wie flachen tropischen Korallenriffen bleibt jedoch eine Herausforderung. Traditionelle Hypothesen zur Trennung von Korallenstämmen basierten lange Zeit auf morphologischen Merkmalen, die keine Auflösung auf Artniveau ermöglichten. Die plastische Reaktion der Korallen auf Umweltvariationen und Berichte über intermediäre Morphotypen in der Praxis haben diese Abgrenzung ebenfalls erschwert und lassen Zweifel an den derzeitigen Artenabgrenzungen aufkommen. Während also anthropogene Störungen die Ökosysteme der Korallenriffe bedrohen, bleibt die Taxonomie der gefährdeten Gattungen, die sie bewohnen, unklar.

Die Schwierigkeit, sich in dieser komplizierten taxonomischen Landschaft zurechtzufinden, wird durch die Korallengattung *Acropora* veranschaulicht, die mehr als hundert Morphospezies aufweist und ein hervorragendes Übungsfeld für die Erprobung und Validierung neuer Verfahren bietet. Versuche der Artabgrenzung bei *Acropora*-Korallen waren bisher aufgrund der weit verbreiteten genealogischen Inkongruenz zwischen genetischen Markern und zwischen morphologischen Gruppierungen, Paarungsversuchen und molekularen Kladen, die in phylogenetischen Analysen ermittelt wurden, erfolglos. Ziel dieser Arbeit war es daher, die derzeitige Artabgrenzung bei Skleraktinischen Korallen zu überprüfen, einen integrativen Ansatz zu skizzieren und eine Methodik zu entwickeln, die den Weg für eine taxonomische Revision am Beispiel der Gattung *Acropora* ebnet.

Zu diesem Zweck wurde ein historischer Überblick über die Entstehung der wichtigsten Probleme der Korallentaxonomie erstellt (**Kapitel I**). Anschließend wurden molekulare Methoden angewandt, um sympatrische Arten von ökologisch wichtigen und eng verwandten tafelförmigen *Acropora*-Arten abzugrenzen. Die Übereinstimmung der sich daraus ergebenden molekularen Artabgrenzung mit zusätzlichen Beweisen, wie Paarungsversuchen und Morphologie, wurde dann geprüft, um die solideste taxonomische Hypothese (**Kapitel II**). Das Potenzial dieser integrativen Methodik wurde erweitert, indem die Unterscheidungskraft neuartiger 3D-basierter morphometrischer Ansätze bei *Acropora*-Arten bewertet wurde, die anhand anderer Nachweise zuverlässig abgegrenzt wurden (**Kapitel III**). Schließlich werden die Diskussion, die Perspektiven und die Schlussfolgerungen dieser Arbeit vorgestellt (**Kapitel IV**).

Die Ergebnisse dieser Arbeit zeigen, dass es möglich ist, eine robuste Korallentaxonomie zu entwickeln, wenn man Ansätze kombiniert, die empfindlich genug sind, um Artdivergenz inmitten der Komplexität von Speziationsszenarien zu erkennen. Die Übereinstimmung zwischen den *Acropora*-Artengrenzen, die mit Hilfe verschiedener Nachweismethoden bestimmt wurden, widerlegt die weit verbreitete Ansicht, dass Korallenmorphospezies aufgrund von Hybridisierung auf molekularer Ebene nicht unterschieden werden können. Aufgrund ungerechtfertigter Erwartungen übertrafen die auf Allel-Sharing und Koaleszenz basierenden Multilocus-Ansätze die gängigen molekularen Ansätze. Im Gegensatz dazu war die Trennschärfe der 3D-basierten quantitativen Morphologie bei der Unterscheidung eng verwandter Arten vergleichbar mit traditionellen morphometrischen Analysen. Letztendlich ist der Vergleich mehrerer Beweislinien entscheidend für die Abgrenzung von Arten und eine wertvolle Unterstützung für die Erhaltungsbemühungen von taxonomisch verwirrten und bedrohten Schlüsselorganismen wie Korallen der Gattung *Acropora*.



Resumen (ESP)

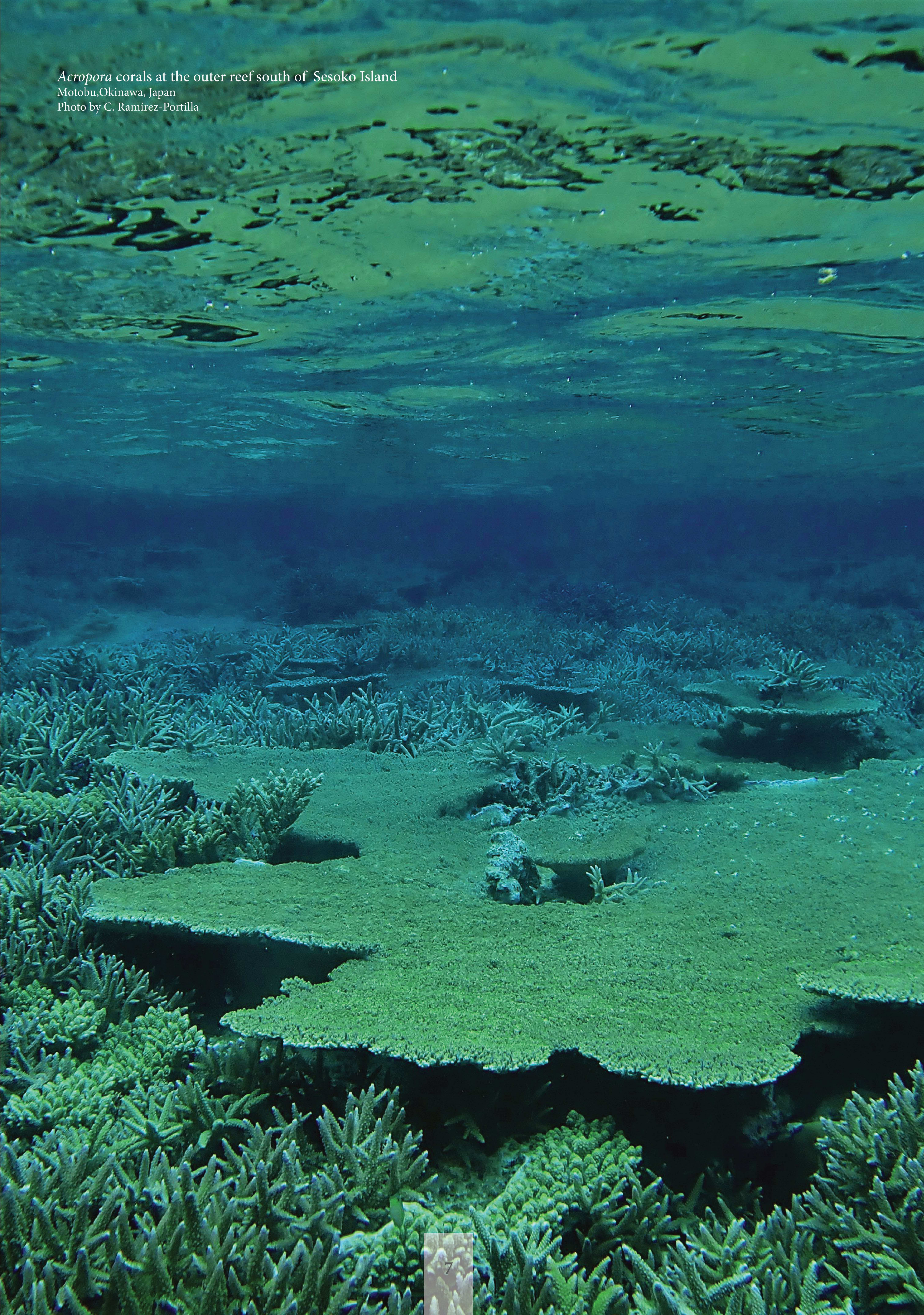
PALABRAS CLAVE | taxonomía de corales · delimitación de especies · métodos integrativos · *Acropora*

La delimitación de las especies no sólo es una cuestión central en la biología evolutiva y sistemática, sino también un requisito previo para los estudios fisiológicos, ecológicos y de genética de poblaciones. No obstante, la delimitación de las especies en ecosistemas diversos, como los arrecifes coralinos tropicales, sigue siendo un reto. Durante mucho tiempo, las hipótesis tradicionales que separan los grupos de corales se han basado en rasgos morfológicos que no han aportado resolución a nivel de especie. La respuesta plástica de los corales a las variaciones ambientales y los reportes sobre morfotipos intermedios en el campo también han dificultado esta tarea, poniendo en duda las delimitaciones actuales de las especies. En consecuencia, mientras las perturbaciones antropogénicas amenazan los ecosistemas de arrecifes coralinos, la taxonomía de los géneros vulnerables que los habitan sigue siendo confusa.

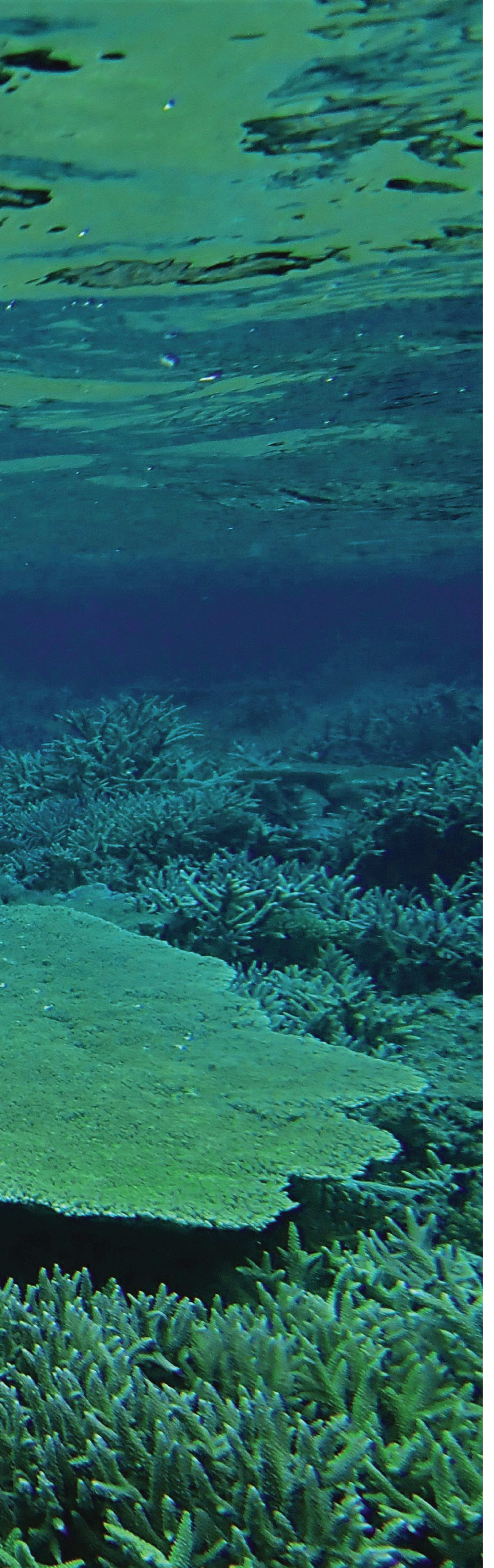
La dificultad de navegar por este intrincado paisaje taxonómico está personificada por el género coralino *Acropora*, que presenta más de cien morfoespecies y proporciona un excelente campo de entrenamiento para evaluar y validar nuevas prácticas. Hasta ahora, los intentos de delimitación de especies en este género han sido poco exitosos debido a la incongruencia generalizada entre las genealogías de los marcadores genéticos y entre las agrupaciones morfológicas, los ensayos de apareamiento y los clados moleculares obtenidos en los análisis filogenéticos. Por lo tanto, el objetivo de esta tesis fue poner a prueba la actual delimitación de especies en los corales escleractinios, trazando un enfoque integrativo y estableciendo una metodología que prepare el camino para una revisión taxonómica utilizando el género *Acropora* como caso de estudio.

Con este propósito, se elaboró un recuento histórico destacando la aparición de los principales problemas a los que se enfrenta la taxonomía de los corales (**Capítulo I**). A continuación, se aplicaron enfoques moleculares para delimitar especies ecológicamente importantes de corales *Acropora* tabulares que habitan en simpatría. Posteriormente, se evaluó la correspondencia entre la delimitación molecular de estas especies con otras líneas de evidencia, como los ensayos de apareamiento y la morfología, para seleccionar la hipótesis taxonómica más robusta (**Capítulo II**). El potencial de esta metodología integrativa se amplió evaluando el poder discriminativo de nuevos métodos morfométricos 3D en especies de *Acropora* previamente delineadas utilizando otras líneas de evidencia (**Capítulo III**). Por último, se presentan la discusión, perspectivas y conclusiones (**Capítulo IV**).

Los resultados de esta tesis apoyan la viabilidad de desarrollar una taxonomía coralina robusta cuando se combinan enfoques suficientemente sensibles para detectar la divergencia de especies en medio de la complejidad de los escenarios de especiación. La congruencia entre los límites de las especies de *Acropora* delineados utilizando diferentes líneas de evidencia desafía la noción generalizada de que las morfoespecies de coral no pueden distinguirse a nivel molecular debido a la hibridación. Los enfoques multilocus basados en la coocurrencia de alelos y la coalescencia superaron a los enfoques moleculares convencionales debido a expectativas injustificadas de estos últimos. En contraste, el poder discriminativo de la morfología cuantitativa basada en 3D fue comparable al de los análisis morfométricos tradicionales a la hora de diferenciar especies estrechamente relacionadas. En última instancia, la comparación de múltiples líneas de evidencia es crucial para abordar la delimitación de especies y proporcionar un valioso apoyo a los esfuerzos de conservación de organismos clave taxonómicamente confusos y amenazados como los corales del género *Acropora*.



Acropora corals at the outer reef south of Sesoko Island
Motobu,Okinawa, Japan
Photo by C. Ramírez-Portilla



1 | Chapter I

1.1 | Introduction

"The first step of science is to know one thing from another"

(Linnaeus, 1750)

Discovery and validation of species boundaries are paramount for the reliability of physiological, ecological, evolutionary, and population genetic studies, as well as for the conservation of endangered ecosystems across the globe (Sites and Marshall, 2003, 2004; Wiens, 2007; Bortolus, 2008). Yet, the significance of delineating species for attaining a consequential picture of the diversity on the planet is comparable to the challenge it represents (Dayrat, 2005; Mora et al., 2011). As nomenclatural or taxonomic ranks, species represent the currency taxon for biodiversity assessments (Agapow et al., 2004). As taxonomic groups or "specions" (Dubois, 2007, 2011), species constitute key biological research hypotheses that rely on the criteria used to delineate their boundaries (Gaston and Mound, 1993; Sluys, 2013). Hence, specions may not necessarily agree with nomenclature and different specion types (i.e., morphospecies, biological species, phylogenetic species) may not agree with each other (Dubois, 2011), which not only complicates the landscape relevant to species delimitation but also widens the existing gap between science and conservation management policies.

While more than forty-thousand species currently face threats of extinction (i.e., are listed as critically endangered, endangered or vulnerable; IUCN, 2022), the prevailing gap in taxonomic knowledge (i.e., the "taxonomic impediment") thwarts the prospects of preserving the "dark-matter" of biodiversity and is likely to contribute to an underestimation of the on-going loss (Wilson, 1985; Agnarsson and Kuntner, 2007; Evenhuis, 2007). In the marine environment, it has been estimated that at least one-third of eukaryotic species are undescribed (Appeltans et al., 2012). Thus, if we are to understand the global implications of biodiversity shifts, reweighting the value of taxonomy and consequently increasing the pace at which species are delineated is timely needed (Drew, 2011; Scheffers et al., 2012).

In the past two decades, the quest for "*identifying species-level biological diversity*" has exploded thanks to advances in the collection and analysis of molecular data (Carstens et al., 2013). However, more than 260 years after the dawn of zoological nomenclature and taxonomy (Linnaeus, 1758), delineation of species boundaries remains a challenge, particularly in species-rich genera and morphologically diverse taxa such as stony corals (a.k.a., scleractinian corals; Hoffmeister, 1926; Kitahara et al., 2016). Traditional hypotheses used to distinguish species in these anthozoans, have long been based on morphological traits that do not provide significant resolution (Filatov et al., 2013; Fontaneto et al., 2015). The low number of informative characters compared and their frequent homoplasy due to the independent evolution of similar features in different lineages (i.e., convergent evolution) accounts for the dearth of morphological characters informative to distinguish species (Daly et al., 2003; Budd et al., 2010). The plastic response of corals to environmental variations, along with the occurrence of intermediate morphotypes in nature and the fossil record, have also hindered this delineation by casting doubts on current species delimitations (Budd and Pandolfi, 2004; Hatta and Matsushima, 2008; Todd, 2008; Zlatarski, 2010; Isomura et al., 2013; Paz-García et al., 2015b). Indeed, morphological groups often exhibit little agreement with molecular data (e.g., van Oppen et al., 2001; Flot et al., 2011; Forsman et al., 2017). This lack of comparability suggests that the boundaries inferred from morphological characters do not correspond to independently evolving lineages (Fukami et al., 2004b; Flot et al., 2008a).

Despite anthropogenic threats to coral reef ecosystems (Pandolfi et al., 2003; Carpenter et al., 2008; Hughes et al., 2017, 2018a) the taxonomy of several endangered reef-building coral genera remains unresolved (Fukami et al., 2004b; Forsman et al., 2010; Richards et al., 2016). Overall, the trend to favor a single line of evidence and the lack of comparison between examined specimens, original descriptions, and type material have become the primary sources of misinformation (Veron, 2013; Bonito et al., 2021). Yet, due to the different levels of susceptibility and tolerance that corals display to stressors, delineating species boundaries is critical to understand the response of coral reefs to, for example, climate change (Stat et al.,

2012; Gómez-Corrales and Prada, 2020; Burgess et al., 2021). Therefore, there is an urgent need to develop and test methods to delineate corals to effectively address coral reef management and conservation.

Consequently, the aim of this thesis is to pave the way for the taxonomic revision of stony corals. To this aim, I first present a brief historical account of coral species delineation, highlighting the criteria used to delineate coral species boundaries through time (section **1.1.1**). These elements constitute the basis for the ensuing overview of species delimitation approaches performed in reef-building corals to date (section **1.1.2**), emphasizing the coral species delimitation conundrum as one of the main issues faced by coral taxonomy (section **1.1.3**) and the features of the coral genus *Acropora* used in this thesis as a case study (section **1.1.4**). Then, I introduce the scope and specific aims of the thesis (section **1.2**), followed by the outlines (section **1.3**) and the corresponding chapters containing the complete versions of the associated publications (**Chapters II** and **III**). Finally, I conclude with a general discussion of the results, remarking on how to move forward in coral taxonomy (**Chapter IV**).

1.1.1 | A seemingly never-ending story: two hundred years of advancing coral taxonomy

The underlying challenge of exploring the ocean and the organisms inhabiting it has been decisive for delimitating coral species over time (Knowlton, 1993). In addition to the prevailing shortfalls related to the number of formally described species and their geographic distribution (i.e., respectively the Linnean and Wallacean shortfalls; Hortal et al., 2015), the constraints to survey and study species from accessible environments (i.e., Racovitzan impediment; Ficetola et al., 2019) shaped the first part of coral taxonomy history. Corals, for instance, were not regarded as animals until after the mid-1700s and such interpretation only became relevant a century later (Vaughan and Wells, 1943; Zlatarski and Stake, 2012). Indeed, coral taxonomy has experienced extensive debate around the concept of species and how to best delineate specions (Willis, 1990). The different stages of coral taxonomy have reflected this progression (Fig. 1); from earlier species descriptions to the ongoing reexamination of coral taxonomy, the existing knowledge gap has been channeled by breakthroughs in oceanic exploration and tool development.

In the early days of coral reef research (see "**Exploring**" in Fig. 1, approx. 1800–1960), species descriptions were based on observations derived from limited numbers of skeletal fragments collected by marine expeditions to shallow tropical coral reefs (Veron, 2011). Coral specimens were collected primarily from a single locality and later deposited in museums on the basis of their conspicuity instead of representativity across the distribution range (Veron, 2013). Technological limitations also hampered the documentation of coral features in their natural habitats (Veron, 2013). Indeed, information necessary for robust comparison was limited and most taxonomic studies at the time lacked a species concept (Wallace and Dale, 1978; Willis, 1990). Consequently, type material descriptions were generally qualitative and each novel specimen with a certain degree of variation was considered representative of a new taxon (Veron, 2013).

Eventually, the challenge that skeleton morphological variability represented for coral taxonomy was acknowledged when significant specimen collections became available (Quelch, 1886; Vaughan, 1907; Wood-Jones, 1907). Initially, phenetic studies at higher taxonomic ranks highlighted the importance of comparing different traits to reduce subjectivity and pointed towards characters with the potential to discriminate species (Powers and Rohlf, 1972; Lang, 1984). Therefore, the phenetic species concept based mainly on morphological resemblance became used to substantiate the description of new species or morphospecies (i.e., phenons or phena *sensu* Mayr, 1969; Sokal and Crovello, 1970; Sokal, 1973).

The development and rise in popularity of diving using SCUBA (self-contained underwater breathing apparatus), led to the proliferation of field data collection (see "**Documenting**" in Fig. 1, post-World War II approx. 1960–1990) changing coral reef research irrevocably (Willis, 1990; Wallace, 1999). Notably, the examination of coral morphological variation and its link with habitats and ecological features was enabled by overcoming the limitations of exploring further and in real-time (Veron, 2011). In consequence, coral species descriptions became

enriched and intraspecific morphological variability started to be formally acknowledged in taxonomic works (Wijsman-Best, 1972; Veron and Pichon, 1976; Best et al., 1984). These developments prompted extensive species synonymization, particularly in speciose coral genera such as *Acropora* (Veron and Pichon, 1976; Wallace, 1978). Likewise, the documentation of coral reproductive phenology (i.e., timing and seasonality of reproductive activity and key events in coral life cycles), particularly of phenomena such as mass broadcast-spawning, expanded the frontiers of experimental research with consequences that impacted the state of knowledge in coral ecology, physiology and consequently, taxonomy (Harrison et al., 1984; Wallace, 1985).

Owing to the increasing knowledge of coral reproductive biology (e.g., Harrison and Wallace, 1990), the biological species concept (Mayr, 1942) based on the mixiological criterion to delineate species as reproductively cohesive genetic pools replaced the phenetic concept (Willis, 1990). Although the 'biological species' terminology is of widespread use, the mixiological term (from the Greek "μιξιζ" mix; Coutagne, 1895) conveys the property of delineating specions as groups of organisms that breed or cross-fertilize in natural conditions (i.e., mayrons; Dubois, 2007, 2011). In corals, the widespread use of mayrons as taxonomic categories was reinforced by the prevalent assumption that they were congruent with phena (Wallace and Willis, 1994). However, following the discovery of mass spawning events in tropical coral reefs, cross-fertilization experiments to assess reproductive compatibility within and between morphospecies revealed variable degrees of interspecific breeding in corals, thus challenging traditional morphology-based species hypotheses (e.g., Heyward and Babcock, 1986; Miller and Babcock, 1997; Szmant et al., 1997; Willis et al., 1997).

Later on, the emergence of molecule-based approaches (see "**Analyzing**" in Fig. 1, approx. 1990–2000) conveyed a new perspective on coral taxonomy previously focused on morphology (Wallace and Willis, 1994; Veron et al., 1996). Evolutionary independent lineages (i.e., simpsons; Dubois, 2007, 2011) emerged as coral species hypotheses in line with the concept of phylogenetic species (Cracraft, 1983; reformulated from the evolutionary species concept originally proposed by Simpson, 1961). In addition, broader geographical sampling provided a new perspective on coral species boundaries (e.g., Fukami et al., 2004a, 2004b; Combosch et al., 2008).

While the concept of phylogenetic species endorsed overall diagnosability, results from standard molecular approaches failed to delineate coral species congruent with other lines of evidence (e.g., van Oppen et al., 2001; Flot et al., 2008a). Specions emerging from these approaches conflicted with previous taxonomic hypotheses based on morphology, most notably when assessing closely related species that lacked monophyly in most gene trees (e.g., Huang et al., 2009; see section **1.1.3**), when contrasting incongruent results obtained with mitochondrial and nuclear genes (e.g., Souter, 2010), and when evaluating species boundaries among unrecognized cryptic species (e.g., Knowlton et al., 1992). Thus, robust delineation of coral species became fuzzy while the breach between coral taxonomists and molecular systematists widened.

The early days of integrative taxonomy (see "**Integrating**" in Fig. 1, approx. 2000–2010) strived to contrast several lines of evidence searching for an effective strategy to delineate coral species (see Stat et al., 2012). The increasing availability of independent approaches led to a reassessment of coral species boundaries in several study systems (Kitahara et al., 2016). To this end, novel genomic approaches integrated with morphometry emerged as the most common trend (Pante et al., 2015b). Alternative lines of evidence stemming from coral life histories as symbiosis, reproduction, ecology, and phenology, also provided additional clues to unravel species boundaries (Pinzón and Lajeunesse, 2011; Schmidt-Roach et al., 2013; Boulay et al., 2014).

Integrative taxonomic studies have explicitly or implicitly worked under approaches stemming from the evolutionary species concept (Simpson, 1961; Wiley, 1981), such as the general lineage concept (GLC; de Queiroz, 1998, 2005). The underlying assumption of such a concept is that no definition encompasses "species" other than being independently evolving lineages or metapopulations (de Queiroz, 2007). In this scenario, the support provided by different lines of evidence is used as operational or secondary species

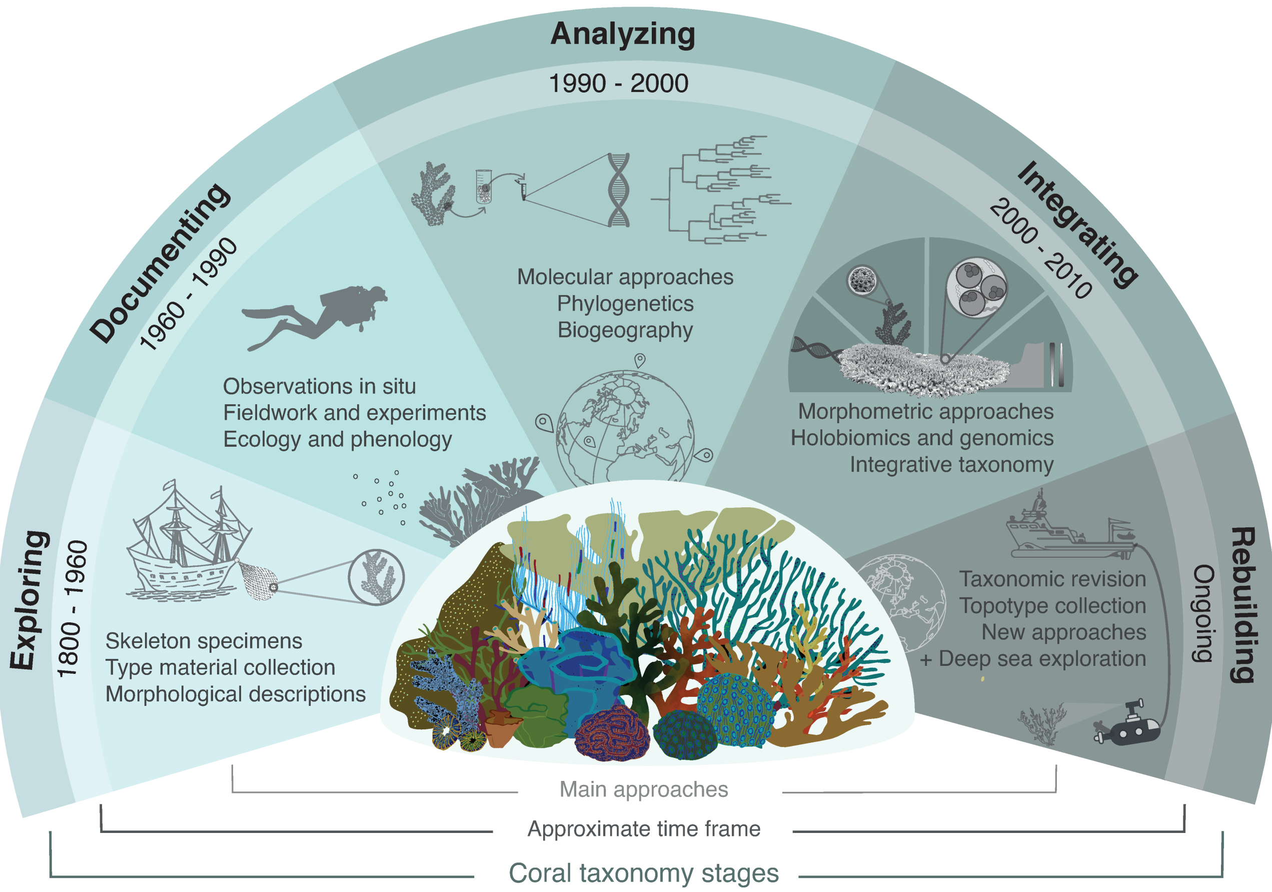


Figure 1 | More than two hundred years of advancing coral taxonomy

Schematic representation of the "brief" history of coral taxonomy, where the main stages and approaches used through time are summarized. The approximate time frames are provided for each stage. However, the emergence and later establishment of some approaches may span more than a single stage, and most developments and implementations overlap. See section **1.1.1** for a detailed description of each stage. Illustrations by C. Ramírez-Portilla or otherwise modified from Canva (Free Media License, 2022).

criteria to assess lineage separation (Naomi, 2011). As a result, species delimitation issues were detached from the problem of the species concept and previously conflicting operational criteria became relevant only for providing evidence of species boundaries (de Queiroz, 2007; Carstens et al., 2013). Although this resulted in the widespread use of the GLC in coral taxonomy (e.g., Schmidt-Roach et al., 2013; Gélin et al., 2017), alternative concepts (i.e., phenetic, mixiological, and phylogenetic) are still in use, which provides a broader operational spectrum to delineate coral species in a variety of study systems where different data types and life history trait information is available.

Coral taxonomy is entering a new era (see "**Rebuilding**" in Fig. 1, ongoing). Although it may have seemed like a "never-ending story", technological limitations previously hindering our understanding of coral species boundaries have mostly been overcome (Benzoni et al., 2010; Wheeler et al., 2012). Extensive sampling and observation of coral species in underexplored marine environments, such as the mesophotic and deep-sea, epitomize some of the recent breakthroughs (e.g., Kitahara et al., 2010; Janiszewska et al., 2011; Miller et al., 2011; Luck et al., 2013; for a review see Altuna and Poliseno, 2019). Species boundaries are being reassessed in relation to type specimens, thus enriching original descriptions and contrasting with integrative studies that often stopped short of providing taxonomic recommendations (Pante et al., 2015b; Bonito et al., 2021). For instance, in addition to collection and analysis of topotypes, sequencing of old museum specimens is now feasible (Untiedt et al., 2021; Scott et al., 2022). Therefore, it is plausible that multidisciplinary knowledge from independent lines of evidence in light of type material will enable reconciling all dimensions of coral taxonomy (Benzoni

et al., 2010). Still, concerted efforts stemming from field, lab, museum, and data repositories are needed to refine coral taxonomy (Bonito et al., 2021; Voolstra et al., 2021).

Ultimately, taxonomic boundaries of coral species constitute testable hypotheses, and therefore are likely to change over time and in the light of newly available data emerging from novel approaches (Willis, 1990; Wallace and Willis, 1994; Pante et al., 2015a). Hence, it is key to ground species hypotheses using all lines of evidence at hand. Once robust taxonomic hypotheses are available, the outstanding criteria to delineate species for the focal group of study can be defined under a particular species concept so that required taxonomic revisions and formal species description can follow (Pante et al., 2015b; Bonito et al., 2021).

1.1.2 | Treading thin lines: approaches for delineating coral species boundaries

The wide range of shapes of scleractinian corals, the main reef-builders, led researchers to outline several hypotheses to explain what drives and maintains their diversity patterns (Connell, 1978; Stanley, 1981, 2003; Knowlton and Jackson, 1994; Veron, 1995). However, the testing of these hypotheses has been hindered by the lack of a robust and efficient approach to delineate species boundaries (Fontaneto et al., 2015). Here, I present an overview of species delimitation approaches performed in scleractinian corals to date, highlighting their main strengths and limitations with the aim of staging the background of this thesis.

Morphology-based approaches

Morphology has traditionally been used as the primary approach to document and describe coral species diversity as in many other branches from the tree of life (Wills, 2001; Stat et al., 2012). Morphological characters are useful not only to preliminary sort specimens, but also to establish primary taxonomic hypotheses and evolutionary hypotheses in relation to extinct life forms (Budd and Olsson, 2006; Schlick-Steiner et al., 2007; Mitteroecker, 2021). Moreover, in study systems for which limited information is available, morphology provides the basis for an operational concept that enables delineating phena or morphospecies (Dubois, 2011). Therefore, morphology is the connecting key between specimens used for a variety of approaches, the matching of type material for taxonomic placement and the relation of extant species to fossils (MacLeod, 2002; Wheeler, 2004; Saraswati and Srinivasan, 2016).

Traditional taxonomic hypotheses in corals have long relied upon skeletal characters documented from bleached specimens (Lang, 1984). Although other morphological traits have been used to characterize and differentiate coral species (e.g., polyp size and shape; Pichon and Veron, 1980), most species descriptions are based on skeleton morphology (Kitahara et al., 2016). However, due to potential homoplasy, intraspecific variation, morphological plasticity, and cryptic diversity documented in coral study systems, skeletal traits have been deemed as potential sources of taxonomic uncertainty (Veron, 1995; Todd, 2008; Bongaerts et al., 2021). For instance, character homoplasy (i.e., character states that are acquired or reverted to convergently by different lineages) has called into question the adequacy of using skeletal characters to sustain coral species hypotheses (Webb, 1996; Romano and Cairns, 2000; Fukami et al., 2004b, 2008; Budd et al., 2010; Kitahara et al., 2010; Budd and Stolarski, 2011). Indeed, most macromorphological or colony-level skeletal characters have not proved species diagnostic in light of phylogenetic evidence (e.g., Budd and Stolarski, 2009; Forsman et al., 2010; Marti-Puig et al., 2014).

The high intraspecific variability of coral morphology along with evidence of environmentally-induced morphological plasticity has reinforced the uncertainty in coral species boundaries, with consequences potentially spanning not only coral taxonomy but also ecological studies of coral adaptability and resilience to climate change (Powers and Rohlf, 1972; Miller, 1994a; Todd, 2008; Paz-García et al., 2015a). In taxonomically intricate taxa, coupling between high intraspecific variability and interspecific similarity has hindered accurate coral species delimitation, particularly when intermediate forms are found both in the field and in the fossil record (Szmant et al., 1997; Budd and Pandolfi, 2004; Zlatarski, 2010; Isomura et al., 2013). Moreover, evidence of corals intergrading from one morphospecies to another in response to environmental disturbances

and incongruence between phena and molecularly delineated species brings evidence of the extent to which morphological plasticity can confound morphology-based taxonomy (Flot et al., 2011; Schmidt-Roach et al., 2014; Paz-García et al., 2015b; Johnston et al., 2017).

The detection of cryptic species (i.e., species difficult or impossible to distinguish based solely on morphological characters; Knowlton, 1986) has exposed both the limitations and prospects of morphology-based taxonomy (Knowlton and Jackson, 1994). Thanks to the implementation of lines of evidence different of morphology, several cryptic coral species have been discovered (e.g., Boulay et al., 2014; Sheets et al., 2018; Arrigoni et al., 2019; Oury et al., 2020, 2022; Bongaerts et al., 2021). Contrastingly, coral species in other study systems have been readily distinguished once the appropriate morphological characters are assessed (e.g., micromorphological features), rendering them pseudo-cryptic species (e.g., Knowlton et al., 1992; Knowlton, 1993; Forsman et al., 2015; Terraneo et al., 2016; Arrigoni et al., 2020). Thus, many uncertainties in coral morphology-based taxonomy stem from the paucity of diagnostic characters at the species level, concealing a large fraction of diversity (Wolstenholme et al., 2003; Stefani et al., 2008; Benzoni et al., 2010).

In this context, the use of novel approaches and new diagnostic characters may enable delineating more robust coral phena, which could be used as primary species hypotheses (PSHs) to be corroborated using additional lines of evidence (Puillandre et al., 2012b; Pante et al., 2015a). For instance, alternative methods such as three-dimensional (3D) morphological analyses have the potential to examine complex coral features that are highly variable and lack homologous landmarks or distinctive outlines (Kruszyński et al., 2007; Reichert et al., 2017). In addition, the implementation of phenetic and more objective statistical strategies may improve modelling of morphological variation in agreement with evolutionary theory (Powers and Rohlf, 1972; Cadena et al., 2018).

Reproduction-based approaches

According to the mixiological criterion (renamed as the biological species concept by Mayr, 1942), species can be delineated as reproductively cohesive gene pools or mayrons (Dubois, 2011). Thus, evidence from interbreeding natural populations that are reproductively isolated from others is considered the "*ultimate test of conspecificity*" in sexually reproducing organisms (Wood-Jones, 1907; Lang, 1984). Consequently, available information of reproductive characters, fertilization systems and the existence of pre- and post-mating barriers needs to be assessed in order to substantiate the occurrence of reproductively isolated units in a study system (Templeton, 1981; Willis, 1990).

Broadcast spawning (i.e., external gamete fertilization and subsequent embryo development) is the reproductive mode of most scleractinian species (Baird et al., 2009; Harrison, 2011). The occurrence of mass spawning in these corals, when gametes of different species are released within a few hours from each other into the water column, has provided an outstanding opportunity to collect relevant data to validate coral species boundaries (Harrison et al., 1984; Willis et al., 1985; Babcock et al., 1986; Wallace and Willis, 1994). For example, substantial interspecific differences in gamete release time have been used to suggest the presence of pre-zygotic barriers to hybridization or vice versa (Kojis, 1986; Knowlton et al., 1997; Levitan et al., 2011; Fogarty et al., 2012; Rosser, 2015). Therefore, documenting coral spawning synchrony in nature contributed to the wealth of field-derived data about potential reproductive barriers and mayrons (Kenyon, 1992; Babcock, 1995; Fukami et al., 2003; Taylor and Friesen, 2017).

However, reproductive evidence has been mainly gathered from experimental breeding crosses, which usually involve the use of sperm from one morphospecies and the eggs from another (i.e., no-choice fertilization trials), thus serving as *in vitro* proxies to evaluate potentially existing pre-zygotic barriers and hybridizing potential (Knowlton, 1993; Willis et al., 2006). For instance, significant fertilization success has been achieved within morphospecies (i.e., using gametes of different colonies of the same morphospecies) and between morphologically similar

species in breeding crosses performed with *Acropora* corals (Willis et al., 1997; van Oppen et al., 2002a). As such, results from fertilization trials challenge taxonomic hypotheses based on morphology and defy widespread notions on coral species boundaries (Wallace and Willis, 1994; Miller and Babcock, 1997; Szmant et al., 1997; Hatta et al., 1999).

Still, evidence arising from cross-fertilization experiments suggests complex intra- and interspecific breeding compatibility patterns in corals, which might lead to overestimate hybridization rates in natural conditions (Willis et al., 1997; Márquez et al., 2002a; Suzuki et al., 2016; Morita et al., 2019). Factors such as gamete dilution can drive differences between crosses produced in the lab and the field, particularly under sperm-limited and sperm-choice conditions (Heyward and Babcock, 1986; Oliver and Babcock, 1992; Levitan and Petersen, 1995; Yund, 2000; Willis et al., 2006; Kitanobo et al., 2016). Conflicting results regarding the viability of the embryos produced from experimental breeding crosses and multispecies spawning in the field, also highlight the complexity of post-zygotic barriers to hybridization and the difficulties of rearing larvae to reproductive ages to test their fertility (Hodgson, 1988; Miller, 1994b; Miller and Babcock, 1997; Isomura et al., 2013; Chan et al., 2019). Yet, cases where F1 (i.e., first-generation) larvae produced from interspecific crosses reached reproductive maturity and spawned have been reported, thus enabling the assessment of the hybrid potential for backcrossing and fecundity (Isomura et al., 2016; Kitanobo et al., 2022). Regardless, carrying out cross-fertilization experiments in corals that do not overlap in distribution ranges or that reproduce in different seasons is generally not possible due to reproductive timing and the limited viability period of the gametes (Heyward and Babcock, 1986; Heyward, 1987; Oliver and Babcock, 1992; Kitahara et al., 2016). Although using cryopreserved sperm for cross-fertilization experiments has substantially overcome this limitation (Ohki et al., 2015; Zuchowicz et al., 2021), validation of fossil species and coral systems without reproductive information available is still precluded (Willis, 1990).

Studies of a few other reproductive characters that may shed light on the nature of coral species boundaries have also been performed (Lang, 1984). For instance, gamete ultrastructure in scleractinian corals has revealed species differences in sperm morphology (Harrison, 1985; Willis, 1990). Yet, most of these studies have been performed between distantly related taxa, so a comparison between congeneric species remains to be addressed (Steiner, 1993). In addition, potential conspecific sperm attractants have been detected in coral eggs, suggesting the existence of partial barriers to hybridization by conferring the sperm certain degree of specificity (Coll et al., 1994; Babcock, 1995). However, comprehensive molecular assessments of the proteins governing egg-sperm interactions are still needed (Clark et al., 2006; Willis et al., 2006; Hobbs et al., 2022).

Molecular-based approaches

The emergence of molecular-based approaches revolutionized taxonomy and systematics across the tree of life branches (Pennisi, 2003; Tautz et al., 2003; Carstens et al., 2013). Owing to their operationality, molecular-based approaches have largely circumvented the difficulties associated with assessing species boundaries across distribution ranges and in taxa with limited biological information (Agapow and Sluys, 2005; Padial and De La Riva, 2006; but see Isaac et al., 2004; Mace, 2004; Zachos, 2013, 2015). In particular, the availability of genetic data and the feasibility of applying the phylogenetic species concept expedited the progress of molecular species delimitation approaches in many study systems, including corals (Blaxter, 2004; Stat et al., 2012). Yet, molecular approaches in species delimitation extend well beyond the delineation of simpsons according to the phylogenetic species concept.

Existing molecular approaches to delineate species boundaries can be broadly categorized as distance-based, tree-based, and allele sharing-based (Flot, 2015; Dellicour and Flot, 2018). Comparably to the delineation of phena, distance-based implementations rely on the computation of genetic distances between samples to identify groups (i.e., putative species) that respectively minimize and maximize the within and between species genetic distances (i.e., “barcode” gap; Puillandre et al., 2012a, 2021). Tree-based methods implement sequence evolution models to infer

historical relationships between taxa to delineate simpsons, either using reciprocal monophyly and fixed differences or coalescent-based approaches (Pons et al., 2006; Yang and Rannala, 2010; Fujita et al., 2012; Zhang et al., 2013). In contrast, allele sharing-based approaches use mutual allelic exclusivity to identify common genetic pools and act as indirect evidence for reproductive isolation to delineate mayrons (Doyle, 1995; Flot et al., 2010). Other methods that combine strategies, such as genetic distance and trees, have also been proposed and implemented but not yet applied to delineate coral species (e.g., K/θ; Birky et al., 2005, 2010; Birky, 2013; Spöri et al., 2021).

Each approach type carries limitations stemming from assumptions related to the parameter space relevant to species delimitation (Carstens et al., 2013). Distance-based approaches, for instance, rely on intraspecific genetic distances to be substantially smaller than interspecific ones (Fontaneto et al., 2015). Therefore, if genetic distances overlap due to factors such as short divergence time between species, these approaches are bound to fail (Dellicour and Flot, 2015, 2018). Lack of monophyly and gene tree heterogeneity has largely confused tree-based approaches for species delimitation (see Funk and Omland, 2003). Among the various reasons that can account for para- and polyphyly in resulting gene trees, incorrect identification of specimens, incomplete lineage sorting, and hybridization account for most intricate cases of coral species delineation (Flot et al., 2011). Likewise, allele sharing-based approaches are prone to error when molecular markers' mutation rates and effective population sizes are extreme (i.e., either high or low; Dellicour and Flot, 2018). Hence, all reasonable scenarios have to be adequately gauged before dismissing possible explanations of intricate patterns, notably because several coral species are likely to have large effective population sizes with potentially large dispersal abilities and interspecific gene flow might have occurred to some extent (Willis et al., 2006; Hobbs et al., 2022).

Regarding molecules, early studies in corals used predominantly the electrophoretic mobility of allozymes (i.e., structural variants of enzymes encoded by different alleles) for genotyping species (Ohlhorst, 1984; Ayre et al., 1991; Knowlton et al., 1992; Van Veghel and Bak, 1993; Stobart and Benzie, 1994; Miller and Benzie, 1997). As an allele sharing-based approach, allozyme electrophoresis leverages the information from heterozygous individuals to delineate putative species using mutual allele exclusivity to identify potential fields for recombination (FFRs; Doyle, 1995). Overall, allozymes showed good agreement with morphological data and, to some extent, with behavioral responses or breeding trials (Ayre et al., 1991; Knowlton et al., 1992; Weil, 1992; Garthwaite et al., 1994; Stobart, 1994, 2000; Stobart and Benzie, 1994). Contrastingly, conflicts between different lines of evidence arose when sequences from molecular markers were examined using Sanger sequencing (e.g., McMillan et al., 1991; Odorico and Miller, 1997). Most of such studies used a single-locus (or a mitochondrial and a nuclear gene at most) and applied tree-based approaches using reciprocal monophyly and fixed differences to establish molecular species hypotheses (e.g., Hatta et al., 1999; Medina et al., 1999; van Oppen et al., 2000, 2001, 2002a, 2004; Márquez et al., 2002b; Vollmer and Palumbi, 2002; Fukami et al., 2003). Disagreement between results obtained with both methodologies (i.e., allozymes and DNA-sequenced markers) could stem from the higher sensitivity of the mutual allelic exclusivity over the reciprocal monophyly criterion to delineate species boundaries (Flot et al., 2010). Still, using mitochondrial markers presented additional challenges to achieving sufficient species-level resolution in corals (Hellberg, 2006).

In contrast with bilaterian animals and ctenophores, coral mitochondrial DNA exhibits slower nucleotide substitution rates than nuclear DNA (Shearer et al., 2002; Hellberg, 2006; Chen et al., 2009; Lavrov and Pett, 2016). Accordingly, mitochondrial markers have been deemed generally uninformative to assess coral species boundaries despite the extensive use of some of the mitogenome regions (i.e., cytochrome *c* oxidase subunit 1 or COI) as taxonomic barcodes in other taxa (van Oppen et al., 1999; Concepcion et al., 2006; but see Flot et al., 2008b; Pinzón and Lajeunesse, 2011). Although a variety of molecular markers have been developed through time (e.g., microsatellites and a number of nuclear markers; Wang et al., 1995; Lopez et al., 1999; Maier et al., 2001; Miller and Howard, 2004; Severance et al., 2004; Flot and Tillier, 2007;

Eytan et al., 2009), a paucity of species-level molecular markers that can be applied across coral clades has made species delimitation particularly challenging in species-rich coral genera (Kitahara et al., 2016; Quattrini et al., 2018).

More recently, the development of molecular markers and the novel implementation of approaches have promoted the reexamination of species boundaries in several coral study systems (see Stat et al., 2012; Kitahara et al., 2016). For example, studies have strived to use multiple loci and distance-based, tree-based, and allele sharing-based approaches alone or in combination (e.g., Ladner and Palumbi, 2012; Adjeroud et al., 2014; Prada et al., 2014; Arrigoni et al., 2016a, 2016b; Terraneo et al., 2016; Gélin et al., 2017; Sinniger et al., 2017; Smith et al., 2017; Chiazzari et al., 2019). In addition, markers derived from high-throughput sequencing (HTS), such as restriction site-associated DNA (RAD) sequencing and target enrichment, have delivered data to assess species boundaries with sufficient resolution and potential for application to a broad range of coral taxa (Johnston et al., 2017; Quattrini et al., 2018; Arrigoni et al., 2020; Cowman et al., 2020; Wepfer et al., 2020; Grinblat et al., 2021; Aurelle et al., 2022). HTS limitations, however, such as the amount of missing data due to inconsistent locus recovery, the low taxon occupancy levels and the resulting incomplete data matrices, need additional consideration as they can impact the accuracy of species delimitation (Davey et al., 2013; Lemmon and Lemmon, 2013).

In this context, the best strategy is to analyze molecular evidence using a wide variety of approaches to find specions that are consistently supported (Dellicour and Flot, 2018). Indeed, a robust delineation of molecular species boundaries can only be achieved by evaluating the spectrum of intra- and interspecific variation through sampling closely related species, assessing several populations over broad geographic ranges, and using multiple molecular markers particularly when species likely share polymorphisms (Stat et al., 2012; Pante et al., 2015a). Ultimately, molecular-based approaches can complement other lines of evidence but not substitute them when delineating species boundaries within an integrative framework.

Other potentially diagnostic features and clues to disentangle coral species boundaries

Alternative methods to morphology, reproduction and molecular-based approaches have been gauged as independent lines of evidence that could shed light on obscure taxonomic boundaries. Niche diversification, for instance, was long disregarded as an important factor in maintaining coral reef diversity due to the assumption that most coral species presented wide distribution ranges (Goreau, 1959; Connell, 1978). However, reexamination of species boundaries in study systems, such as those comprised by sympatric cryptic species, has shown that distinct habitat preferences can suggest otherwise undetectable boundaries (see Knowlton, 1993). Microhabitat selection, for instance, may drive differentiation even since early life-history stages (Van Moorsel, 1983; Morse et al., 1988, 1996; Baird et al., 2003; Gélin et al., 2018). Still, cryptic species niches seem to overlap despite displaying different relative abundances according to habitat (Warner et al., 2015; Bongaerts et al., 2021; Johnston et al., 2022b). Similarly, although symbiont shifts have been documented in reef-building corals (e.g., Silverstein et al., 2012), a certain degree of specificity of symbiont-host associations has been observed in coral cryptic species and continues to be explored in light of new molecular methodologies (Pinzón and Lajeunesse, 2011; Johnston et al., 2022a). While these ecological characters may not be diagnostic of coral species (i.e., present in all specimens of a species and absent from others), they provide diversification signatures that could be explored further (Knowlton, 1993; Wiens and Servedio, 2000).

Approaches such as physicochemical coral-coral interactions assessed in the field or through grafting histocompatibility assays have also been used to hint at species boundaries (Lang, 1971, 1984; Wells, 1971, 1973; Hildemann et al., 1975; Logan, 1984). Although behavioral features evaluated through these means have proved to be indicative of species owing to their congruence with other lines of evidence (e.g., Knowlton et al., 1992; Weil and Knowlton, 1994), the broad range of outcomes that result from such tests point towards highly complex responses rather than clear clues (Heyward and Stoddart, 1985; Ayre and

Table 1 | Main lines of evidence used in coral taxonomy

Overview of the lines of evidence used to delineate species boundaries in corals to date, highlighting their main limitations and strengths. See section **1.1.2** for extended descriptions and relevant references.

Line of evidence	Strengths	Limitations
Morphological	Preliminary assessment can be performed during sampling for sorting specimens	Homoplasy of the characters
	Useful to establish phena as primary species hypotheses	High intraspecific variability coupled to interspecific similarity and geographical variation
	Link to type specimens and fossils	Morphological plasticity in response to environmental factors
	New approaches may uncover diagnostic taxonomic characters	Cryptic diversity due to morphological stasis or pseudo-cryptic species due to lack of characters
Reproductive	Proxy to gauge pre-zygotic breeding barriers and hybridizing potential *in vitro*	Potential overestimation of interbreeding potential in nature
	Enables assessing reproductively isolated units or mayrons	Difficulties in rearing embryos to sexually mature stages to test for fertility
	Applicable to a wide range of broadcast spawning corals	Unfeasible to be applied on dark taxa or lesser known species
	Useful to evaluate species boundaries when species distribution ranges overlap	Not feasible across the distribution range due to differences in breeding timing/season
Molecular	Feasibility of application to samples collected across the range of distribution	Dearth of species-level universal markers tested across a wide range of coral taxa
	Main input data for several different species delimitation approaches	Limitations of each category of species delimitation approaches
	Potential link to type specimens thanks to topotype collection	Difficulties in obtaining material from aged specimens
Other	Provide alternative lines of evidence to assess species boundaries	Challenges and difficulties of standardizing approaches
	Potential discovery of diagnostic species characters	Not enough information available for most coral species

Willis, 1988). An example is the array of morphological and cytological responses elicited through xenogeneic grafts derived from interspecific interactions and allogenic grafts stemming from conspecific interactions (Potts, 1976; Chadwick-Furman and Rinkevich, 1994; Rinkevich et al., 1994; Frank et al., 1997; Hidaka et al., 1997; Amar et al., 2008; Amar and Rinkevich, 2010; Puill-Stephan et al., 2012; for a review see Rinkevich, 2003). Overall, conflicting results and difficulties in standardizing such behavioral approaches have limited their application to establish species-specific trends, if any. Yet, the increasing availability of coral genomic resources may be on the way to revealing the molecular mechanisms behind responses to coral-coral interactions (Oren et al., 2010).

Karyological differences, particularly regarding chromosome numbers, were suggested as potential indicators of species due to their role as proxies of reproductive incompatibility (Lang, 1984; Willis, 1990; Knowlton, 1993). Still, most coral species assessed to date have displayed the same number of chromosomes (2n = 28; Heyward, 1985, 1987; Flot et al., 2006)

with the exception of some *Acropora* species, which unusual chromosome numbers were used to propose models of reticulate evolution through polyploidy and aneuploidy in this coral genus (Kenyon, 1997; Flot et al., 2006; Taguchi et al., 2014). More recently, detailed karyotypes using techniques as Giemsa staining (G-banding) and fluorescent in situ hybridization (FISH) have revealed more characteristics of coral chromosomes that can aid taxonomic comparisons in the future (see Taguchi et al., 2017b). Still, evaluation of cytogenetic features such as chromosomes may be hampered by their small size and the difficulties of obtaining mitotic cells required for karyotyping (Flot et al., 2006). Indeed, breeding crosses to obtain coral embryos for these assessments is restricted by the short time span in which corals spawn and precludes cytogenetic studies in coral species for which reproductive mode and/or season is still unknown. However, advancing molecular cytogenetic approaches may not only elucidate new features to differentiate coral species, but also promote the understanding of chromosome evolution in corals (Taguchi et al., 2017a, 2020; Vacarizas et al., 2021).

In contrast with the nearly invariable pattern of chromosome numbers, preliminary studies show a significant degree of intraspecific variation in coral genome sizes (Rañises, 2022). Paucity of data to assess intra- versus interspecific values hampers further comparative studies as only data for six scleractinian coral species has been reported in the Animal genome size database to date (Gregory et al., 2007; Gregory, 2022). Moreover, standardization issues and incongruence between outcomes obtained from different methodological approaches in other data rich taxa, highlight the overall uncertainty of genome size estimations (Doležel and Greilhuber, 2010; Pflug et al., 2020). Still, estimations of holoploid genome size (i.e., half of the total DNA content in a diploid somatic cell or C-value; Greilhuber et al., 2005) will likely benefit from growing amount of genomic data. Ultimately, interspecific genome size variation may provide valuable insights in evolutionary hypotheses and potentially into species boundaries as it has provided for taxa with extensive hybridization and taxonomic issues (Ekrt et al., 2010; Kabatova et al., 2014; Dąbrowska et al., 2015).

1.1.3 | The coral species delimitation conundrum: morphological and molecular evidence at odds

The widespread incongruence between morphologically and molecularly delineated species has been a pervasive conundrum in coral taxonomy (Huang et al., 2009). Closely related morphospecies, in particular, have been found interspersed in mitochondrial and nuclear gene trees, which has largely been interpreted as evidence for interspecific hybridization in corals (Odorico and Miller, 1997; Hatta et al., 1999; Medina et al., 1999; van Oppen et al., 2000, 2001, 2004; Márquez et al., 2002b). Hence, the notion that coral species frequently integrate “syngameons” (i.e., complexes of species that can interbreed; Veron, 1995) became widespread (Veron, 2011). Yet, observed nonmonophyletic patterns can result from alternative processes and issues. For instance, the incorrect identification of specimens can likely cause polyphyletic patterns in morphologically diverse groups with obscure taxonomy (Funk and Omland, 2003). Also, deep coalescence or the failure of alleles to sort after speciation can produce nonmonophyletic patterns, particularly in recently diverged groups or species with large effective population sizes (Maddison, 1997).

In corals, high rates of interspecific breeding *in vitro*, considerable overlap in spawning times and observation of intermediate morphotypes have further supported the hypothesis of porous species boundaries and reticulate evolution (Knowlton, 1993; Veron, 1995; Willis et al., 1997, 2006). Studies in a few systems have documented lab-reared coral hybrids surviving into adulthood and displaying reproductive compatibility for self-fertilization and backcrossing (e.g., Isomura et al., 2013, 2016; Fukami et al., 2019). Yet, other evidence challenges the role and incidence of hybridization in nature. For instance, the large variability in hybridization potential suggests that several elements are at play in natural conditions and that coral species may be more discrete than cross-fertilization experiments suggest (Willis et al., 2006; Kitanobo et al., 2016; Morita et al., 2019).

Notably, robust evidence of an F1 coral hybrid (a.k.a., ‘*Acropora prolifera*’) has revealed the significance of introgressive hybridization in the

evolution of the involved species for at least the past 120.000 years in the Caribbean (van Oppen et al., 2000; Vollmer and Palumbi, 2002; Miller and van Oppen, 2003; Palumbi et al., 2012; Kitchen et al., 2019; Modys et al., 2020). Indeed, permeable barriers to gene flow between the morphologically contrasting parental species *A. palmata* and *A. cervicornis* throughout their distribution ranges are consistent with the syngameon hypothesis (Veron, 1995; Fogarty, 2012; Fogarty et al., 2012; Nylander-Asplin et al., 2021). However, evidence of hybridization remains debatable in other marine provinces and coral study systems (Willis et al., 2006; Richards and Hobbs, 2015; Hobbs et al., 2022).

In the Indo-Pacific, for instance, nonmonophyletic tree patterns are often found in recently diverged groups such as the *Acropora* (~6 Ma; Fukami et al., 2000). However, no incontrovertible evidence supporting a clear hypothesis for such patterns has been found yet (Fukami, 2008; Richards and Hobbs, 2015). Studies have provided molecular evidence supporting the occurrence of hybridization and suggesting its role in the diversification and adaptation of the Indo-Pacific *Acropora* (Richards et al., 2008; Richards and van Oppen, 2012; Mao et al., 2018). Research also shows that although some *Acropora* species may hybridize *in vitro*, the frequency of hybridization in the field is likely much rarer than expected (Morita et al., 2006, 2019; Kitanobo et al., 2016). Hence, quandaries still surround one of the greatest taxonomical conundrums since Linnaeus.

1.1.4 | The genus *Acropora* as a case study of coral species delimitation

> *"Large, diverse, and abundant genera have the greatest potential to provide new systematic information and yet are the potential source of greatest inaccuracies"*
>
> (Wallace and Willis, 1994)

Acropora Oken, 1815 is the largest extant reef-building coral genus, with more than 400 nominal species widespread on shallow reefs throughout the tropical and subtropical oceans (Oken, 1815; Wallace and Willis, 1994). The *Acropora* genus is also one of the most taxonomically challenging groups of corals (Fig. 2), which is best highlighted by the fact that the latest genus revisions validated only a third of the nominal species (Wallace, 1999; Wallace et al., 2012). Since the establishment of modern coral reefs during the Pleistocene, the *Acropora* have remained ecological keystones in the ecosystem (Alvarez-Filip et al., 2013; Ortiz et al., 2021; Siqueira et al., 2022). Yet, *Acropora* species have been considerably affected by global warming and anthropogenic threats in the last decades (Marshall and Baird, 2000; Carpenter et al., 2008; Hughes et al., 2018b). Therefore, assessing species boundaries in this genus is crucial to understand coral reefs response to climate change.

Among scleractinian corals, the *Acropora* displays an unparalleled morphological diversity that stems from its particular dimorphic branching pattern (Wallace, 1999). Two cuplike structures with distinctive morphologies form the polyp skeleton (Fig. 2A): an axial corallite that outlines the branch and several budding radial corallites arranged around the axis. By themselves, the radial structures exhibit a variety of forms and can potentially become axial corallites budding into new branches (Wallace and Dale, 1978). Due to such corallite dimorphism, the *Acropora* presents high levels of colony integration, which can lead to incredibly diverse patterns in colony shape (Gladfelter, 1983; Soong and Lang, 1992; Wallace and Willis, 1994; Wallace, 1999; Baird and Marshall, 2002).

Remarkably, skeletal features that provide diagnostic species traits in other corals are absent or reduced in *Acropora* (Wallace and Dale, 1978). For instance, they lack macromorphological characters such as the dissepiments (i.e., thin plates of the skeleton beneath the polyps) and columella (i.e., vertical skeletal formation at the center of a corallite), which are found and used for descriptions in coral genera as *Porites* and *Pocillopora*, and families as the Faviidae (Wallace, 1999; Veron, 2000; Fukami et al., 2004b; Budd et al., 2012; Zhao et al., 2021). Due to such skeletal feature reduction, few diagnostic characters remain for morphology-based taxonomy in the *Acropora* (Wallace and Willis, 1994). Among them, qualitative diagnostic characters derived from the corallites have been known for providing greater resolution when compared to quantitative ones (Veron and Wallace, 1984; Wallace et al., 1991; Wolstenholme

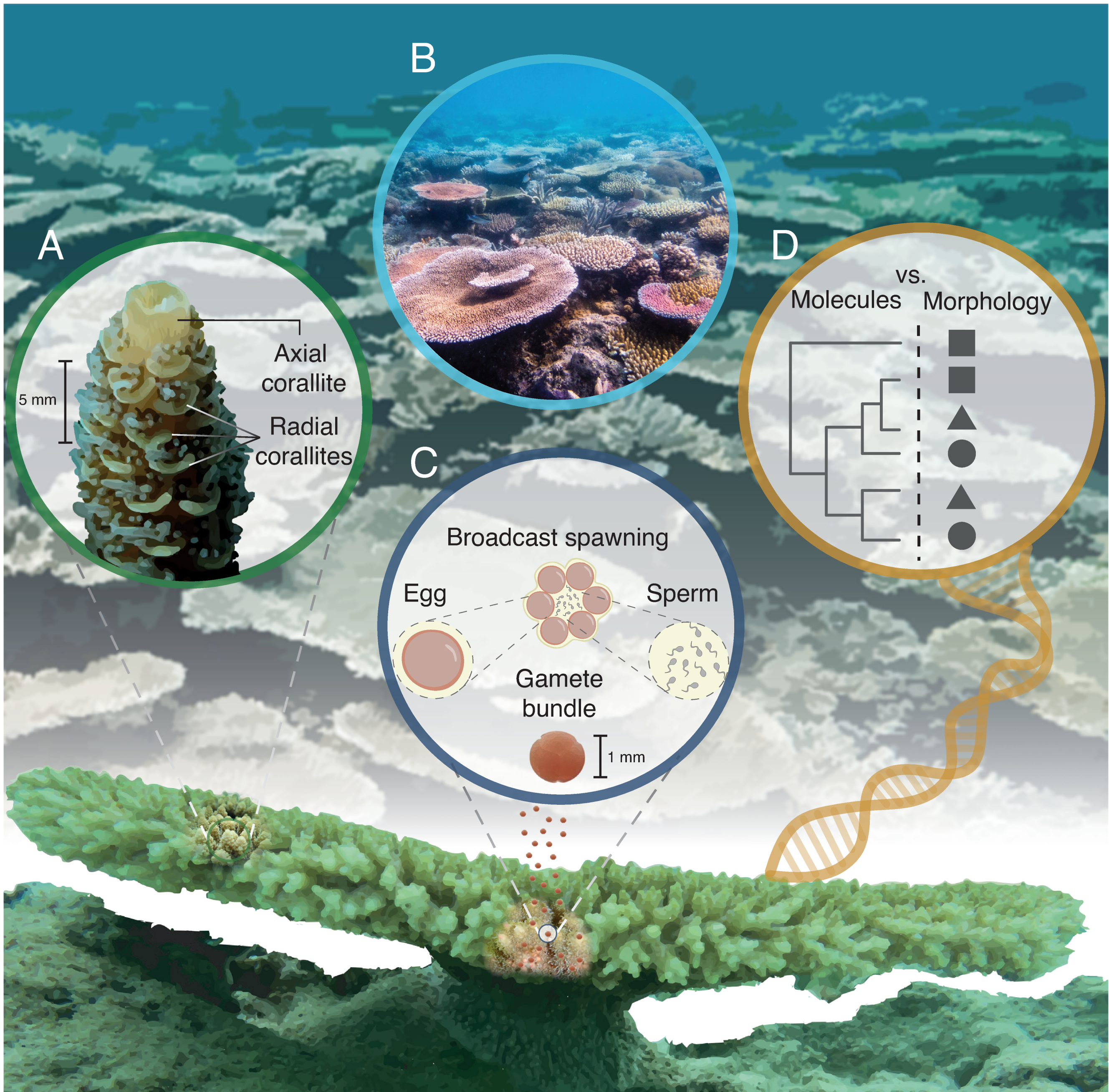


Figure 2 | *Acropora* corals as a study case for coral taxonomy

Graphical summary of four of the main features that make the *Acropora* coral genus an exceptional training ground to evaluate approaches to delineate coral species boundaries: (A) Corallite dimorphism has led to diverse patterns in colony shape, where high morphological intraspecific variability coupled with interspecific similarity has hampered morphospecies discrimination. (B) The occurrence of tens of morphospecies in sympatry enables comparative research to resolve obscure species boundaries. (C) Multiple species overlap in spawning time and potentially permeable cross-breeding barriers inferred from interspecific fertilization success. (D) Morphology-based species hypotheses are frequently found at odds with the results of mainstream molecular approaches. Illustrations and modified images by C. Ramírez-Portilla. Reef photograph by E. Gress at Lizard Island, Australia.

et al., 2003).

In addition to the conspicuous morphological features of the *Acropora*, the frequent occurrence of tens of its morphospecies in sympatry (Fig. 2B) and the substantial overlap in their spawning times (Fig. 2C) make this genus a compelling case study to evaluate the evidence that supports its current taxonomy in the light of molecular evidence (Harrison et al., 1984; Wallace, 1985; Willis et al., 1985; Babcock et al., 1986; Wallace and Willis, 1994). However, the lack of species-level molecular markers has made species delimitation particularly challenging in this genus

(Márquez et al., 2002a, 2003; van Oppen et al., 2002b; Chen et al., 2009; Cowman et al., 2020). For instance, *Acropora* morphospecies usually end up interspersed in mitochondrial and nuclear gene trees (Fig. 2D; (Odorico and Miller, 1997; van Oppen et al., 2001; Márquez et al., 2002b; Suzuki et al., 2016). Although there are alternative interpretations of such widespread nonmonophyly, incongruence between molecular and morphological groupings has reinforced the widespread assumption that interspecific hybridization might be responsible for this seeming conundrum (see section **1.1.3**; Odorico and Miller, 1997; Hatta et al., 1999; van Oppen et al., 2002a).

Tabular or table-like *Acropora* species (a.k.a., the *Acropora hyacinthus* species group) display several features that embody a taxonomic challenge and, thus, an exemplar case study reflecting the challenges that affect coral taxonomy as a whole: high intraspecific variability and interspecific similarity (Wallace, 1999), wide distribution range over the Indo-Pacific (Veron, 2000), potentially permeable cross-breeding barriers (Willis et al., 1997), relatively long larval lifespan in the water column (Nozawa and Okubo, 2011), high reef coverage (Denis et al., 2013), significant species synonymization in the latest taxonomic revisions (Wallace, 1999), likely recent diversification (~2.58 Ma; Wallace, 1999), and incongruence between molecular and morphological groupings that suggest they may be actually cryptic species connected by introgressive hybridization (Márquez et al., 2002b; Ladner and Palumbi, 2012; Suzuki et al., 2016; Sheets et al., 2018)

1.2 | Scope and aims

Improving coral taxonomy is crucial for performing accurate physiological, ecological, or population genetic studies of these keystone organisms and designing effective management and conservation plans in ecosystems populated by these marine invertebrates. Therefore, the main aim of this thesis was to test and refine the current morphology-based species boundaries in tabular *Acropora* corals, paving the way for a taxonomic revision of the genus and a methodology applicable to scleractinian corals in general. This was achieved by:

i | assessing the congruence between morphology (phenons), the fertilization success of cross-breeding trials (mayrons), and different molecular approaches for species delimitation (namely, distance-based, tree-based, and allele sharing-based), thereby providing independent assessments of species boundaries with *Acropora* from Okinawa, Japan (**Chapter II**);

ii | evaluating the applicability and discriminative power of 3D-morphological analyses to delineate phenons congruent with *a priori* delimited species and/or to discriminate *a posteriori* morphogroups among specimens of complex shaped and taxonomically intricate *Acropora* corals from Okinawa, Japan (**Chapter III**);

iii | outlining perspectives for assessing species boundaries in the light of the evidence collected using the *Acropora* as a case study to solve the coral species delimitation conundrum (**Chapter IV**).

1.3 | Publication outlines

Chapter II | Tabular *Acropora* corals as a case study of coral species delimitation

Systematic Biology OXFORD ACADEMIC

Ramírez-Portilla, C., Baird, A. H., Cowman, P. F., Quattrini, A. M., Harii, S., Sinniger, F., and Flot, J.-F. (2022a). Solving the coral species delimitation conundrum. *Syst. Biol.* 71, 461–475. https://doi.org/10.1093/sysbio/syab077.

The effective management and reliability of physiological, ecological, and evolutionary studies focused on threatened reef ecosystems depend on delineating accurately the coral species that integrate them. Still, traditional morphology-based hypotheses used to delineate coral species based on coral skeleton traits have not provided enough resolution at the species level. Indeed, they are frequently at odds with mainstream molecular approaches that hinge on criteria such as reciprocal monophyly and genetic distance to delineate species. Moreover, a lack of adequate molecular markers has made species delimitation particularly challenging in speciose coral

genera, leading to the widespread assumption that interspecific hybridization might be responsible for this apparent conundrum. Therefore in this paper, Ramírez-Portilla et al. (2022a) used three lines of evidence (i.e., morphology, breeding trials, and molecular approaches) to identify species boundaries in a group of sympatric tabular *Acropora* corals.

Although morphological plasticity, potential homoplasy, and cryptic diversity can confound morphology-based species delimitation, multivariate morphological analyses using both quantitative and qualitative characters (either from the coral skeletons or recorded at the field) enabled establishing three primary species hypotheses (PSHs). By comparison with the relevant type material, these three morphospecies, were tentatively identified as *A.* cf. *cytherea*, *A.* aff. *hyacinthus*, and *A.* cf. *bifurcata* using open nomenclature qualifiers according to the degree of uncertainty in each case. Consistent with how closely related these species are and the expected relatively short time since their divergence, tree- and distance-based molecular approaches were not able to recover groups congruent with these PSHs. In contrast, genetic clustering, allele sharing- and coalescent-based approaches were able to differentiate groups that matched both morphology and experimental crosses, regardless of using target-enrichment followed by genomic sequencing or traditional PCR amplification followed by Sanger sequencing.

The results from this paper, support tabular species of the genus *Acropora* as reproductively isolated and independently evolving units that can be also distinguished on a morphological basis. Comparison between molecular techniques allowed to delineate species both using traditional and HTS sequencing strategies by using approaches sensitive enough to detect divergence in light of potential incomplete lineage sorting between closely related species (**Aim ii**). Congruence between the molecular species delimitations, the fertilization success of breeding crosses, and the morphological classification was also established (**Aim iii**). In consequence, these findings outline a path to address species delimitation in a wide variety of organisms by comparing evidence from multiple independent sources, which in this particular study system improved confidence in an intricate case of coral species boundaries.

Personal contribution: CR-P helped conceiving the ideas behind the manuscript, performing experiments in the field, collecting data, performing the morphological analyses, completing the molecular analyses, writing, and revising the manuscript.

Chapter III | New approaches to long-standing challenges in coral species delimitation

Ramírez-Portilla, C., Bieger, I. M., Belleman, R. G., Wilke, T., Flot, J.-F., Baird, A. H., Harii, S., Sinniger, F., and Kaandorp, J. A. (2022b). Quantitative three-dimensional morphological analysis supports species discrimination in complex-shaped and taxonomically challenging corals. *Front. Mar. Sci.* 9, 955582. https://doi.org/10.3389/fmars.2022.955582

While morphological characters often play an important role in species descriptions, quantifying morphological traits and assessing their intra- and interspecific variation in complex-shaped organisms lacking characteristic features remains a problem. For such organisms, 3D imaging, model rendering, and informative variable selection might provide alternative measures to differentiate between morphogroups and potentially aid the delineation of species boundaries. Therefore, in this paper Ramírez-Portilla et al. (2022b) assessed the potential of 3D-based quantitative morphology for discriminating morphogroups of complex-shaped and taxonomically challenging organism.

Variables derived from triangulated polygon meshes and medial axis skeletons of the 3D models of coral specimens were extracted by Ramírez-Portilla et al. (2022b) from three closely related taxa previously delimited using other lines of evidence (namely, *A.* cf. *cytherea*, *A.* aff. *hyacinthus*, and *A.* cf. *bifurcata*; Ramírez-Portilla et al., 2022a). For quantifying overall shape, 3D-based variables assessing curvature, branching, and complexity were examined using univariate and multivariate analyses. The informative power of these 3D variables was then assessed to either delineate a priori and/or to discriminate a posteriori

coral morphospecies.

Although morphospace overlap hindered the non-guided delineation of species boundaries, results revealed significant interspecific differences in the means of a set of 3D-based variables. These results highlight potentially informative characters that provide sufficient resolution to discriminate morphogroups congruent with an independent species identification based on other lines of evidence (**Aim iii**). A linear combination of representative features, particularly curvature-related, yielded alternative measures that assisted in differentiating closely related species. This paper shows that a well-justified combination of 3D measures may aid species discrimination of complex-shaped organisms such as corals. Yet, variable screening and selection is determinant to achieving sufficient resolution for species boundaries validation. In the end, integrating informative morphological features with other independent lines of evidence will not only advance taxonomy but also our understanding of morphological variation in complex-shaped organisms.

Personal contribution: CR-P helped conceiving the ideas behind the manuscript, collecting and analyzing data, and leading the writing and the revision of the manuscript.

Additional co-authored publications

www.nature.com/scientificdata

SCIENTIFIC DATA

Baird, A. H., Guest, J. R., Edwards, A. J., Bauman, A. G., Bouwmeester, J., Mera, H., et al. (2021). An Indo-Pacific coral spawning database. *Sci. Data* 8, 35. doi:10.1038/s41597-020-00793-8.

In the late 80s, the discovery of multi-species mass spawning in scleractinian corals encouraged field-research efforts to document spawning times around the globe. Regrettably, most coral spawning data remain unpublished, thereby limiting our understanding of reproductive patterns at a local and global scale. To cover this knowledge gap, the publication by Baird et al. (2021) presents the Coral Spawning Database (CSD) initiative. The up-to-date status of this repository will provide open access to coral spawning data. Thus, in parallel with the advancement of coral reproductive biology research, integrative species delimitation approaches can leverage this information for future implementations (**Aim iv**).

Personal contribution: CR-P contributed unpublished data and commented on the manuscript.

Baird, A. H., Edwards, A. J., Guest, J. R., Harii, S., Hatta, M., Lachs, L., et al. (2022). A coral spawning calendar for Sesoko Station, Okinawa, Japan. Galaxea, *J. Coral Reef Stud.* 24, G2021_S10O. doi:10.3755/galaxea. G2021_S10O.

Observation and documentation of coral phenology and life histories are crucial to understand diversity evolution and how to preserve it in the face of climate change and anthropogenic disturbances. Indeed, developing efficient and informed management plans in coral reef ecosystems requires a grasp of coral reproductive biology and ecology. Moreover, documenting natural history is relevant to incorporate knowledge of coral biology and evolution into integrative approaches to delineate species boundaries (**Aim iv**). As such, informed species delimitation provides robust strategies for facing the main challenges of assessing species boundaries in intricate taxa. In this paper, Baird et al. (2022) present a spawning calendar for a coral research hot spot: the Tropical Biosphere Research Center (TBRC) of the University of the Ryukyus, based on Sesoko Island, Okinawa, Japan. For more than 50 years, this research station has provided the scenario for coral reef research. This publication summarizes coral reproductive observations of the last 30 years, including data on the spawning date for 87 species and the spawning time for 58 species. Additional work is needed to understand the patterns from all the species recorded around the station.

Personal contribution: CR-P contributed unpublished data and commented on the manuscript.

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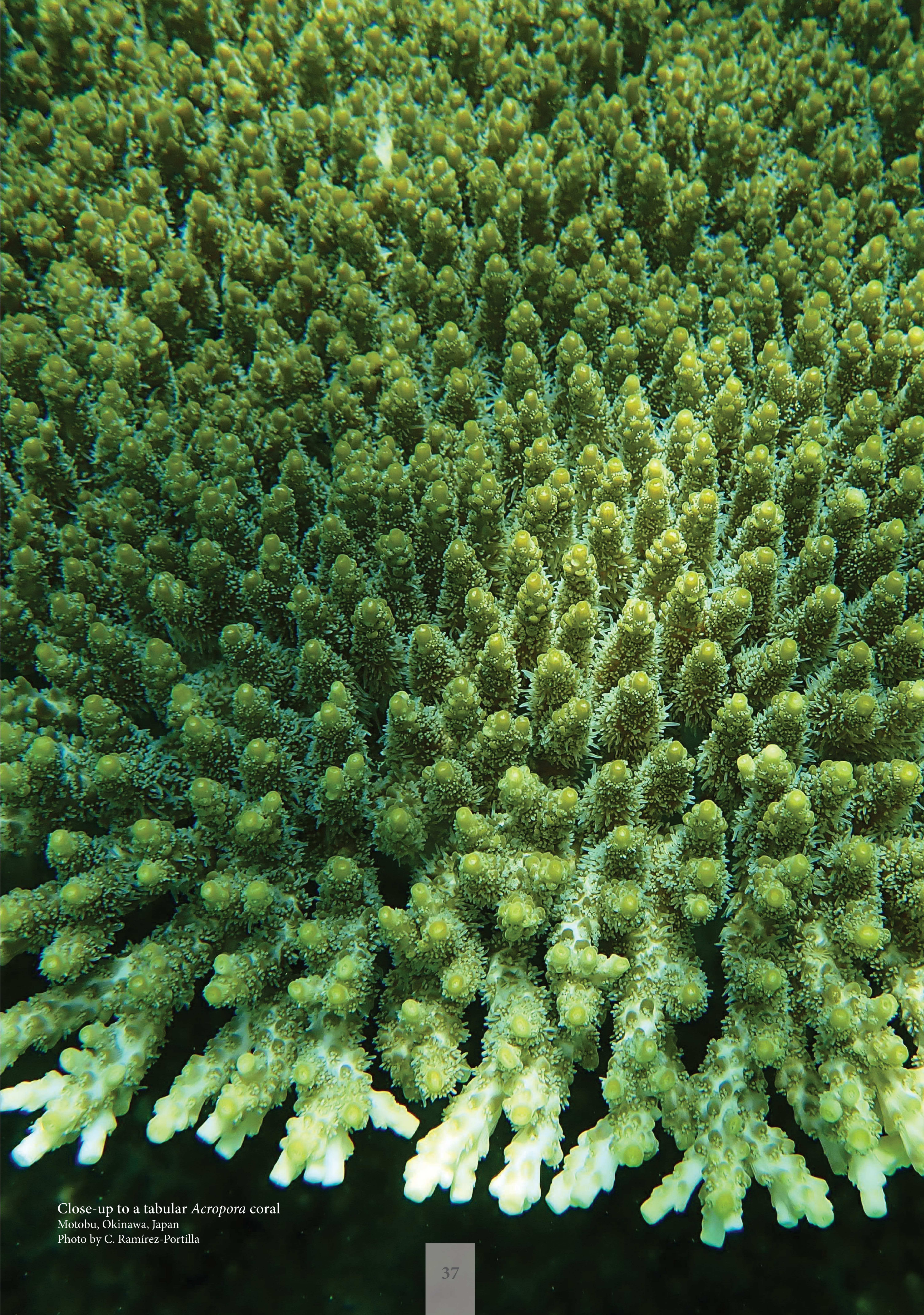
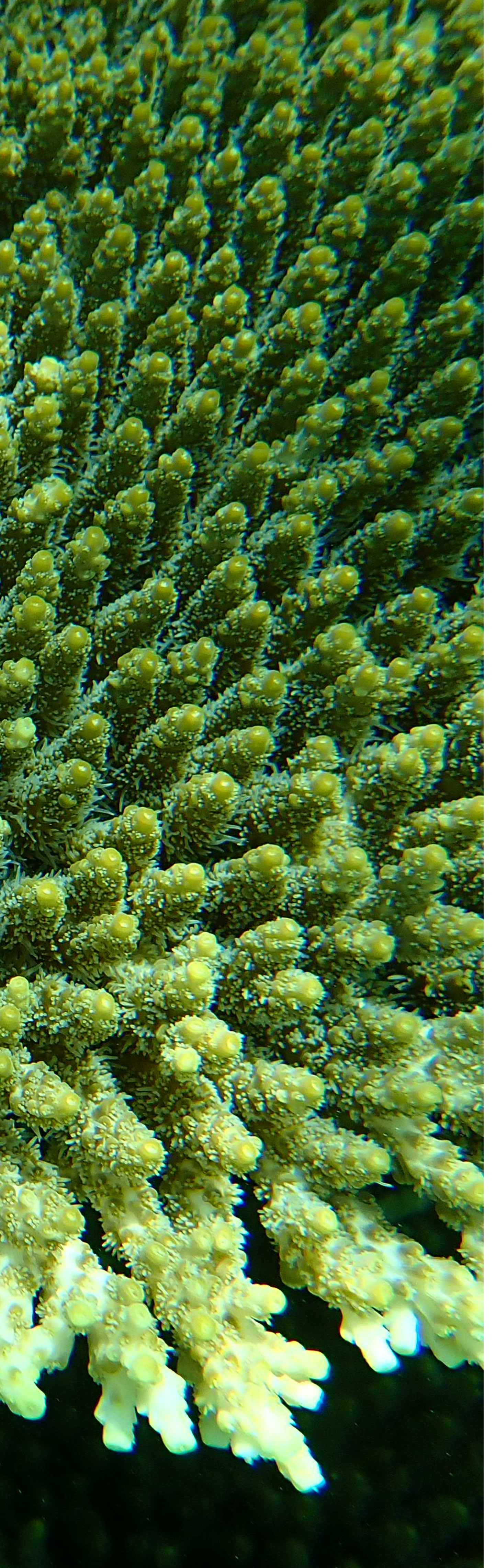


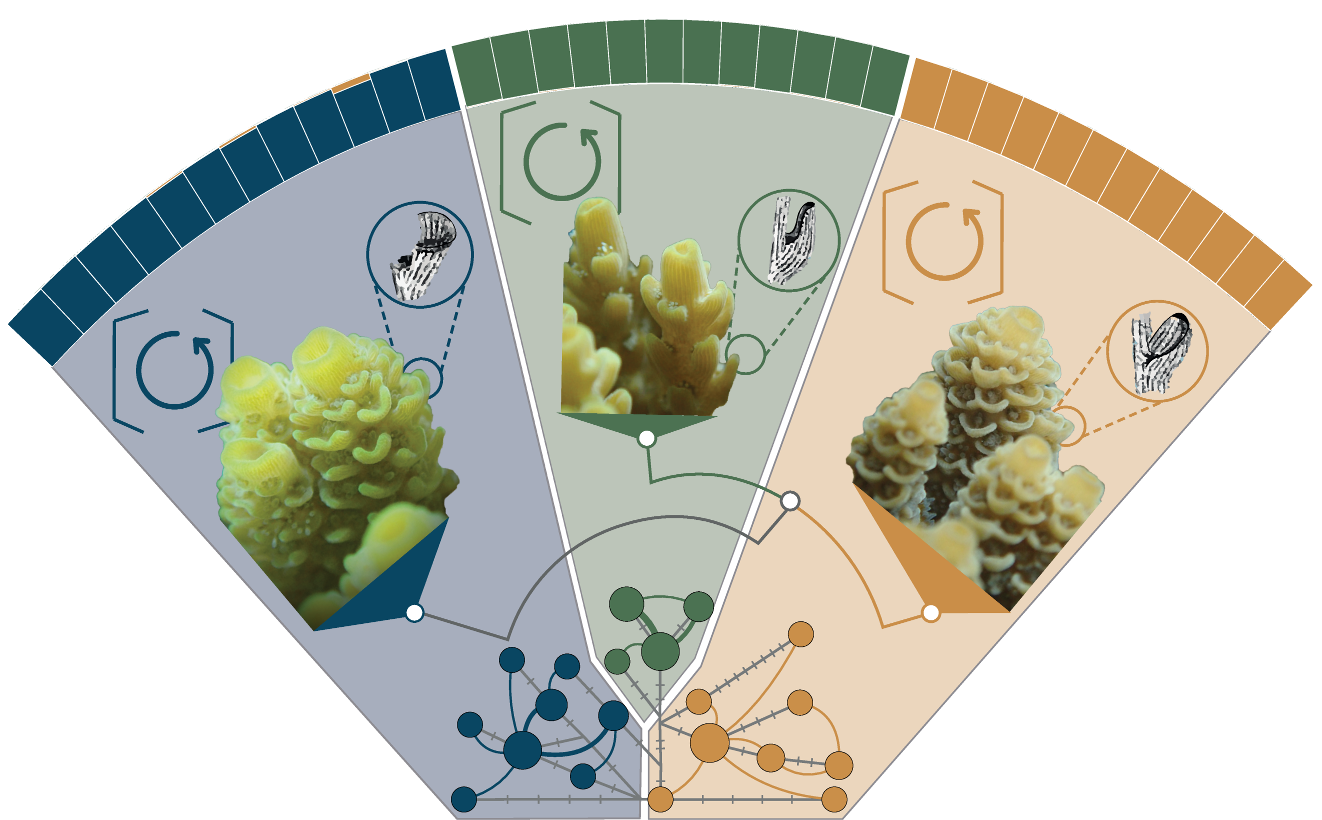
Close-up to a tabular *Acropora* coral
Motobu, Okinawa, Japan
Photo by C. Ramírez-Portilla



2 | Chapter II

Chapter II: The tabular *Acropora* as case study for coral species delimitation

Ramírez-Portilla, C., Baird, A. H., Cowman, P. F., Quattrini, A. M., Harii, S., Sinniger, F., and Flot, J.-F. (2022). Solving the coral species delimitation conundrum. *Syst. Biol.* 71, 461–475. https://doi.org/10.1093/sysbio/syab077.



Graphical abstract Chapter II | Comparing three lines of evidence —morphology, breeding trials, and molecular approaches— improves confidence in species boundaries among corals from the genus *Acropora*

Coral pictures and drawings from skeletal details represent morphology-based taxonomy and representative traits as radial corallite shape and distribution. Loop arrows surrounded by brackets represent that only crosses performed with gametes of individuals from the same morphospecies were significantly successful in breeding trials. Genetic clustering (e.g., STRUCTURE bar plots at the top), allele sharing-based approaches (e.g., haploweb at the bottom), and coalescent-based approaches (e.g., unrooted collapsed species tree in the middle) illustrate results obtained in molecular species delimitation. Congruence between the lines of evidence demonstrates that closely related *Acropora* species are reproductively isolated and independently evolving units that can be distinguished morphologically and that an integrative approach is key to develop an accurate taxonomy in corals.

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Solving the Coral Species Delimitation Conundrum

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Abstract.—Distinguishing coral species is not only crucial for physiological, ecological, and evolutionary studies but also to enable effective management of threatened reef ecosystems. However, traditional hypotheses that delineate coral species based on morphological traits from the coral skeleton are frequently at odds with tree-based molecular approaches. Additionally, a dearth of species-level molecular markers has made species delimitation particularly challenging in species-rich coral genera, leading to the widespread assumption that interspecific hybridization might be responsible for this apparent conundrum. Here, we used three lines of evidence—morphology, breeding trials, and molecular approaches—to identify species boundaries in a group of ecologically important tabular *Acropora* corals. In contrast to previous studies, our morphological analysis yielded groups that were congruent with experimental crosses as well as with coalescent-based and allele sharing-based multilocus approaches to species delimitation. Our results suggest that species of the genus *Acropora* are reproductively isolated and independently evolving units that can be distinguished morphologically. These findings not only pave the way for a taxonomic revision of coral species but also outline an approach that can provide a solid basis to address species delimitation and provide conservation support to a wide variety of keystone organisms. [*Acropora*; coral reefs; hybridization; reproductive isolation; taxonomy.]

A working coral taxonomy is crucial for meaningful physiological, ecological, and population genetic studies of these keystone organisms, as well as for the effective management and conservation of the ecosystems they support (Knowlton et al. 1992; Knowlton 2001). Even though climate and anthropogenic disturbances represent substantial threats to these ecosystems (Pandolfi et al. 2003; Carpenter et al. 2008; Hughes et al. 2017, 2018a), the taxonomy and systematics of some of the most vulnerable and diverse genera remain obscure (Fukami et al. 2004b; Richards et al. 2016). Such is the case for corals of the genus *Acropora*, the species of which are among those most affected by global warming (Marshall and Baird 2000; Carpenter et al. 2008; Hughes et al. 2018b). The genus is abundant on most reefs throughout the world's tropical and subtropical oceans and with more than 400 nominal species it is the most diverse extant reef-building coral genus (Wallace and Willis 1994; Wallace 1999). Consequently, ascertaining species boundaries in this genus will not only advance approaches to delineate species in corals but is also critical to understand the global response of coral reefs to climate change.

Distinguishing scleractinian coral species has always been a challenge, particularly in species-rich genera (Kitahara et al. 2016). The genus *Acropora* is emblematic of these difficulties: traditional morphological taxonomy has been mired in confusion, best highlighted by the fact that only 122 of approximately 400 nominal species were considered valid in the most recent revision of the genus (Wallace et al. 2012). Similarly, standard molecular approaches based on genetic distances or species-level monophyly have failed to delineate species. Indeed, closely related *Acropora* morphospecies usually turn out to be interspersed in mitochondrial and nuclear gene trees (Odorico and Miller 1997; van Oppen 2001; Márquez et al. 2002; Suzuki et al. 2016), which has been widely interpreted as evidence for ongoing hybridization between coral species (the "syngameon" concept; Veron 1995).

However, there are alternative interpretations of such widespread nonmonophyly (Wallace 1999; Funk and Omland 2003; Miller and van Oppen 2003). Polyphyletic patterns observed in gene trees can result from the incorrect identification of specimens, which is highly likely in morphologically diverse groups with an intricate taxonomy (Funk and Omland 2003). In addition, the failure of alleles to sort after speciation can produce nonmonophyletic species with intraspecific distances as large as or even larger than interspecific distances (Flot et al. 2010). Such incomplete lineage sorting is more likely in species groups that have recently diversified (e.g., *Acropora* ~6 Ma; Fukami et al. 2000), as well as in species with large effective population sizes. In such

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cases, single-locus species delimitation approaches that require monophyly are bound to fail (Dellicour and Flot 2018).

Tabular morphospecies of *Acropora* have several features that make them an intriguing group on which to propose and validate novel taxonomic approaches (Wallace and Willis 1994): a high overall morphological similarity (Wallace 1999); the occurrence of multiple morphospecies in sympatry (Wallace 1985); and a substantial time overlap in gamete release across multiple described morphospecies (Harrison et al. 1984). *Acropora hyacinthus* (Dana 1846) is considered the epitome of tabular morphospecies and is regarded as the senior synonym for eight other nominal species (e.g., *A. bifurcata* Nemenzo 1971, *A. conferta* (Quelch 1886), *A. pectinata* (Brook 1892), *A. surculosa* (Dana 1846); Wallace 1999; Veron 2000), whereas genetic analyses suggest that it is a complex of several species (Ladner and Palumbi 2012; Suzuki et al. 2016). In addition to incongruence between molecular markers and morphological groupings (Márquez et al. 2002; Suzuki et al. 2016), the possibility of permeable cross-breeding barriers between morphospecies (as documented in *A. hyacinthus* vs. *A. cytherea* (Dana 1846)) casts further doubts on current species boundaries (Willis et al. 1997) and makes this group an exemplar system reflecting the challenges that affect coral taxonomy as a whole.

Traditional coral taxonomy is based on features of the skeleton that can confound species delimitation due to morphological plasticity, potential homoplasy, and cryptic diversity (Fukami et al. 2004b; Budd et al. 2010). However, morphology provides baseline information to identify primary species hypotheses that can be subjected to further analyses (PSHs; Puillandre et al. 2012). In addition, if the evidence supports such groups as independently evolving lineages, morphological analyses can help single out characters that are taxonomically informative (Wolstenholme et al. 2003). In this study, we compared three independent lines of evidence (i.e., morphology, breeding trials, and molecular approaches) to delineate species boundaries and assess hybridization in three sympatric tabular *Acropora* (Supplementary Fig. S1 available on Dryad at https://doi.org/10.5061/dryad.k98sf7m5x), inhabiting the outer reef of Sesoko Island, Okinawa, Japan (Fig. 1a).

Materials and Methods

Colony Sampling

In the days preceding the full moon of May 2018 (29/05/2018), fragments (~8 cm × 8 cm) from living tabular colonies ($n=36$) of reproductively mature *Acropora* (min. diameter >20 cm) were collected from the outer reef (26° 37′44" N, 127° 51′44" E) located south of the Tropical Biosphere Research Center (TBRC) at Sesoko Island (Okinawa, Japan). The reproductive condition of the colonies was assessed by breaking branches to expose developing oocytes (Harrison et al. 1984; Baird and Marshall 2002), and avoiding peripheral areas of the colony and tips of branches, as they usually present no gametes (Wallace 1985). Tissue samples (~2 cm^3) from each colony were preserved in a guanidium thiocyanate solution (4M guanidine thiocyanate, 0.1% N-lauroylsarcosine sodium, 10mM Tris-HCl pH 8, 0.1 M 2-mercaptoethanol; Fukami et al. 2004a) and alternatively in 95% ethanol for subsequent DNA extraction.

Morphological Taxonomy Assessment

Each colony was photographed in the field using an Olympus Tough TG-5 waterproof compact digital camera at the time of fragment collection (Olympus, Japan). After spawning, the fragments were bleached with a commercial solution of ~3–6% sodium hypochlorite (NaOCl) for morphometric assessment and then stored as vouchers at the Sesoko Station (specimen photos available on Morphobank Project 4065 at http://morphobank.org/permalink/?P4065). In addition to the collected specimens, 38 tabular *Acropora* skeletons deposited in the station from other field campaigns (2015, 2019) were also used for morphometric assessment ($n=74$ colonies in total, Data set S1—Morphological data available on Dryad). Qualitative and quantitative characters adapted from previous studies were recorded and measured from the coral skeletons (Supplementary Table S1 available on Dryad, see Wallace 1999; Wolstenholme et al. 2003; Wallace et al. 2012).

In order to provide a quantitative evaluation of the morphological taxonomic units (morphospecies), multivariate analyses of descriptive (qualitative), and morphometric (quantitative) characters were performed in R v3.6.2 (R Core Team 2018) through the Rstudio console v1.2.5033 (RStudio Team 2017). Qualitative characters along with categorized quantitative variables (Supplementary Tables S1 and S2 available on Dryad) were analyzed using hierarchical clustering analysis (HCA) with simple match coefficient distances (nomclust package, v2.1.4) and the Ward clustering method (cluster package, v2.1.0, Fig. 1b). Quantitative variables with a normal distribution and homogeneity of variance (Supplementary Table S2 available on Dryad) were analyzed using linear discriminant analysis (LDA) with the maximum likelihood (ML) estimator method (MASS v 7.3-51.5 and flipMultivariates v1.0.0), and a multivariate analysis of variance (MANOVA, stats package v3.6.2) to test for significant differences (Supplementary Fig. S2 a available on Dryad). Finally, using the complete morphological data set, a factor analysis of mixed data (FAMD) was performed (FactoMineR, v2.3 and factoextra v1.0.7) to identify morphological groupings supported by all features and to determine how much each variable contributed to the differentiation (Fig. 1c and Supplementary Fig. S2 b available on Dryad). Morphospecies (groups) obtained from this morphological assessment were used as primary species hypotheses (PSHs; Puillandre et al. 2012).

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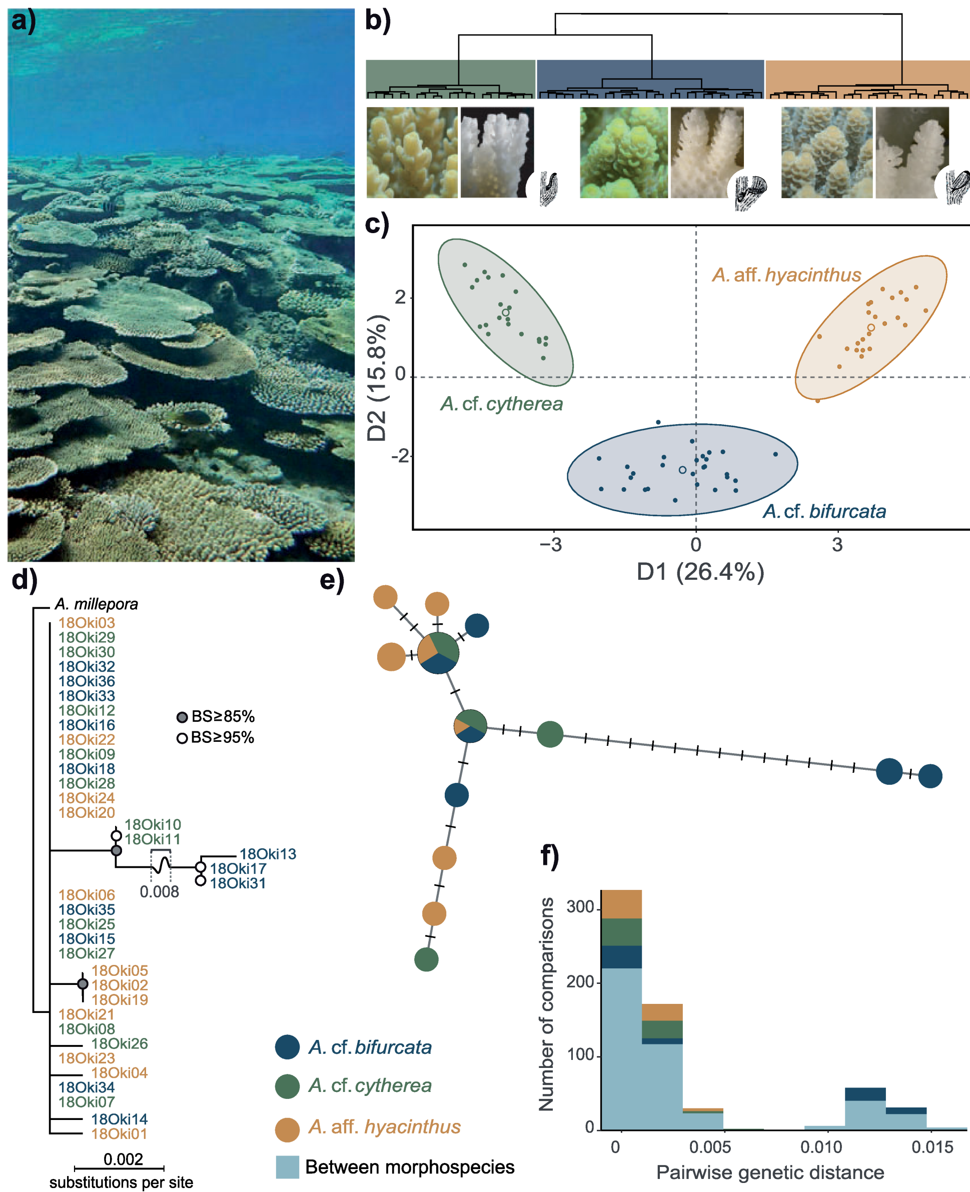

FIGURE 1. Morphology yields primary species hypotheses that are at odds with the mitochondrial phylogeny. a) Tabular *Acropora* at Sesoko Island outer reef (Okinawa, Japan); photo by A.H. Baird). b) Hierarchical clustering analysis (HCA, agglomerative coefficient = 0.95), along with the main morphological features that contributed to the differentiation for each morphospecies: color of colonies in the field (left) and shape and crowding of radial corallites along branches (right). c) Factor analysis of mixed data (FAMD) based on both qualitative and quantitative characters, distinguishing three morphospecies: *A.* cf. *bifurcata*, *A.* cf. *cytherea*, and *A.* aff. *hyacinthus*. See also Figure S2 and Table S1 available on Dryad for additional information. d) Maximum likelihood (ML) phylogeny of the mitochondrial control region (AcroCR) using ultrafast bootstrap. Branches with less than 85% of bootstrap support (BS) were collapsed. e) Haplotype network of the AcroCR region shaded according to morphospecies, with gaps recoded as single base changes. f) Histogram of the pairwise genetic distances of the AcroCR sequences within and between morphospecies.

Field Identification and Taxonomic Identity of the Morphospecies

Acropora bifurcata, *A. cytherea*, and *A. hyacinthus* were identified in the field following Veron (2000). The main field characters for each species are as follows; *A. hyacinthus* has tapered (gradually narrowing) branches with labellate (liplike) radial corallites with a flaring lip and colonies are orange-red; *A. cytherea*

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TABLE 1. A summary of the research into the taxonomic identity of the species used in the study

Nominal species, authority, accepted name (if different), type locality	Type material (ID) and current location	Type material vs. specimens in this study (ON qual)	Ongoing and future perspectives
Madrepora hyacinthus Dana 1846, *Acropora hyacinthus*, Fiji	Lectotype (USNM 246) designated by Wallace (1999), deposited at the NMNH, SI (Washington D.C., US)	Distinctive morphological differences between specimens and type material, particularly in branch shape and width ("aff.", affinity with a known species)	Ongoing molecular and morphological comparison to topotypes and other material from the Indo-Pacific
Madrepora cytherea Dana 1846, *Acropora cytherea*, Tahiti	Lectotype (USNM 423) designated by Wallace (1999), deposited at the NMNH, SI (Washington DC, USA)	Similar morphology, including that of the radial corallites ("cf.", specimens closely resemble type material, but this needs to be confirmed)	Ongoing morphological comparison to lectotypes and molecular comparison to other material from the Indo-Pacific
Acropora bifurcata Nemenzo 1971, Philippines	Holotype (UP C-1295), collected by Nemenzo (1971), deposited at UP, ZD (Quezon City, PH)	Geographical proximity to type location and similar morphology, including radial corallite shape ("cf.", specimens closely resemble type material, but this will require confirmation)	Ongoing molecular and morphological comparison to topotypes and other material from the Indo-Pacific

Catalog numbers from type material (ID) are depicted. Open nomenclature qualifiers (ON qual) were attributed according to the degree of uncertainty in identification following Sigovini et al. (2016) and Cowman et al. (2020): *affinis* (aff.) and *confer* (cf.). Refer to "Field Identification and Taxonomic Identity of the Morphospecies" in Materials and Methods for further information.
USNM = United States National Museum; NMNH = National Museum of Natural History; SI = Smithsonian Institution; UP = University of the Philippines; ZD = Zoology Department. Country codes: United States (US), Philippines (PH).

has terete (cylindrical) branches with labellate radial corallites with an extended outer lip and colonies are dark brown; *A. bifurcata* has terete branches with labellate radial corallites with a square lip and colonies are light brown (see images in Fig. 1b, color of colonies in the field and shape of radial corallites). Most local coral researchers would readily agree with the field identifications of *A. cytherea* and *A. hyacinthus*, however, *A. bifurcata* is not generally accepted as a valid species as it was considered a junior synonym of *A. hyacinthus* in the last major revision of the genus (Wallace 1999). In contrast, Veron (2000) accepted the species as valid but did not record it in Japan. However, field images in Nishihira and Veron (1995, see middle panel at p. 128) indicate that this species does occur in Japan but was identified by these authors as *A. hyacinthus*. Further information regarding type material and ongoing research into the taxonomic status of these species is presented below and summarized in Table 1.

Acropora hyacinthus (Dana 1846) has a type location in Fiji. A comparison of the colonies collected in this study to the lectotype designated by Wallace (1999, USNM 246; see http://n2t.net/ark:/65665/3fdf539df-6f98-4b91-a91c-53aa88a67457) indicates that there are significant differences in morphology. For example, the branches of the colonies at Sesoko are wider with more of a taper, which suggests that the species is distinct from that in Fiji. Consequently, the open nomenclature "aff." is used to indicate that the colonies found in Sesoko have affinities with *A. hyacinthus* but most likely belong to a distinct species.

Acropora cytherea (Dana 1846) has a type location in Tahiti. A comparison of the colonies from Japan to the lectotype designated by Wallace (1999; USNM 423, see http://n2t.net/ark:/65665/367cd18b6-2f69-4451-a32b-6ae18bacd0ab) suggests that the species is morphologically similar to *A. cytherea*. In particular, colonies of both species have labellate radial corallites with an extended lip. Here we use the open nomenclature qualifier "cf." to suggest that this species is possibly *A. cytherea* but further information is required to confirm this assessment.

Acropora bifurcata Nemenzo 1971 has a type location in the Philippines. Given the proximity of Okinawa to the Philippines and the morphological similarity of the colonies to the holotype (UP C-1295, see http://www.coenomap.org/fact-sheet/acropora-bifurcata/), notably the labellate radial corallites with a squared margin, we used the open nomenclature qualifier "cf." to suggest the species is probably *A. bifurcata* but further information is required to confirm this.

Breeding Compatibility Experiments

Half of the collected colonies ($n=18$) were kept in running seawater tanks and separated in individual buckets a few hours before the predicted time of spawning. Immediately after spawning, buoyant gamete bundles containing eggs and sperm were collected at the water surface of each container for the first two colonies that spawned from each morphospecies ($n=6$). Once the eggs and sperm were separated, eggs were collected and

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FIGURE 2. Cross-fertilization experiments suggest no hybridization potential. a) Gamete combinations (sperm × eggs) performed between representative colonies of each tabular *Acropora* morphospecies. b) Fertilization success (%) in each category of breeding trial, including the "eggs only" [top cells with bold border in a)] and the "self" fertilization controls [diagonal cells with bold font in a)] to account for sperm contamination and potential self-compatibility, respectively.

serially washed in 0.2 μm-filtered seawater to remove sperm and decrease the potential for self-fertilization. A portion of the eggs ("eggs only"—control) was kept aside in order to control for gamete separation and fertilization that may arise from leftover sperm in the eggs sample (Willis et al. 1997). The concentrated sperm obtained from the bundles was diluted approximately to 1:50 by adding filtered seawater before performing the crosses. In order to evaluate fertilization compatibility between the different morphospecies, approximately 100 washed eggs of each individual were added to each sperm dilution according to the breeding trial matrix (Fig. 2a, Data set S2—Breeding trials data available on Dryad).

Briefly, crosses were performed with gametes from 6 colonies for a total of $n=6$ eggs only controls and $n=36$ crosses: 6 self-control, 6 within morphospecies, and 24 between morphospecies, with at least two replicates for each combination. The numbers of regularly shaped embryos (prawn chip stage) and unfertilized eggs were counted under a stereomicroscope approximately 10 h after the breeding trials started. Mean fertilization success (%) was calculated as the average proportion of embryos divided by the number of embryos plus the remaining unfertilized eggs (Data set S2—Breeding trials data available on Dryad). Nonparametric Kruskal–Wallis rank sum test (stats package v3.6.2) was performed to test for significant differences in the mean proportion fertilized, and further posthoc tests (PMCMR v4.3 and PMCMRplus v1.4.4) were implemented in R (RStudio Team 2017; R Core Team 2018) to determine which particular crosses had significantly different fertilization success (Fig. 2b).

Preliminary Screening of Available Molecular Markers

To assess the species-level resolution of previously reported loci (Supplementary Table S3 available on Dryad), DNA was extracted from the 36 tissue samples preserved in guanidium thiocyanate solution using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) and following the manufacturer's protocol. DNA integrity

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was assessed on agarose gels (1%) and quality checked with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). We used the primers and protocols detailed in Supplementary Table S4 available on Dryad to perform PCR-based amplification then Sanger sequencing of the mitochondrial putative control region (AcroCR) and two nuclear exon-primed intron crossing (EPIC) markers (Ladner and Palumbi 2012): a plasma membrane calcium-transporting ATPase (PMCA) and a frizzled-4 like homolog (FZD or exon 5491). Due to the relatively short span of these markers (545 and 639 bp, respectively), we redesigned primers to extend the product length of the FZD marker. For this purpose, we mapped FZD sequences previously obtained for tabular *Acropora* (Ladner and Palumbi 2012), to the available genome assemblies (see Supplementary Table S5 available on Dryad) of *Acropora digitifera* (Shinzato et al. 2011), *Acropora millepora* (Ying et al. 2019), *A. hyacinthus* (ReFuGe 2020 Consortium 2015; Liew et al. 2016), *Acropora cervicornis* and *Acropora palmata* (Kitchen et al. 2019) using Bowtie2 v2.3.4.3 (Langmead and Salzberg 2012) in local configuration. The unambiguously mapped contigs of each genome were recovered and converted to BAM files using SAMtools v1.9 (Li et al. 2009), then transformed into BED formatted files with BEDtools v2.26.0 (Quinlan and Hall 2010). The mapped regions in the BED files were extended at least 200 bp upstream and downstream, to be then recovered from the contig FASTA files using Seqkt v1.3 (Li 2013). Alignment between the extended mapped regions and FZD original sequences was performed using Mafft (E-INS-i method; Katoh et al. 2008). The consensus sequence for FZD (including ambiguities) was obtained from the alignment using SeaView v4.6.4 (Gouy et al. 2010) and used as a target to design primers using Primer3web v4.1 (Untergasser et al. 2012), by maximizing product length and allowing for a difference of 2° C in melting temperature between primers.

Sanger sequencing of the products was performed at GenoScreen (Lille, France). Sequencher v5.4.6 (GeneCodes, USA) was used to edit the chromatograms (Data set 3—Chromatograms available on Dryad). Multiple sequence alignments for each locus were generated using the E-INS-i method (Katoh et al. 2008) in the online implementation of Mafft v7.471 (available at https://mafft.cbrc.jp/alignment/server/; Katoh et al. 2008). For the mitochondrial putative control region (AcroCR), alignments were used directly for the downstream analyses. For the sequences obtained of the EPIC markers, two different complementary phasing approaches were used. Sequences of heterozygous individuals displaying alleles of the same length (without indel), were phased using SeqPHASE (Steps 1 and 2 available at https://eeg-ebe.github.io/SeqPHASE/; Flot 2010) PHASE v2.1.1 (Stephens et al. 2001; Stephens and Donnelly 2003). When length-variant heterozygotes were found in the data set, Champuru v1.0 (Flot et al. 2006; Flot 2007) was used to phase those sequences in a first step. Subsequently, they were inputted as "known haplotype pairs" during SeqPHASE's step 1, thereby contributing to the phasing of the other individuals. Allele pairs with posterior probability $\geq$ 0.9 were chosen, except when more than one possible pair with similar posterior probabilities was found. In such cases, alleles shared with the highest number of individuals or that were connected with the most frequent haplotypes in the network were selected.

Model-based genetic clustering of the phased EPIC sequences was performed using STRUCTURE v2.3.4 (Pritchard et al. 2000), with admixture model, correlated allele frequencies, and no prior. Implementing StrAuto v1.0 (Chhatre and Emerson 2017), values from 1 to 10 for the inferred number of populations (K) were used (20 runs per K, 250,000 burnin, 1,000,000 MCMC generations) to compute in parallel the probabilities of membership of each individual. Runs were further aligned, combined and finally merged using CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) and the Pophelper package v2.3.0 in R (Supplementary Fig. S3 a,b available on Dryad; Spöri and Flot 2020; R Core Team 2018). Various species delimitation approaches were performed.

For the allele sharing-based approach (Flot et al. 2010), the EPIC markers phased sequences were input directly into the online program HaplowebMaker (available at https://eeg-ebe.github.io/HaplowebMaker/; Spöri and Flot 2020), from which haplowebs and the corresponding putative species or fields for recombination (FFRs; Doyle 1995) were obtained (Supplementary Fig. S3 c available on Dryad). For the distance-based approach, the best model of evolution was identified using the Bayesian information criterion (BIC) value criterion in ModelFinder (Kalyaanamoorthy et al. 2017). After converting the DNA alignments to bins using *fasta2DNAbin* (adegenet package v2.1.2), pairwise genetic distances using the closest available model to the best BIC score list were computed by *dist.dna* function (ape package v5.3) and histograms were plotted using ggplot2 v3.3.0.9 in R. Further phylogenetic analyses were performed under maximum likelihood (ML) with IQ-TREE v2.0.3 (Nguyen et al. 2015), using 1000 ultrafast bootstrap replicates (-B 1000) and an additional step to optimize trees by nearest-neighbor interchange (-bnni; Minh et al. 2013). Branches of the consensus trees with nodes with less than 85% of bootstrap support were collapsed using *multi2di* function (ape package v5.3). Trees (Supplementary Fig. S3 e available on Dryad) were visualized and formatted using FigTree v1.4.4 (Rambaut 2018). Genomic regions of *A. millepora* (Ying et al. 2019) that mapped to each nuclear loci with Bowtie2 v2.3.4.3 (–local; Langmead and Salzberg 2012), were used as outgroup for the phylogenies. For AcroCR, the closest *A. millepora* match found using megaBLAST (against the nr/nt database, available at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch; Altschul et al. 1990) with GenBank accession number KY408102.1 was used for that purpose instead (100% query coverage, 99.85% identity and E-value=0).

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Overall, the mitochondrial AcroCR (Fig. 1d–f) and the nuclear EPIC markers (Supplementary Fig. S3 available on Dryad) from the literature did not provide enough resolution at the species level. Model-based genetic clustering of the two EPIC markers was only able to recover two clusters (Supplementary Fig. S3 a available on Dryad), neither congruent with the primary species hypotheses (PSHs) inferred from morphological species delimitation (Fig. 1b,c), nor with the fertilization success in breeding trials (Fig. 2b). Similarly, haplowebs obtained from these markers (Supplementary Fig. S3 c available on Dryad) were not able to resolve them. Each morphospecies showed some private alleles but shared alleles connected individuals from different morphospecies into single fields for recombination (FFRs). Pairwise genetic distances and gene trees did not recover groups congruent with the other lines of evidence either (Supplementary Fig. S3 d,e available on Dryad). For these reasons, we explored target enrichment followed by high-throughput sequencing to assess more accurately the species boundaries in this case study and to target for loci with enough resolution at the species level that could be amplified in a larger data set.

Target-Enrichment Using the Scleractinian Bait Set

To find molecular markers that provide better resolution at the species level (Supplementary Table S3 available on Dryad), we performed target-capture sequencing for nine of the samples ($n=9$, three from each morphospecies) preserved in 95% ethanol (Supplementary Table S6 available on Dryad). DNA was extracted, its quality assessed and then sent to Arbor Biosciences (Ann Arbor, USA) for library preparation (following Quattrini et al. 2018) and target capture sequencing (detailed in Cowman et al. 2020). For target enrichment of conserved elements (derived from exonic loci and ultraconserved elements (UCEs); Faircloth et al. 2012), we implemented a new set of baits (Cowman et al. 2020), that was redesigned from a set that originally targeted anthozoans (Quattrini et al. 2018). The new bait set targets hexacorallians (hexacoral-v2 bait set, scleractinian subset—2,476 target loci) and has been successfully tested in a comprehensive sample of acroporids (Cowman et al. 2020). Demultiplexing, trimming, and assembly were performed according to the parameters and software previously tested in *Acropora* (Cowman et al. 2020). Subsequently, the contigs assembled for the nine tabular samples (Supplementary Table S4 available on Dryad) were matched to the baits employing PHYLUCE (Faircloth 2016) with default parameters (*phyluce_assembly_match_contigs_to_probes*). As a result, 2060 loci (1026 exons and 1034 UCEs) were extracted into FASTA (*phyluce_assembly _get_match_counts* & *phyluce_assembly_get_fastas_from_match_counts*) to proceed with allele phasing using two different pipelines, described in the following sections (see Supplementary Table S3 available on Dryad for a summary).

Genetic Clustering and Preliminary Species Trees Using the Target-Enrichment Data Set

First, to generate a broad subset of loci that could be used to evaluate genetic clustering and estimate a preliminary species tree, loci were aligned (*phyluce_align_seqcap_align –incomplete -matrix –no-trim –aligner mafft*) and globally trimmed using Gblocks (Castresana 2000; Talavera and Castresana 2007) with default parameters (*phyluce_align_get_gblocks_trimmed_alignments_from_untrimmed*). Phasing of the aligned loci was performed following the phase_everyone v0.1 or "Laninsky" pipeline (Baca et al. 2017; Alexander 2018a). Once alleles were obtained, they were aligned and processed following Steps 5–8 from the pipeline reference_ aligning_to_established_loci v0.0.3 (Baca et al. 2017; Alexander 2018b). Then, single nucleotide polymorphisms (SNPs) were pulled out from each locus and filtered to ensure that only loci with data for at least one individual per morphospecies were included in the downstream analyses. SNPs for the resulting 1889 loci (1022 exons and 867 UCEs) were used to perform a STRUCTURE analysis (Pritchard et al. 2000) parallelized through StrAuto (Chhatre and Emerson 2017), with K values from 1 to 9, admixture model, correlated allele frequencies and no prior (20 runs per K, 250,000 burnin, 1,000,000 MCMC generations). CLUMPP (Jakobsson and Rosenberg 2007) and pophelper package tutorial (available at http://www.royfrancis.com/pophelper/articles) were used to align, combine and merge the runs. Evanno ΔK plots (Evanno et al. 2005) were used to determine the most likely number of clusters (K, Fig. 3a). Using ggplot2 v3.3.0.9 in R, the corresponding bar plots depicting the probability of individual membership to each cluster were obtained for the suggested K values ($K=3$ or $K=5$), from which K=3 depicted better stratification of the samples according to their allele frequencies and suggested that there does not seem to be population structure within the putative species (Fig. 3b).

In addition, the most likely species tree was estimated with SNAPP v1.5.1 (Bryant et al. 2012) through the CIPRES gateway (Miller et al. 2010). SNPs were extracted from the concatenated FASTA of a subset of 210 loci present in all the samples (128 UCEs and 82 exons) using *fasta2DNAbin* (adegenet package v2.1.2) and storing them in a Nexus file using the *write.nexus.data* function (ape package v5.3). This file was used to create the XML input file in the Bayesian Evolutionary Analysis Utility (BEAUti) v2.6.3 (Bouckaert et al. 2014, 2019). Five independent runs of BEAST were performed with MCMC length of 10,000,000, preburnin of 100,000, sampling frequency of 1000, and default model parameters. Output trees and log files were combined using LogCombiner v2.6.3 (Bouckaert et al. 2014, 2019). After 10% burnin, combined logs were input into Tracer v1.7.1 (Rambaut et al. 2018) to check MCMC convergence and effective sample sizes (ESS) $>$ 200. TreeAnnotator v2.6.2 (Bouckaert et al. 2014, 2019) was used to generate maximum clade credibility trees and DensiTree v2.2.7

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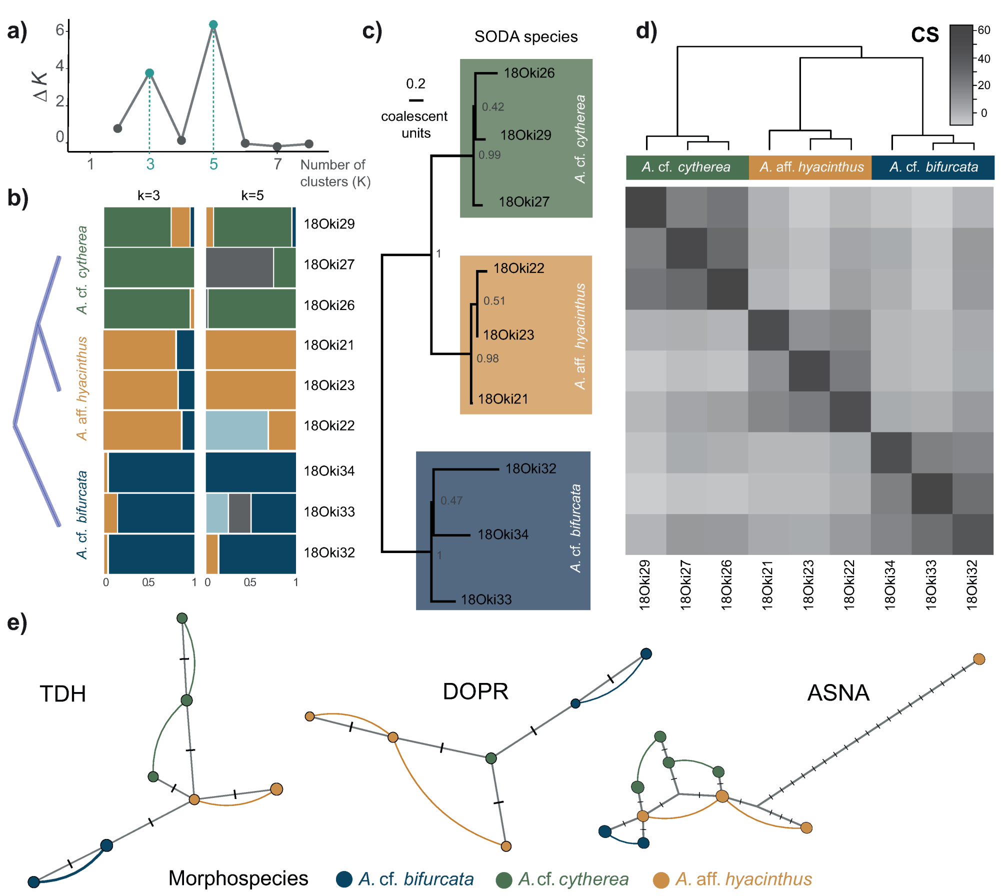

FIGURE 3. Screening of target capture-derived markers. a) Evanno ΔK plot depicting two possible optimal cluster (K) values (dashed lines) for STRUCTURE analyses. b) Bar plots displaying the individual probability of membership assigned using model-based clustering for each K value (1889 loci). The most frequent SNAPP tree (using 210 loci present in all samples) is depicted on the left side of the plots. c) ASTRAL resolved extended species tree with phased sequences according to the molecular species delineated by SODA, where alleles were mapped to individuals and nodes with less than 10% of local posterior probability (LPP) or low branch support were collapsed. d) Conspecificity score (CS) matrix for a subset of 79 target-enrichment sequenced loci used to perform a preliminary allele sharing-based species delimitation. e) Haplowebs of three loci displaying putative species delimitation under mutual allelic exclusivity criterion, congruent with model-based genetic clustering, species trees and the primary species hypotheses (PSHs) based on morphology and breeding trials.

(Bouckaert 2010) to plot the corresponding consensus tree (Fig. 3b, left).

Estimation of a Resolved Extended Species Tree Using the Target-Enrichment Data Set

Loci were first aligned and edge trimmed (*phyluce_align_seqcap_align* –taxa 9 –incomplete-matrix) using PHYLUCE (Faircloth 2016). Subsequently, following the phasing tutorial (available at https://phyluce.readthedocs.io/en/latest/tutorial-two.html; Andermann et al. 2019), loci were phased into alleles for each individual. Allelic sequences were aligned (*phyluce_align_seqcap_align* –no-trim –ambiguous –incomplete-matrix) and globally trimmed (*phyluce_align_get_gblocks_trimmed_alignments_from_ untrimmed*). To remove sequences with unphased bases (N) that could cause problems in downstream analyses, loci alignments were further screened and filtered (*phyluce_align_screen_alignments_for_problems*). The resulting subset of 79 loci (TC79loci hereafter) was used to perform species delimitation from the data available for the target-enriched samples. To achieve this, we estimated a resolved extended species tree using the frequency of the quartet topologies of the individual gene trees build from the phased loci alignments. However, instead of mapping individuals to species as in an extended species tree (Rabiee et al. 2019), the resulting guide tree was obtained by mapping alleles to individuals.

Similar to the preliminary screening, IQ-TREE (Nguyen et al. 2015) was implemented to obtain individual ML trees from the phased FASTA alignments

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obtained from the TC79loci data set. Those trees were used as input to run ASTRAL-III v5.7.3 (Zhang et al. 2018; Rabiee et al. 2019) and to estimate a resolved extended species tree following the ASTRAL tutorial (available at https://github.com/smirarab/ASTRAL/blob/master/astral-tutorial.md#running-astral). After pruning branches with low support or local posterior probability (LPP) < 10% (Junier and Zdobnov 2010), the gene trees were used to generate a resolved extended species tree without constraining each morphospecies to be monophyletic and incorporating a mapping file that assigned each allele sequence to an individual (-a option, Fig. 3c). Moreover, we used the same data set to perform multilocus species delimitation using quartet frequencies implementing Species bOundary Delimitation using Astral (SODA) v1.0.1 (Rabiee and Mirarab 2020) with the default alpha (α) threshold of 0.05 (Fig. 3c, right).

Screening for Loci with Species-Level Resolution in the Target-Enrichment Data Set

To screen for markers providing resolution at species-level, we used the mutual allelic exclusivity criterion to define species boundaries. This criterion is always met before or at the same time as reciprocal monophyly; thereby it provides a more sensitive criterion to delineate species (Flot et al. 2010). Consequently, allele sharing-based species delimitation was performed on the TC79loci data set using both haplowebs and the corresponding conspecificity matrix (Debortoli et al. 2016) obtained using the online programs HaplowebMaker and CoMa (Spöri and Flot 2020). There, a conspecificity score (CS) was calculated for each pair of individuals by subtracting the number of markers/loci that do not support them being conspecific (H, different species or heterospecific) from the number of markers/loci for which they are considered conspecific (C, same species or partition) [CS = C-H]. According to these scores, the matrix was then clustered and plotted (Fig. 3d) using the R package heatmap3 v1.1.7 with the Ward agglomeration method from the *hclust* function (stats package v3.6.2). Loci with at least one individual per genetic cluster (as identified in STRUCTURE) were kept, and their corresponding haplowebs were individually explored to assess their congruence with the conspecificity matrix, and the primary species hypotheses (PSHs) inferred from the morphological assessment and supported by the breeding trials. We selected loci with haplowebs depicting partitions (FFRs) congruent with the PSHs, and that provided resolution (genetic clusters containing different PSHs did not lump in the same FFR) even when gaps were considered as missing data. From this reduced subset, three loci with different variability degrees were chosen as candidate regions to develop markers at species level (2 exons and 1 UCE loci, Fig. 3e). GenBank megaBLAST (Altschul et al. 1990) searches were implemented (nr/nt database) to find the closest annotated match for each locus and code them accordingly (TDH, DOPR, and ASNA, see Supplementary Table S7 available on Dryad).

Developing Target-Enrichment Derived Markers for Larger Data Sets

To delineate species boundaries without resorting to high-throughput techniques, we used an identical approach to that previously employed to extend the length of the EPIC markers (see primer redesign performed in the section *Preliminary Screening of Available Molecular Markers* and Supplementary Table S5 available on Dryad). From the DNA extracted of the 36 tissue samples of *Acropora* preserved in the guanidium thiocyanate solution (Fukami et al. 2004a), PCR-based amplification followed by Sanger sequencing of the three target-enrichment derived loci was performed (Supplementary Tables S3 and S4 available on Dryad). Sequences obtained from GenoScreen (Lille, France) were processed and phased as for the preliminary screened EPIC markers (PMCA and FZD).

In a first step, genetic clustering, potential population structure, and admixture within the sympatric putative species was assessed using model-based genetic clustering for the derived target-capture markers (TDH, DOPR, and ASNA). The corresponding ΔK plot and the bar plots to evaluate individual probability membership were performed using K = 1 – 10 and the same parameters as before (Fig. 4a,b). Additionally, to detect clusters based on genetic similarity and without relying in evolution models, a discriminant analysis of principal components (DAPC; Jombart et al. 2010) was completed using the package adegenet v2.1.2 in R (following Quattrini et al. 2019; Fig. 4c,d).

Molecular Delineation of Species Boundaries Using the Target-Enrichment Derived Markers

Sampling pattern, speciation rate, species richness, mutation rate, and effective population size tend to exert widely different effects and biases onto species delimitation methods (Dellicour and Flot 2018). To overcome these issues, we performed different approaches to delineate species boundaries in this tabular *Acropora* study case. Distributions of pairwise genetic distances were first evaluated in search of a barcode gap (see Supplementary Fig. S4 available on Dryad, right). As such, distance-based approaches might not work for recently diverged species, on which intraspecific distances may not be substantially smaller than interspecific ones. Consequently, we also used haplowebs and their corresponding conspecificity matrices to delineate species under the mutual allelic exclusivity criterion (Fig. 4e, Supplementary Table S8 available on Dryad). Instead of taking into account the genetic distances, such allele sharing-based approaches aggregate individuals based on the haplotypes they share, providing a more sensitive criterion to delineate closely related species (Flot et al. 2010).

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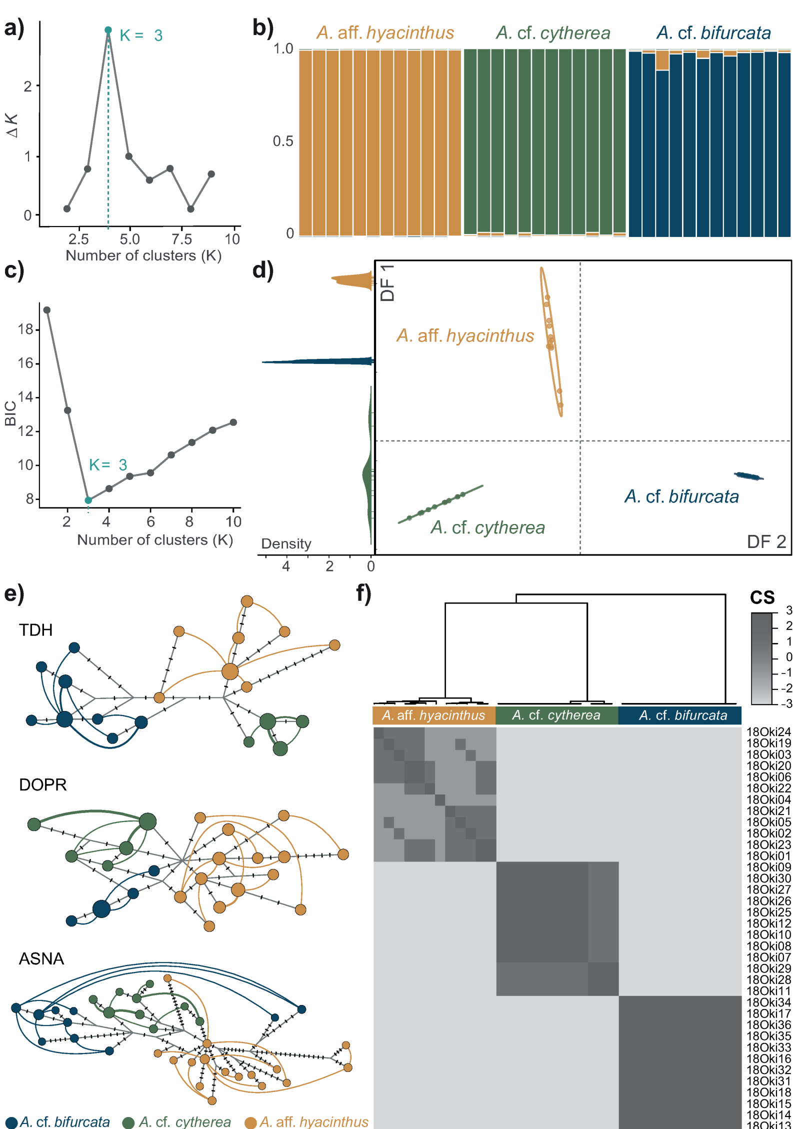

FIGURE 4. Molecular evidence from three target capture-derived loci supports the primary species hypotheses in *Acropora* corals. a) Evanno ΔK plot highlighting the most likely number of genetic clusters. b) Bayesian model-based genetic structure plot depicting the probability of individual membership to each cluster when $K = 3$. c) Optimal cluster number for the Discriminant analysis of principal components (DAPC) according to the Bayesian Information Criterion (BIC) statistic with the most likely K value highlighted ($K = 3$). d) DAPC scatterplot depicting clustering based on genetic similarity among the individuals using two discriminant functions (DF). e) Haplowebs delineating putative species based on the co-occurrence of alleles for each one of the nuclear markers defined from target-capture sequencing, coded according to morphospecies. f) Conspecificity score (CS) matrix summarizing the fields for recombination (FFRs) found using the allele sharing-based approach to delineate species with the three target-capture defived loci. The conspecific groups delineated by the FFRs of the three markers are congruent with the morphospecies and with the results from breeding trials (see Figs. 1 and 2).

To evaluate species boundaries under the reciprocal monophyly criterion, maximum likelihood phylogenies of individual (left in Supplementary Fig. S4 available on Dryad) and concatenated genes (Supplementary Fig. S5 a available on Dryad) were performed on the target-capture derived loci as described for the preliminary screening of available molecular markers. Additionally, the CIPRES gateway (Miller et al. 2010) was used to perform SNAPP and estimate the posterior distribution of trees from the SNPs extracted from the three loci. Independent runs of BEAST v2.6.3 (Bouckaert et al. 2014, 2019) were performed with MCMC length of

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10,000,000, preburnin of 100,000, sampling frequency of 1000, and default model parameters. After 10% burnin, the output trees and log files were combined and examined for MCMC convergence. A cloudogram depicting the most frequently recovered species trees with individuals as terminal tips (Supplementary Fig. S5 b available on Dryad) was generated from this analysis. In addition, individual ML trees obtained from the phylogenetic analyses performed with IQ-TREE, were used in a resolved extended species tree estimation using ASTRAL on the three loci, both when constraining each morphospecies to be monophyletic (top left inset in Supplementary Fig. S5 c available on Dryad), and without such constraint (main resolved extended species tree in Supplementary Fig. S7 c available on Dryad). In both cases, alleles were mapped to individuals to obtain the final tree (using ASTRAL's -a option). Due to the small number of loci, species delimitation with SODA was not performed on this data set.

To test for alternative species models a SNAPP coalescence-based analysis was performed (Supplementary Table S9 available on Dryad). The alternative models tested were: 1) a single species-model that includes individuals from the three morphospecies, in one complex; 2) the two species-model supported by the current taxonomy in which *A. hyacinthus* and *A. cytherea* are considered different species but *A. bifurcata* is a synonym of the former; and 3) a three species-model (*A. hyacinthus*, *A. cytherea*, and *A. bifurcata*), supported by the morphological and breeding trial approaches from this study. Five runs of SNAPP were performed using BEAST, with 48 path sampling steps, 100,000 MCMC and 10,000 of preburnin (following Herrera and Shank 2016; Quattrini et al. 2019). Finally, ranking of the models was performed using Bayes factor delimitation (BFD; Grummer et al. 2014; Leaché et al. 2014) by comparing the marginal likelihood estimates (MLE) obtained for each model by calculating the Bayes factor (BF; Kass and Raftery 1995) between the current taxonomy model (model 1, i.e., two accepted species) and the alternative species models (model x) , as suggested in the tutorial (BF= 2 * [model 1 - model x]; Leaché and Bouckaert 2018).

Additionally, a joint Bayesian analysis of species delimitation and species tree estimation was performed using Bayesian Phylogenetics and Phylogeography v4.2 (BPP; Yang 2015). We performed the A11-type analysis (Flouri et al. 2020), using Phylip alignments for each target-capture derived loci obtained with the *fas2phy* function of the R package chopper v0.1.8. BPP was run for 200,000 generations, with a burnin of 20,000, and a sample frequency of 1 (following McFadden et al. 2017). Comparison of replicate runs performed with each rjMCM algorithm, different starting tree topologies and initial seeds was performed to assess overall convergence. The influence of prior distributions of the ancestral population size (θ) and root age (τ_0), was evaluated under three scenarios (similar to Leaché and Fujita 2010): 1) large ancestral population size and deep divergence, 2) small ancestral population size and shallow divergence, and 3) large ancestral population size and shallow divergence among species (Supplementary Table S10 available on Dryad).

Results and Discussion

Morphology Yields Primary Species Hypotheses

We first examined our collected specimens for a series of morphological characters (Tables S1, S2 and Data set S1 available on Dryad; Wallace 1999; Wolstenholme et al. 2003; Wallace et al. 2012). Multivariate analysis clearly distinguished three morphospecies (Fig. 1b,c and Supplementary Fig. S2a available on Dryad; $n=74$, $P \leq 0.001$), tentatively identified by comparison with the relevant type material as *A.* cf. *cytherea*, *A.* aff. *hyacinthus* and *A.* cf. *bifurcata* (see Table 1, Wallace 1999; Veron 2000; Wallace et al. 2012). The main features that contributed to the discrimination achieved by this analysis were the color of the colonies in the field, the shape, and extent of crowding of the radial corallites (Fig. 1b) and the median length and width of the branches (Supplementary Fig. S2b available on Dryad).

Mitochondrial Marker Analyses are at Odds with Morphology

As in previous studies of the genus *Acropora* (van Oppen 2001; Márquez et al. 2002), neither maximum-likelihood phylogeny (Fig. 1d) nor pairwise genetic distances (Fig. 1e) obtained from the mitochondrial putative control region (AcroCR) recovered groups congruent with the morphological analyses. Instead, specimens from the three morphospecies were scattered throughout the tree, a pattern that may result from incorrect identification of the colonies (caused for instance by morphological stasis or by phenotypic plasticity), incomplete lineage sorting, or hybridization (Funk and Omland 2003).

Due to considerable overlap in the time of spawning among *Acropora* species (Harrison et al. 1984; Baird et al. 2009) and their high rates of interspecific breeding in vitro (Willis et al. 1997), hybridization has often been evoked as the most likely cause for the lack of species-level monophyly in this genus (Miller and van Oppen 2003; Ying et al. 2019). However, in groups with relatively recent diversification and significant population size, such as the *A. hyacinthus* species group (~2.58 Ma; Wallace 1999), shared ancestral polymorphisms caused by large expected coalescent time should be considered as an alternative explanation. Distinguishing among these competing hypotheses requires several independent markers, which is impossible using only mitochondrial sequences (Sang and Zhong 2000).

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Cross-fertilization Experiments Suggest no Hybridization Potential

In such a situation, breeding trials not only supply an important layer of biologically relevant information for delimiting sympatric species but also provide a litmus test to assess hybridization potential based on *in vitro* fertilization success (Wallace and Willis 1994). Consequently, we evaluated mating compatibility by performing cross-fertilization experiments using representative colonies from each of the three morphospecies (Fig. 2a). Significant fertilization success only occurred in crosses performed within morphospecies (Kruskal–Wallis *chi-squared* $=23.26$, $df=3$, $P=$ 3.565e-05), whereas all the other crosses resulted in almost no fertilization (Fig. 2b). The reproductively isolated groups delineated using this approach comprised only individuals of the same morphospecies, thereby supporting the boundaries inferred from morphology.

Molecular Evidence Supports the Primary Species Hypotheses

Since breeding compatibility experiments can only be performed between colonies that reproduce synchronously or within a few hours of difference (Willis et al. 1997), we extended the scope of the cross-fertilization trials by looking at patterns of genetic clustering and allele sharing, i.e. using genetic similarity and mutual allelic exclusivity as indirect evidence for reproductive isolation (Supplementary Table S3 available on Dryad). Molecular approaches stemming from high-throughput techniques have recently overcome long-standing methodological limitations of molecular studies such as the small number of markers available and lack of species-level resolution (Cowman et al. 2020; Erickson et al. 2021). Here, three individuals per morphospecies ($n=9$) were analyzed applying an enrichment procedure designed to capture conserved elements (derived from UCEs and exonic loci) with a set of baits targeting hexacorals (Quattrini et al. 2018; Cowman et al. 2020). Using this approach, more than two thousand phased loci were recovered (1026 exons and 1034 UCEs, Supplementary Table S6 available on Dryad).

Model-based genetic clustering using STRUCTURE (Fig. 3a,b), as well as an ASTRAL resolved extended species tree (Fig. 3c) of subsets of these loci (1889 and 79 loci, respectively), identified groups that were consistent with both morphology and breeding trials. To verify this across a larger number of specimens, we screened the captured loci for candidate markers displaying allelic exclusivity for each cluster (79 loci, Fig. 3d). As a result, three nuclear loci—L-threonine 3-dehydrogenase (TDH), dopamine receptor 2 (DOPR), and ATPase ASNA-1 (ASNA) (Fig. 3e)—were selected for PCR-based amplification of the 36 individuals in the tabular *Acropora* data set followed by various molecular species delimitation approaches (Supplementary Tables S3, S4, and S7 available on Dryad).

Genetic clustering of the specimens ($n=36$) differentiated three groups that were congruent with both morphospecies hypotheses and breeding compatibility results (Fig. 4a–d). As previously observed with the mitochondrial control region, the pairwise genetic distances between and within morphospecies overlapped for each marker (Supplementary Fig. S4 available on Dryad, right), and neither the individual gene phylogenies (Supplementary Fig. S4 available on Dryad, left), a concatenated tree (Supplementary Fig. S5 a available on Dryad) nor a cloudogram (Supplementary Fig. S5 b available on Dryad) inferred from these loci supported the reciprocal monophyly of the three species.

By contrast, each of the three species was recovered as monophyletic in the resolved extended species tree obtained using ASTRAL (Supplementary Fig. S5 c available on Dryad), albeit with uncertain topology and low support for some clades. In addition, the haplowebs inferred from these three loci (Fig. 4e) and the conspecificity matrix summarizing them (Fig. 4f and Supplementary Table S8 available on Dryad) all unequivocally supported the grouping of our samples into three reproductively isolated units. Similarly, coalescence-based (Supplementary Table S9 available on Dryad) and Bayesian species delimitation analyses (Supplementary Table S10 available on Dryad) supported the three-species model with decisive values (Bayes factor >10 and posterior probability >0.95, respectively).

These results challenge the generally accepted idea that morphospecies of *Acropora* cannot be distinguished using molecular approaches because of hybridization. On the contrary, despite being closely related these species appear to be reproductively isolated. It was possible to delineate them using target-enrichment followed by genomic sequencing (which probes thousands of markers but can yield incomplete data matrices) as well as using traditional PCR amplification followed by Sanger sequencing (which targets only one marker/individual at a time but yields high-quality, complete data sets). Hence, our results are different from other examples of successful molecular species delimitation based exclusively on high-throughput genomic sequencing (Quattrini et al. 2019; Erickson et al. 2021).

Conclusions

By using approaches sensitive enough to detect divergence at both the morphological and molecular levels, congruence between the three lines of evidence (i.e., morphology, breeding trials, and molecular approaches) demonstrates that it is possible to develop a robust coral taxonomy, thus helping to solve one of the greatest taxonomical conundrums since Linnaeus (Kitahara et al. 2016). Comparing evidence from multiple independent sources improved confidence in coral species boundaries by illustrating that *Acropora* species, once considered a taxonomic nightmare, are actually reproductively isolated and independently evolving units that can be distinguished morphologically.

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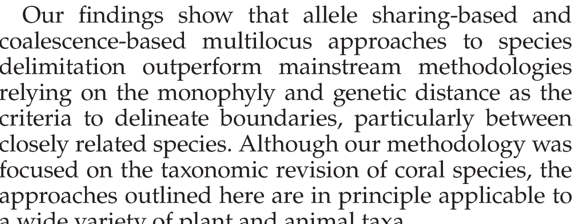

Our findings show that allele sharing-based and coalescence-based multilocus approaches to species delimitation outperform mainstream methodologies relying on the monophyly and genetic distance as the criteria to delineate boundaries, particularly between closely related species. Although our methodology was focused on the taxonomic revision of coral species, the approaches outlined here are in principle applicable to a wide variety of plant and animal taxa.

Supplementary Material

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.k98sf7m5x

The main sequence data sets generated for this study have been placed in GenBank and SRA repositories (see Supplementary Tables S4 and S5 available in Dryad). All photographical records of the specimens used for this study have been deposited in MorphoBank (Project 4065, http://morphobank.org/permalink/?P4065). Alignments, trees and examples of scripts and commands used can be found in the GitHub repository (https://github.com/catalinarp/SpeciesDelimitationTabularAcropora).

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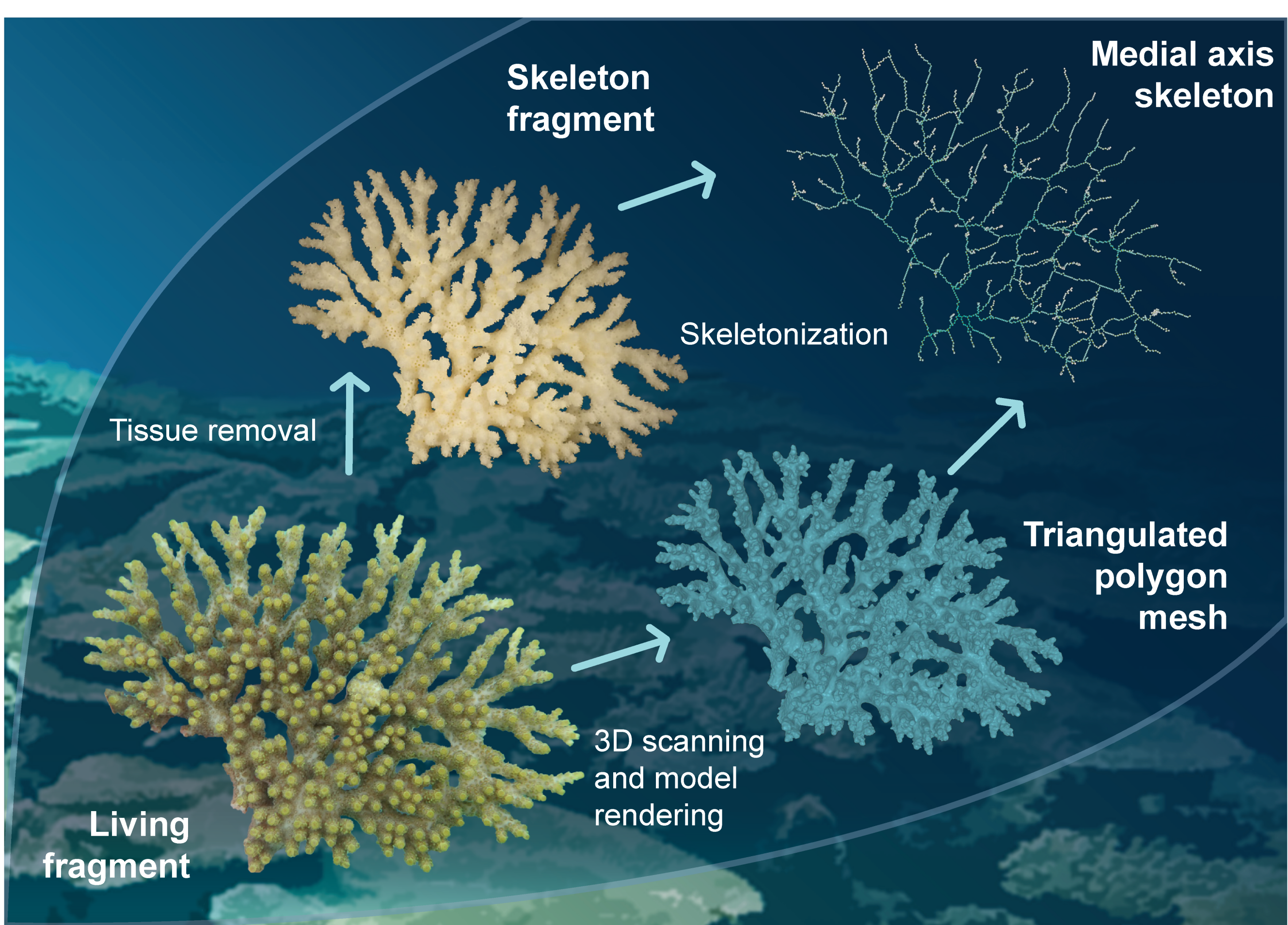
Cape Hedo
Kunigami, Okinawa, Japan
Photo by C. Ramírez-Portilla

3 | Chapter III

Chapter III| New approaches to long-standing challenges in coral species delimitation

Ramírez-Portilla, C., Bieger, I. M., Belleman, R. G., Wilke, T., Flot, J.-F., Baird, A. H., Harii, S., Sinniger, F., and Kaandorp, J. A. (2022). Quantitative three-dimensional morphological analysis supports species discrimination in complex-shaped and taxonomically challenging corals. *Front. Mar. Sci.* 9, 955582. https://doi.org/10.3389/fmars.2022.955582



Graphical abstract Chapter III | Potential of 3D-based quantitative morphology to delineate a priori and/or to discriminate a posteriori morphogroups of complex-shaped and taxonomically challenging organisms

Quantifying morphological traits and delineating phenotypically distinc groups remains challenging in complex-shaped organisms lacking distinctive features. In this study, we harnessed the potential of 3D-morphological analyses to assess their applicability in delineating morphogroups among specimens of complex-shaped and taxonomically intricate organisms such as corals. For this purpose, we extracted a set of variables derived from triangulated polygon meshes and medial axis skeletons of the 3D models. Univariate and multivariate analyses of 3D-based variables quantifying overall shape including curvature, branching, and complexity were conducted, along with informative variable screening and selection.

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Quantitative three-dimensional morphological analysis supports species discrimination in complex-shaped and taxonomically challenging corals

Catalina Ramírez-Portilla[1,2*], Inge M. Bieger[3], Robert G. Belleman[3], Thomas Wilke[1], Jean-François Flot[2,4], Andrew H. Baird[5], Saki Harii[6], Frederic Sinniger[6] and Jaap A. Kaandorp[3*]

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Morphological characters play an important role in species descriptions and are essential for a better understanding of the function, evolution and plasticity of an organism's shape. However, in complex-shaped organisms lacking characteristic features that can be used as landmarks, quantifying morphological traits, assessing their intra- and interspecific variation, and subsequently delineating phenotypically distinct groups continue to be problematic. For such organisms, three-dimensional morphological analysis might be a promising approach to differentiate morphogroups and potentially aid the delineation of species boundaries, though identifying informative features remains a challenge. Here, we assessed the potential of 3D-based quantitative morphology to delineate *a priori* and/or to discriminate *a posteriori* morphogroups of complex-shaped and taxonomically challenging organisms, such as corals from the morphologically diverse genus *Acropora*. Using three closely related coral taxa previously delimited using other lines of evidence, we extracted a set of variables derived from triangulated polygon meshes and medial axis skeletons of the 3D models. From the resulting data set, univariate and multivariate analyses of 3D-based variables quantifying overall shape including curvature, branching, and complexity were conducted. Finally, informative feature selection was performed to assess the discriminative power of the selected variables. Results revealed significant interspecific differences in the means of a set of 3D-based variables, highlighting potentially informative characters that provide sufficient resolution to discriminate morphogroups congruent with independent species

identification based on other lines of evidence. A combination of representative features, remarkably represented by curvature, yielded measures that assisted in differentiating closely related species despite the overall morphospaces overlap. This study shows that a well-justified combination of 3D-based variables can aid species discrimination in complex-shaped organisms such as corals and that feature screening and selection is useful for achieving sufficient resolution to validate species boundaries. Yet, the significant discriminative power displayed by curvature-related variables and their potential link to functional significance need to be explored further. Integrating informative morphological features with other independent lines of evidence appears therefore a promising way to advance not only taxonomy but also our understanding of morphological variation in complex-shaped organisms.

KEYWORDS

species delimitation, quantitative morphology, phenotypic variation, 3D scanning, skeletonization algorithms, feature selection, surface curvature

1 Introduction

The morphological diversity encompassed by the tree of life displays an extraordinary range of forms and shapes. Beyond contributing to characterize the biodiversity of these otherwise "endless forms" (Darwin, 1859), assessing their variation spectrum is key to gaining a better understanding of shape function and evolution (Klingenberg, 2010). Indeed, delimiting groups of individuals based on their morphological resemblance (morphogroups), or more broadly on their phenotypic distinctiveness (phena; sensu Mayr, 1969), has been traditionally the first step in taxonomic approaches and also often a preliminary step for sorting specimens in ecological, physiological, and evolutionary studies (MacLeod, 2002; Pereira et al., 2021). As such, morphology is the tie that connects the samples used for a variety of contemporary approaches, the designated type specimens used for species description, and the placement of extant species in relation to extinct life forms (Budd and Olsson, 2006; Schlick-Steiner et al., 2007; Saraswati and Srinivasan, 2016). Thus, morphological assessments are crucial to disentangle the confused and sometimes obscure categorisation of the diversity of life forms that inhabit the planet (Wheeler, 2005).

Phena, as designated taxonomic units or morphospecies, do not necessarily correspond with taxonomic categories delimited using other criteria (e.g., reciprocal monophyly, reproductive isolation, Mayr, 1969; Dubois, 2011). Indeed, finding out whether two phena are an instance of intraspecific polymorphism (e.g., sexual dimorphism, developmental stages, morphological plasticity) or correspond to two distinct species requires extra information and cannot be deduced from morphological analysis alone (Dayrat, 2005). Besides, the coupling of intraspecific variability and interspecific similarity can hamper the use of morphological features in taxonomically intricate taxa (Sites and Marshall, 2004). Yet, phena can provide primary species hypotheses (PSHs) that can be subjected to validation under a variety of scenarios (e.g., Puillandre et al., 2012).

Different phenetic approaches have been proposed to delineate *a priori* phena as groups of individuals characterized by intra-group diversity lower than inter-group differences (Sokal, 1986; Jensen, 2009). Such quantitative morphological analyses rely on obtaining a set of comparable measurements from all investigated specimens, which is particularly challenging in the case of complex-shaped organisms (Konglerd et al., 2017). Although traditional morphometric approaches excel at quantifying differences across a wide range of forms in the tree of life (e.g., Cardini, 2003; Migicovsky et al., 2018; Chaplin et al., 2020), they struggle to capture and describe complex geometric structures that are highly variable and lack homologous landmarks or distinctive outlines (Kaandorp, 1999; Kaandorp and Kübler, 2001; Konglerd et al., 2017).

The contrast between the morphological diversity of marine invertebrates and the shortage of informative morphological characters exemplifies many of the challenges faced by morphology-aided categorization in complex-shaped organisms (Filatov et al., 2013; Fontaneto et al., 2015). For instance, morphological plasticity in response to environmental factors

such as water flow and light availability in corals can lead to large intraspecific differences in the shape of colonies, hindering unambiguous morphogroups differentiation (Miller, 1994; Todd et al., 2004; Todd, 2008; Paz-García et al., 2015b). Moreover, traditional morphological traits used to delineate coral phena are frequently at odds with molecular analyses (e.g., Forsman et al., 2009; Flot et al., 2011; Keshavmurthy et al., 2013; Erickson et al., 2021), which is particularly evident in species groups with low interspecific morphological differences (e.g., sibling or cryptic species) as well as between recently diverged species (Knowlton, 1993).

In the last decades, substantial progress in three-dimensional (3D) imaging has made it possible to document form and structure of complex-shaped organisms, revolutionizing the way morphological data is collected and analysed (Ziegler et al., 2010; Laforsch et al., 2012). While in the past this was done by hand or extracting data from two-dimensional photos and illustrations, high-throughput techniques such as magnetic resonance imaging (MRI), computed tomography (CT) scanning, structured light scanning, and photogrammetry have made it possible to capture morphology in digital and 3D data sets (e.g., Bythell et al., 2001; Faulwetter et al., 2013; Sigl et al., 2013; Reichert et al., 2016). Alternative descriptors of 3D shape and complexity, such as fractal dimension and alpha shapes, have emerged as potential approaches for quantifying morphology in complex-shaped organisms and structures (Martin-Garin et al., 2007; Reichert et al., 2016; Gardiner et al., 2018; Klinkenbuß et al., 2020; Orbach et al., 2021). Yet, previous frameworks to extract meaningful characters in the absence of identifiable landmarks and characterize phena in complex modular organisms have either gauged only a few variables from 3D-morphological data (e.g., Gutierrez-Heredia et al., 2016; Reichert et al., 2017) or been restricted to two-dimensional analyses (e.g., Reeb et al., 2018). However, in most cases, geometrical complex shapes such as corals can be only represented adequately in three dimensions (Kaandorp and Kübler, 2001; Courtney et al., 2007). Thus, the main objective of this study was to assess the applicability of 3D-morphological analyses to delineate phena among specimens of complex-shaped and taxonomically intricate organisms. For this purpose, specimens from three morphologically similar and closely-related species of *Acropora* corals, robustly delimited using independent evidence (Ramírez-Portilla et al., 2022), were used as a case study. Here, we specifically aimed to:

1. evaluate 3D features and perform variable selection for a prospective combination of representative characters that support morphogroups discrimination;
2. test whether the morphogroups delineated using 3D-based variables are congruent with species boundaries assessed using other sources of information; and
3. test whether the 3D-morphological analyses enable discrimination between *a priori* delimited species.

2 Materials and methods

2.1 Experimental design and data set

We assessed the power of 3D quantitative morphology to discriminate morphogroups using skeleton specimens of three closely related tabular *Acropora* species previously delineated using different lines of evidence (i.e., morphology, breeding trials, and molecular analyses): *A.* cf. *bifurcata* (n = 28), *A.* cf. *cytherea* (n = 21) and *A.* aff. *hyacinthus* (n = 25), hereafter species A, B and C respectively (for further information and comparison to type material see Table 1 at Ramírez-Portilla et al., 2022). Briefly, morphospecies were identified in the field following Veron (2000), particularly using the branch taper (either gradually narrowing or cylindrical) and the radial corallites shape (all labellate either with round, straight or flaring lips, see zoom in branches in Figure 1 in this paper and Figure 33 in Wallace, 1999). Subsequently, multivariate morphological analyses of qualitative and quantitative variables, cross-fertilization experiments, and molecular analyses using target capture and Sanger sequencing were used to identify species boundaries in the data set. For the 3D morphology assessment in this study, we documented a total of 74 skeleton fragments deposited as vouchers at the Sesoko Station, Tropical Biosphere Research Center (TBRC); collected in 2015, 2018, and 2019 from the outer reef south of Sesoko Island (26.6288 North, 127.8622 East, Okinawa, Japan). Documented specimens corresponded to medium-size fragments (min. area 8x8 cm) collected from adult colonies with similar sizes (Supplementary Table S1, photos available in Morphobank Project 4065, http://morphobank.org/permalink/?P4065).

2.2 Data acquisition, model rendering, and processing

The 3D scanning of the coral fragments was performed using a handheld Artec 3D Space Spider Scanner coupled with the software Artec Studio v10 (Artec 3D, Luxembourg). For a preliminary assessment of the 3D model quality, scanning was completed using the real-time fusion mode for all fragments (Supplementary Figure S1). Following Reichert et al. (2016; 2017), the Artec Studio software was used to render and clean up the 3D models for which fusion was performed with 0.2 mm resolution (Supplementary Materials and Methods). Meshes were then exported as triangulated mesh files (either.stl or.obj; available in Morphobank http://morphobank.org/permalink/?P4205) for downstream analyses derived either from triangulated polygon meshes, medial axis skeleton graphs, or a combination of both (Figure 1 and Supplementary Figure S2).

TABLE 1 Outline of the quantitative morphological variables assessed from the 3D data (triangulated polygon meshes and extracted medial axis skeletons) according to their type: branching, complexity, and curvature.

Input	Estimated variables	Type	Abbr.	Output			Units
				G	D	A	
Triangulated polygon mesh	Surface to volume ratio	Complexity	*S/V*	X			cm^2/cm^3
	Fractal dimension	Complexity	*FD*	X			–
	Sphericity	Complexity	φ	X			–
	Gaussian curvature	Curvature	*K*		X	X	cm^{-2}
	Mean curvature	Curvature	*H*		X	X	cm^{-1}
	Maximum curvature	Curvature	*k1*		X	X	cm^{-1}
	Minimum curvature	Curvature	*k2*		X	X	cm^{-1}
Medial axis skeleton graph	Branch spacing:		$br_{spacing:}$		X	X	cm
	- Kruszyński et al., 2007	Branching	*_v1*		X	X	cm
	- Wallace et al., 1991	Branching	*_v2*				
	Branch length	Branching	br_{length}			X	cm
	Branching rate	Branching	br_{rate}		X	X	cm
Polygon mesh and medial axis skeleton graph	Branch width at:		$br_{width:}$		X	X	cm
	- the base	Branching	*da*		X	X	cm
	- the midsection	Branching	*db*		X	X	cm
	- the endpoint (terminal)	Branching	*dc*				
	Average branch width	Branching	*d_avg*			X	cm
	Branch angle	Branching	br_{angle}		X	X	rad
	Curvature at the tip of the branches	Curvature	*K_tip*		XX	X	cm^{-2}
		Curvature	*H_tip*		X	X	cm^{-1}
		Curvature	*k1_tip*		X	X	cm^{-1}
		Curvature	*k2_tip*			X	cm^{-1}

Units, abbreviations (Abbr.), and outputs obtained for each feature are also displayed. Global values (G) were obtained from the complete specimens, density distributions (D) were estimated per branch in the skeleton or per vertex of the polygon mesh when possible. Finally, univariate average measures (A) were calculated including mean values (*_mean*), and variance (*_var*) for both curvature and branching variables, and also skewness (*_skew*) and kurtosis (*_kurt*) for curvature variables.

2.3 Polygon mesh-based estimations

The resulting triangulated polygon meshes were further analysed and visualized using the Visualization Toolkit v9.1.0 (VTK; Schroeder et al., 2006) in Python v3.8 (Van Rossum and Drake, 2009) and the Insight Toolkit v5.1.2 (ITK; Ibáñez et al., 2003) in c++20 (ISO/IEC, 2020). The surface area (*SA*) and the volume (*V*) of the specimens were obtained with *vtkMassProperties* from which the surface-area-to-volume (*S/V*) ratio and the sphericity (φ) were estimated directly (Supplementary Materials and Methods).

Four common characteristics of surface curvature were estimated for each vertex of the polygon mesh following Meyer et al. (2003). First, a discrete approximation of the Gauss-Bonnet theorem and the Laplace-Beltrami operator were implemented to obtain the Gaussian (*K*) and mean (*H*) curvature for all vertices respectively (Supplementary Materials and Methods). The sign of *H* was then determined based on the direction of the normal vectors, which were obtained using *vtkTriangleMeshPointNormals*. Finally, the two principal curvatures, maximum curvature (*k1*) and minimum curvature (*k2*), were estimated considering that the Gaussian curvature (*K*) is defined as the product of the two principal curvatures at that location, and the mean curvature (*H*) corresponds to average of the two principal curvatures (Supplementary Figure S3).

2.4 Medial axis skeleton-derived estimations

To capture the topological branching structure of corals and facilitate the estimation of measures related to this type of morphology, we extracted the medial axis skeleton from the previously rendered 3D models using a voxel thinning algorithm. For this purpose, the polygon mesh was first smoothed using the *vtkWindowedSincPolyDataFilter* module (iterations: 100, pass-band frequency = 0.005), thereby reducing the details of the surface and potential noise while still maintaining the general shape of the coral specimens. Next, the smoothed mesh was transformed into a binary voxel image (resolution = 0.5mm×0.5mm×0.5mm) using *vtkPolyDataToImageStencil* (tolerance = 0). Finally, voxel thinning was performed with *itkBinaryImageThinningFilter3D* (Homann, 2007), an implementation of the algorithm of Lee et al. (1994) that results in single-voxel thin skeletons. The voxel skeletons were then transformed into graphs (*G*) by translating each voxel to a vertex (*v*) with coordinates corresponding to the represented

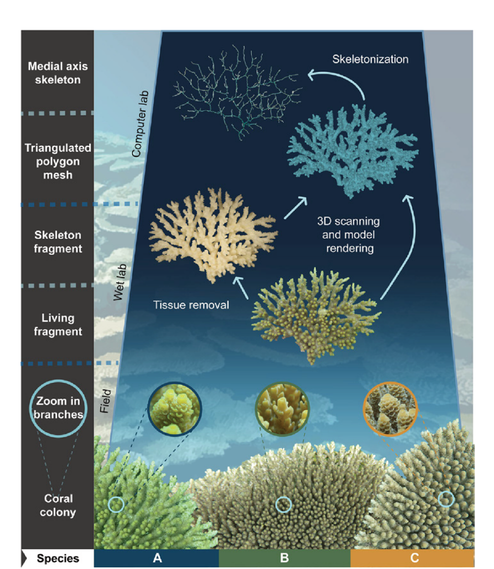

FIGURE 1
Schematic representation of three-dimensional (3D) model rendering and skeletonization workflow from complex-shaped organisms. Coral morphology can be analysed by 3D scanning either live (Reichert et al., 2016) or voucher skeleton specimens (as in this study; see Wet lab section). Here, three species of tabular *Acropora* corals (i.e., A, B, and C) identified using diagnostic characters in the field (see Coral colonies and Zoom to the branches) and later confirmed using different lines of evidence (Ramírez-Portilla et al., 2022) were used as a case study (coral photos by A.H. Baird). Downstream processing rendered 3D models as triangulated polygon meshes or medial axis skeletons (see Computer lab section) from which variable types such as curvature, branching, and complexity were estimated (see Table 1, Figure 2, and Supplementary Figure S2).

location and connected by edges using the method described by Reinders et al. (2000). In this graph, a branch (b) was considered to be the set of neighbouring vertices and edges between two successive junction vertices (with a vertex degree higher than two), or between a junction and a terminal vertex (with a degree of one, Supplementary Materials and Methods).

The branches were then identified from the graph and three different morphological characters were estimated (Supplementary Materials and Methods). Branch length (br_{length}) was calculated as the sum of all the edge lengths. Branching rate (br_{rate}), or how often a coral branches, was defined as the distance between the first (v_0) and last vertex (v_N) of the branch. Then, two definitions were used to estimate branch spacing. First, following Kruszyński et al. (2007), branch spacing ($br_{spacing}_v1$) was defined as the shortest distance between the tip (v_T) of the terminal branches and any vertex in the skeleton graph not belonging to the current branch. Finally, following Wallace et al. (1991), a second proxy of branch spacing ($br_{spacing}_v2$) was defined as the shortest distance between the tip (v_T) of a terminal branch and any other v_T.

2.5 Polygon mesh and medial axis skeleton graph-based estimations

To obtain information about the branch width (br_{width}), once the medial skeleton axis and the smoothed polygon mesh were obtained for each specimen, each vertex of the skeleton graph was associated with a medial thickness parameter ($d(v)$), which represents the diameter of the branch at v. There, the medial thickness was estimated as twice the distance to the closest point on the smoothed polygon mesh. Following Kruszyński et al. (2007), the medial thickness of the individual vertices was translated to three metrics related to the branch width (i.e., the diameter of a sphere at a certain point of the branch): the width at the base of a branch or the junction vertices (*da, a−sphere*), the width adjacent to the junction a, towards the midsection of the branch (*db, b−sphere*), and the terminal width (*dc, c−sphere*) or medial thickness at the tip of the branches (v_T) (Figure 2, upper left). As an additional parameter, the average thickness of each branch (d_{avg}) was obtained by averaging the medial thickness of all vertices in the branch. The location of the *a−sphere* and *b−sphere* were also used to obtain the angle of the branches (b_{angle}). The angles were obtained for all terminal branches (b_T) and were defined as the smallest angle at its associated *a−sphere* between its *b−sphere* and the *b−sphere* of a neighbouring branch.

Curvature features were also estimated at the branch tips where it was defined from the subset of the vertices on the polygon surface that were located on the tips of the branches. To identify the branch tip vertices the skeleton graph was used (Supplementary Figure S2). For each b_T, a cylinder that had a diameter of da and an axis that followed the direction of the vector between v_T and $v_{N/2}$ was placed on v_T together with a plane orthogonal to the cylinder axis. The vertices and faces that were located within the cylinder and exceeded the plane were selected using with the *vtkExtractPolyDataGeometry* module. From this selection, the set of connected vertices closest to v_T (obtained with *vtkPolyDataConnectivityFilter*) were considered to be the branch tip vertices (v_{tip}) of the polygon mesh. For these vertices, curvature values were calculated for the specimens as previously described for the polygon meshes (see section 2.3).

2.6 3D-based morphological variables assessment and feature screening

Three variable types were estimated from the 3D data set; complexity, curvature, and branching (Figure 2 and Table 1). For complexity variables, global values for each one of the coral fragments were obtained (i.e., a single value per specimen). For curvature and branching variables, estimation methods yielded results per branch in the skeleton or per vertex of the polygon mesh. Therefore, to transform these distributions into univariate

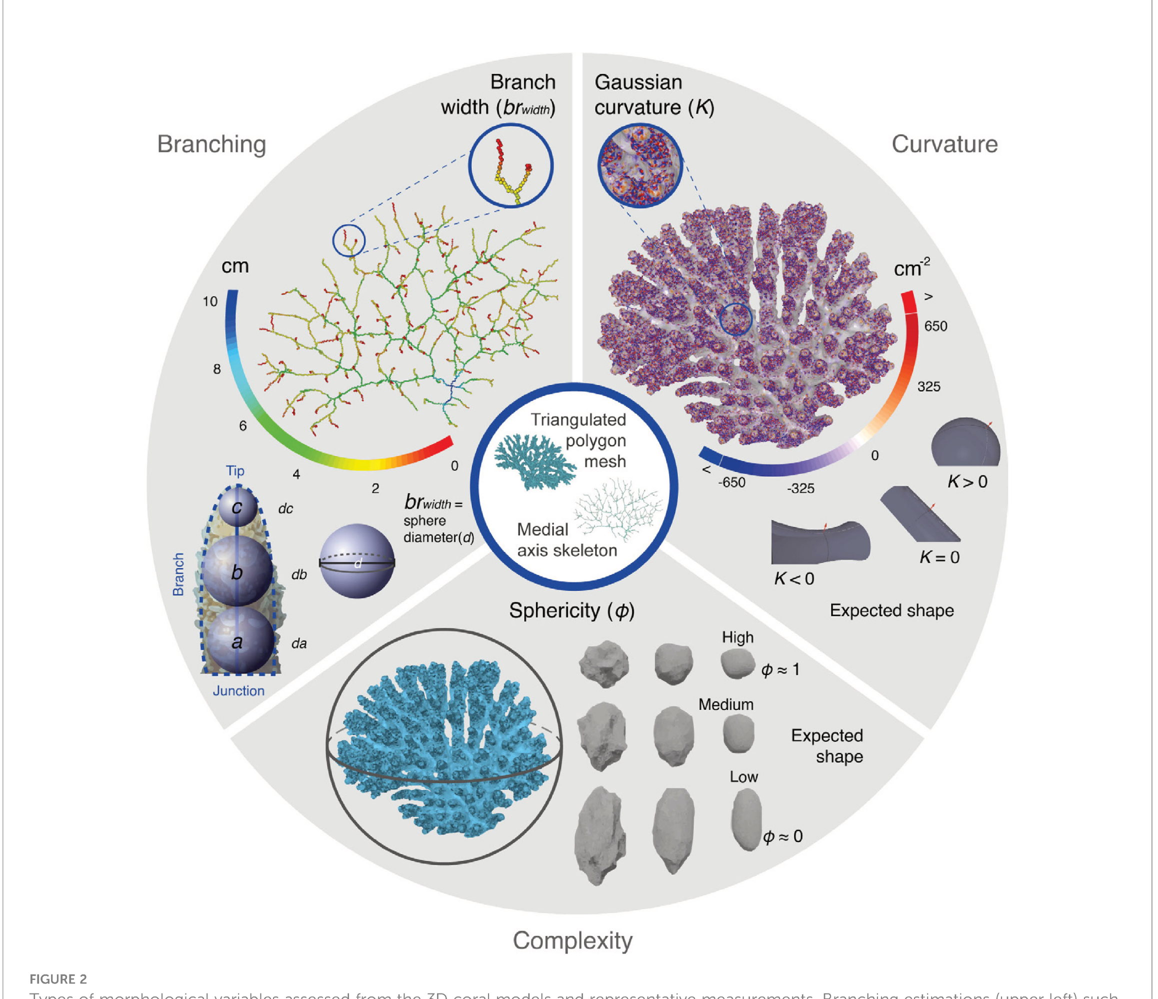

FIGURE 2
Types of morphological variables assessed from the 3D coral models and representative measurements. Branching estimations (upper left) such as the width (br_{width}) at different points between the tip and the branch junction were performed using the diameter of spheres (d) between the medial skeleton axis (solid blue line) and the smoothed polygon mesh (dashed blue line). Measures of the surface curvature (upper right) such as the Gaussian estimations (K) were obtained from the polygon meshes (see expected shapes according to values and Supplementary Figure S3). Complexity shape measures (bottom) were estimated as global values for each coral fragment, like sphericity (φ), which captures the volume compactness by measuring how close is the shape of each coral fragment to a sphere (see expected shapes according to values).

measures and obtain the average values, certain features were assessed. For the branch-related measures, outliers ($|Z| > 3$) of each coral were removed and the mean (*_mean*) and variance (*_var*) of the distributions were obtained. For the general curvature measures, values within the 2.5–97.5th percentiles were analysed to obtain the weighted mean (*_mean*), variance (*_var*), skewness (*_skew*) and kurtosis (*_kurt*) of each distribution. For curvature measures at the branch tips, first the distribution of the number of v_{tip} per b_T was analysed to remove branches with too many v_{tip} ($Z > 3$), as it indicates that reliable estimation of v_{tip} failed. The remaining branch tip vertices were assembled and analysed in a similar fashion as the general curvature distributions.

To provide a quantitative comparison of the estimated morphological variables, both univariate values and distributions were analysed using R v4.1.0 (R Core Team, 2018) through the RStudio console v1.4.1103 (RStudio Team, 2017). The three species previously delineated in this data set (Ramírez-Portilla et al., 2022) were used as a three-level factor for the subsequent analyses.

2.6.1 Variable assessment of global and average values

For assessing differences between species, univariate analysis of variance (ANOVA; $\alpha = 0.05$) and *post-hoc* Tukey tests ($\alpha = 0.05$; stats v4.1.0; R Core Team, 2018) were performed for each

variable (see Supplementary Materials and Methods). In addition, bivariate scatter plots and density plots (ggplot2 v3.3.5; Wickham, 2016) of measures with significantly different mean values between the three species were used to assess the morphospaces overlapping.

2.6.2 Variable assessment of density distributions

To weigh the informative value of measures obtained per branch in the skeleton or per vertex of the polygon mesh (D) in contrast to the univariate measures obtained per specimen (see section 2.6.1), probability density functions (*pdf*) were estimated. Gaussian kernel densities (KD) were estimated using the *scipy.stats.kde.gaussian_kde* function as implemented in SciPy v1.7.1 (Virtanen et al., 2020), where the bandwidth factor of each *pdf* was determined using Scott's rule (Scott, 2015). For curvature-related distributions, the values were weighted by the surface area associated to the vertex of which the curvature values were obtained ($A_{mixed}(v)$) following Meyer et al. (2003). For calculating branching rate, only branches with a minimum of 4 vertices were taken into account. To compare between species, the mean and standard deviation of the *pdf* were obtained within each species per step.

To test for significant interspecific differences between the distributions of the variables, ten replicates of the Mann-Whitney U test were performed using a thousand random samples for each variable measurement (*scipy.stats.mannwhitneyu* function). To sum up the information obtained from these tests, *p*-values obtained from each of the pairwise comparisons were transformed into integers according to an alpha (α) of 0.05 significance: if *p*-value > 0.05, then = 1 (i.e., there is a high probability that the samples come from similar distributions); if *p*-value ≤ 0.05, then = -1 (i.e., there is a high probability that the samples do not come from similar distributions). The integer values of the ten replicates were then added cumulatively to obtain a final value or distribution comparison score (DCS). Finally, heatmaps with samples reorganized according to the similarity displayed in the DCS pairwise comparisons using hierarchical clustering (Ward algorithm, heatmap3 v1.1.9; Zhao et al., 2021) were obtained for each of the variables and three different sets of them: all the variables (n = 15), curvature variables (n = 8), and branching variables (n = 7).

2.6.3 Screening of 3D-based morphological features

To perform feature screening for a prospective combination of representative characters that support interspecific discrimination, variables that exhibited significant differences in the ANOVA and in at least two-species comparisons in the *post-hoc* Tukey test were included in a "preliminary selected" subset (see Supplementary Figure S4 for a complete flow chart). Correlation between the preliminary selected variables was evaluated using Pearson coefficients (Hmisc v4.5-0; Harrell and Dupont, 2021) and a correlation plot (psych v2.1.6; Revelle, 2021). Box plots (ggplot2 v3.3.5; Wickham, 2016) were used to examine this subset of variables.

2.7 Contrasting 3D-based morphogroups and species boundaries assessed using other sources of information

To inspect clustering using the complete set of variables and the preliminary selected subset, the most likely number of groups was estimated according to 30 different indices (NbClust v3.0; Charrad et al., 2014), followed by a hierarchical clustering analysis (HCA; cluster v2.1.2; Maechler et al., 2021) using Euclidean distance and three different clustering methods (i.e., Ward, complete, and average) in which *p*-values were calculated via multiscale bootstrap resampling (pvclust v2.2-0; Suzuki et al., 2019).

A principal component analysis (PCA) was also performed to evaluate the ordination of the subset (stats v4.1.0; R Core Team, 2018). For this purpose, unbiased feature selection was performed using Gaussian model-based clustering (clustvarsel v2.3.4; Scrucca and Raftery, 2018) according to the Bayesian information criterion (BIC). Briefly, a set of variables that best discriminated groups using normal mixture models (NMMs) without *a priori* information was defined using the greedy algorithm both in forward and backward directions (Raftery and Dean, 2006; Scrucca, 2010). This set of variables was then used to reduce the dimensionality of the data using a PCA (Supplementary Figure S4).

Congruence between morphogroups discriminated using these multivariate approaches were contrasted to the three species previously delineated in this data set by mapping each coral specimen to its corresponding taxonomic assignment in each of the analyses (Ramírez-Portilla et al., 2022).

2.8 Discrimination of *a priori* delimited species by 3D-morphological analyses

The discriminative potential of the 3D-based variables was gauged by removing highly correlated features from the complete variable subset according to their variance inflation factor (VIF < 10; usdm v1.1-18; Naimi et al., 2014) to perform a multivariate analysis of variance (MANOVA; stats v4.1.0; R Core Team, 2018), and a linear discriminant analysis (LDA) with the maximum likelihood (ML) estimator method (MASS v7.3-54; Venables and Ripley, 2002). The accuracy of the discriminant approach was assessed by randomly partitioning the data set in a training (n = 50, 67.6% of specimens) and testing (n = 24, 32.4% of specimens) subsets and calculating the corresponding prediction accuracy tables or confusion matrices.

3 Results

3.1 3D-based morphological variables

Overall, 53 univariate variables from one of three types (complexity, curvature, and branching, Figure 2) were estimated from the data rendered by the 3D models of the 74 coral fragments (Table 1). For each specimen, a single measure of its surface to volume ratio (*S/V*), fractal dimension (*FD*), and sphericity (φ) captured the geometric complexity of its colony shape, the irregularity of its surface, and the compactness of its volume (complexity variables). Contrastingly, average values per branch or branch tip either estimated traits such as spacing, length, width, and angle (branching) or characterized the topological concavity/convexity of coral surfaces (curvature). A total of 19 variables were derived from the polygon meshes, 8 from the medial axis skeletons, and 26 using both the polygon meshes and the medial axis skeletons. In addition, probability density functions using kernel estimates were obtained for 15 of these variables: 8 curvature variables and 7 branching variables (Table 1). Forty-one of the univariate variables did not conform one or both of the assumptions of normality and were subsequently transformed (Supplementary Table S2). Four variables were removed from downstream analyses as they did not conform either to the normality or the homogeneity of variance assumption, even after transformation (i.e., *K_tip_mean*, *K_tip_skew*, *k2_tip_mean*, and *k2_tip_skew*).

3.2 Phenotypic differences in central tendencies of 3D-based variables

To test the potential of the variables for species-level differentiation, both univariate values (i.e., global and average) and Kernel density (KD) distributions of the 3D-estimated data were examined. Using an analysis of variance (ANOVA) for univariate values, significant interspecific differences were found in the means of 42 variables when the three species previously delineated in this data set were used as a three-level factor (p-value < 0.05, df = 2; Supplementary Table S3). Further exploration using *post-hoc* Tukey tests (p-value < 0.05; Supplementary Table S4) indicated differences between the means of all species in 10 of the characters and between at least two pairs of species in 21 cases. In summary, more than half of the variables derived from the univariate values exhibited significant differences in the ANOVA in at least two-species comparisons in one of the two-sample tests ($n = 29$; preliminary selected subset).

Although no complexity or branching variable could statistically differentiate the means of all three species, at least 50% of the branching variables could differentiate two species (9/18 according to the *post-hoc* test). Likewise, significant differences in two to three interspecific comparisons were detected in 23 of the 33 curvature variables. In addition, although the interspecific morphospaces tended to overlap, the probability density profiles of each species were visibly distinct for most univariate variables with significant differences (Figure 3 and Supplementary Figure S5). Overall, the pair of species for which more significant differences were found in the central tendencies was A vs. C, with 64% of the comparisons with p-values $< \alpha$ in contrast to 47% between A vs. B and 42% between B vs. C. A high degree of correlation was found between most of these variables (Supplementary Table S5 and Supplementary Figure S8), which mainly corresponded to curvature features (20 curvature and 9 branching variables).

The kernel densities (KD) analysis did not reveal significant interspecific differentiation in the distribution profiles, contrasting with the univariate variables results obtained. Instead, a high degree of overlap was apparent in the distributions of most of the 3D-based morphological variables (Figure 4A and Supplementary Figure S6). The cumulative analysis of the distribution comparison scores (DCS) based on the Mann-Whitney U tests of all variables and curvature variables was able to discriminate two morphogroups (Supplementary Figure S7A and Supplementary Figure S7C, respectively). However, among the individual heatmaps plotted for each variable, there was a considerable degree of clustering between samples of the same species when assessing interspecific differences using the minimum curvature (*k2*) distributions (Figure 4B). Here, three clusters were observed, each comprising mainly individuals of one of the three species in the data set (from top to bottom: cluster 1 = 61.90% of species B, cluster 2 = 56% of species C, cluster 3 = 82.14% of species A).

3.3 Congruence between morphogroups and previously delineated species boundaries

Despite the overall significant differentiation in central tendency of the set of 3D-based morphological features (see section 3.2 and Supplementary Figure S9), none of the clusters identified by the exploratory hierarchical clustering analysis (HCA) were entirely congruent with the species delineation achieved using other lines of evidence (Supplementary Figure S10, see Ramírez-Portilla et al., 2022). Likewise, when feature selection for Gaussian model-based clustering allowed finding the optimal subset of features containing information, only two morphogroups were identified (Supplementary Table S6). Consequently, the ordination recovered by the principal component analysis (PCA), based on such feature selection methodology, showed a considerable degree of overlap between the morphospaces as defined by the reduced dimensions considered in this analysis (Supplementary Figure S11).

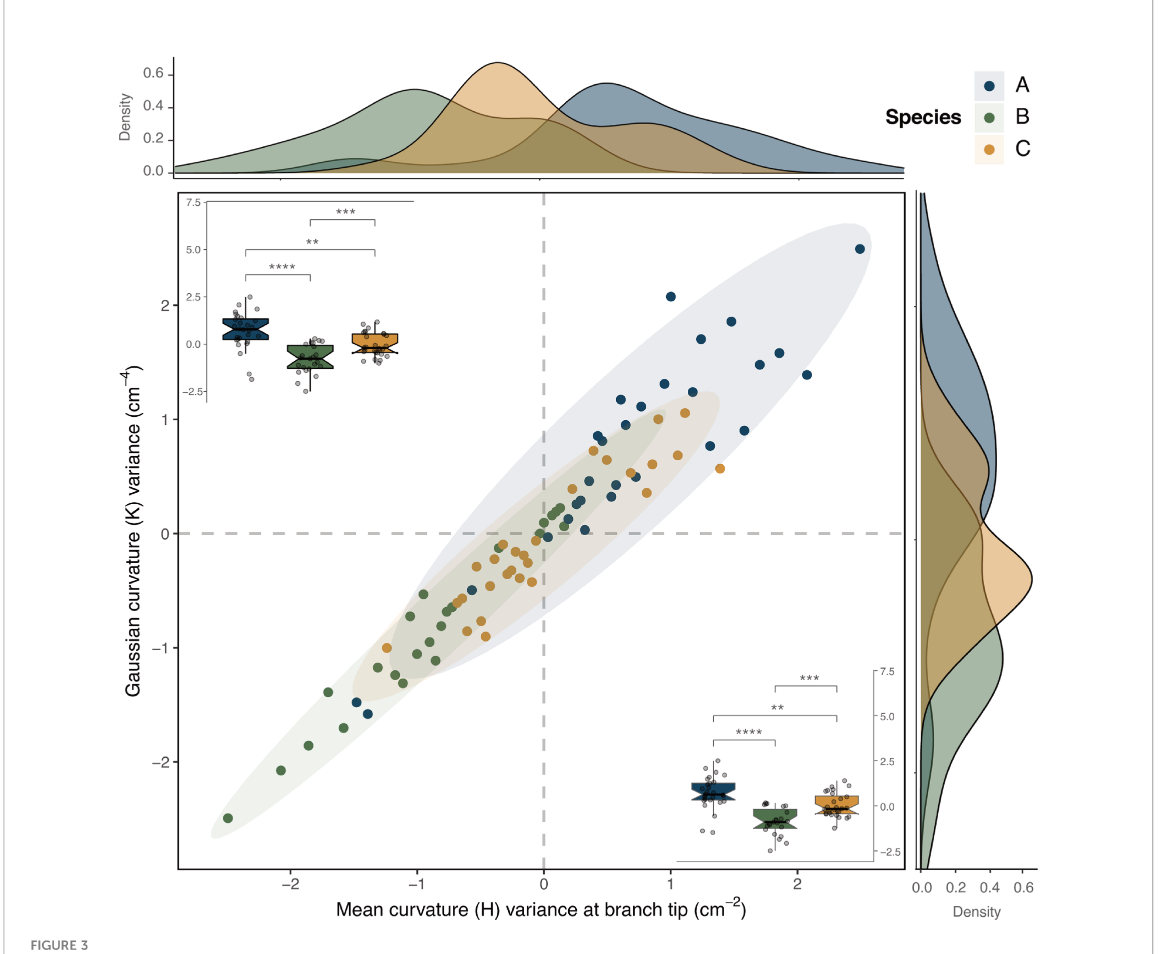

FIGURE 3
Comparison of two correlated curvature variables that displayed significant differences between the three species (ANOVA and *post-hoc* Tukey tests, $\alpha = 0.05$), but where the species morphospaces overlapped: mean curvature variance at branch tip vs. Gaussian curvature variance (*H_tip_var* vs. *K_var*). In the center, a scatter plot depicts the two variables, correlation (Pearson correlation coefficient $\rho = 0.96$), along with their density distributions along each axis. Box plots in each corner display the significant differences in the mean values of the variables according to pairwise interspecific comparisons (*p*-values significance: ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001).

3.4 Potential of 3D-morphological analyses to discriminate between *a priori* delimited species

From the 21 variables that did not present collinearity (Variance Inflation Factor (VIF) < 10; Supplementary Table S7), 10 were also present in the preliminary selected subset and showed significant interspecific differentiation (MANOVA; Supplementary Table S8). The linear discriminant analysis (LDA) using these variables was able to distinguish three groups that matched the previously supported species delimitation with 97.30% of accuracy (Figure 5; scatter plot) and was able to predict correctly at least 75% of the observations once the model was trained and later tested (Supplementary Table S9). Variables such as the skewness of the Gaussian curvature (*K_skew*), the mean curvature (*H_mean*), and the branch angle (*b_angle_mean*) were the ones that mainly contributed to the discrimination according to the ordination coefficients of each linear component (Figure 5, bar plots).

4 Discussion

In this study, we assessed the applicability of 3D-based variables derived from polygon meshes and medial axis skeletons to discriminate morphogroups and potentially

support the delineation of species boundaries in complex-shaped and taxonomically intricate marine organisms. For this purpose, we first evaluated the interspecific morphological differentiation rendered by the 3D variables and performed feature selection for a prospective combination of representative characters that support discrimination of phena. Later, we tested whether the morphogroups delineated using 3D-morphological analysis of these variables were congruent with species boundaries assessed using other sources of information and/or whether the 3D-morphological analyses enabled to discriminate between *a priori* delimited species. Although we used coral skeletons as starting material, previous studies suggested the feasibility of applying our 3D methodology to living specimens, as a minimally invasive method to perform morphological assessments (Figure 1).

FIGURE 4
Kernel density distribution analyses. **(A)** Probability density functions of the terminal branch thickness (*dc*) and the Gaussian curvature at branch tip (*K_tip*) for each of the species. **(B)** Heatmap depicting recoded and summarized *p*-values obtained from two-sample Mann-Whitney U tests for minimum curvature (*k2*). Samples were re-organized using hierarchical clustering according to similarity in pairwise comparisons, calculated as the distribution comparison score (DCS) or the cumulative value of similarity according to the *p*-value significance cut-off (α = 0.05).

FIGURE 5
Discriminant multivariate analysis using a subset of variables. Linear discriminant analysis (LDA) using the variables that present no collinearity (Variance Inflation Factor (VIF) < 10, 21 variables) displaying the percentage of trace for each main discriminant. Distribution densities for each one of the linear discriminants are plotted along the axes, along with the scaling coefficients for each variable.

4.1 Interspecific morphological differentiation achieved by 3D-based variables

While evaluating the performance of individual 3D variables, both univariate and multivariate analyses were able to identify 3D-based morphological features with significant differences between *a priori* delimited species. This trend was particularly evident when using curvature variables, which showed differentiation in their central tendencies in most pairwise species comparisons (Supplementary Tables S3, S4), in discriminant analysis (Figure 5), and partly in comparisons between Kernel density distributions (Supplementary Figure S6 and Supplementary Figure S7C). Indeed, significant differences in attributes such as the skewness, kurtosis, and mean values of these variables suggest that they attain sufficient resolution to capture the morphological differences between the specimens of the three complex-shaped coral taxa used here in for validation. By enabling the characterization of surface profiles and owing to the relationship between curvature variables and functional traits (Hyde et al.,

1997; Ankhelyi et al., 2018), these results suggest that the estimation of curvatures holds promise for improving our understanding of the relationship between morphology and potentially specific ecological traits.

Contrastingly, analyses of branch-related variables did not provide enough resolution at the species level, likely due to the high similarity between the branching patterns of these closely related taxa (Wallace, 1999). Although only branches with a minimum of four vertices were taken into account to reduce the likelihood of including spurious ones in the estimation of branching variables, these features seemed to be highly variable within species and individuals (Supplementary Figure S7). These results suggest that branch-related variables are taxonomically uninformative in this particular case, as it has been observed that species-specific patterns can emerge when comparing such variables between more distantly related taxa (Kaandorp, 1999). Besides, estimating branching variables can be more relevant to understand the function, evolution, and plasticity of an organism's shape, particularly when studying marine taxa and their response to environmental fluctuations (Kaandorp et al., 2003; Kaandorp et al., 2005; Chindapol et al., 2013; Paz-García et al., 2015a).

Overall, analyses based on global and average univariate variables exhibited morphological differentiation consistent with *a priori* delineated species (Supplementary Figures S5, S9). In contrast, most non-averaged density distribution analyses did not display congruent discrimination patterns (Figure 4 and Supplementary Figure S7A), seemingly due to the high intraspecific variation and consequent overlap of the probability density functions between *a priori* delineated species (Supplementary Figure S6). These trends can be related to the different estimations performed in each case. The univariate values rely on average values obtained per specimen, while the probability density distributions were estimated per branch in the skeleton or per-vertex of the polygon mesh using kernel densities. Thus, since kernel densities exhibit high correspondence to data (Pradlwarter and Schuëller, 2008), they can both contain a wealth of information and display a wide range of variance that can potentially conceal the main species-specific trends in quantitative phenotypic data. Moreover, in the case of complex-shaped organisms such as the tabular *Acropora* corals used in this study, the variation of probability density functions can be related to the influence that shape complexity exerts on scanning reproducibility (Bythell et al., 2001). Despite the efforts to avoid the effect of self-shading (Supplementary Materials and Methods and Supplementary Figure S1), it has been observed in previous studies that the coefficient of variation between iterative scans can increase in branching corals due to a higher rate of potentially overlapping structures (Reichert et al., 2016). As a result, the shape complexity of the coral specimens could have affected the variability of the observed data.

4.2 Discriminative power of selected 3D variables

Broadly, species delimitation approaches can be differentiated into validation or discovery tools according to whether or not the samples are partitioned into taxonomic categories before performing the analysis (Carstens et al., 2013). In this study, results showed that a well-justified combination of novel 3D-based variables can aid discrimination of morphogroups of irregularly shaped organisms when based on *a priori* assignment of samples to categories (Ence and Carstens, 2011). In comparison to the quantitative morphological characters previously assessed from the same set of specimens by Ramírez-Portilla et al. (2022), the 3D-based variables evaluated in the current study were able to discriminate three phena congruent with other species delimitation approaches with higher overall accuracy (Figure 5 and Supplementary Table S10; 97.30% in this study vs. 94.94% in the previous). However, when morphogroups were delineated without *a priori* information in this study (Supplementary Figures S9–S11 and Supplementary Table S6), they were not congruent with species boundaries assessed using other sources of information. The high phenotypic heterogeneity detected within the *a priori* delineated species (particularly B and C) using density distributions (Supplementary Figure S5 and Supplementary Table S10) could have hampered the unambiguous and unbiased delimitation of three phena congruent with the species boundaries previously delineated using other lines of evidence (see section 4.4).

Although these results may seem paradoxical given the significant interspecific differences found using 3D-derived variables (see section 4.1), phenotypic differentiation in central tendencies between species defined *a priori* does not necessarily count as evidence of species boundaries when assessed in light of evolutionary theory (Luckow, 1995; Zapata and Jiménez, 2012; Cadena et al., 2018; Cadena and Zapata, 2021). Instead, distinct distributions of phenotypic characters (e.g., those derived from fitting quantitative data to NMMs) can constitute support for species hypotheses as long as they do not result from intraspecific polymorphisms (e.g., González-Espinosa et al., 2018) or morphological plasticity (e.g., Paz-García et al., 2015a). Here, this trend only became evident once the features that yielded information congruent with *a priori* species boundaries were selected and used to perform discriminant analyses (Figure 5 and Supplementary Tables S9, S10).

4.3 Potential of feature selection to improve species discrimination

The quandary of feature selection in multidimensional data sets is often the limiting factor for extending the applicability of approaches such as 3D-derived variables to a wider variety of

organisms (Poon et al., 2013). Certainly, many issues in detecting species boundaries from morphological and phenotypic analyses derive from the potential exclusion of important characters during dimensionality reduction (Cadena et al., 2018). Here, given the large number of variables derived from the 3D analyses and the fact that not all of them provided discriminative and non-redundant information (Supplementary Tables S3–S7), the process of feature screening proved to be key to selecting features used to discriminate between *a priori* delimited species (Ramírez-Portilla et al., 2022). Feature screening and selection, however, would substantially rely on the overall morphology of the studied organisms. Assessing branching features, for example, would be inadequate for describing the 3D morphology of massive or encrusting growth forms.

Although the morphospaces overlapped when performing bivariate comparisons and other multivariate graphical representations (Figure 3 and Supplementary Figure S5), linear combinations of features after variable screening were useful to identify potentially informative characters and a combination of them that enabled discrimination of three morphogroups congruent with the species delineated *a priori* in the data set (Figure 5). These results support the notion that not only technological advances in 3D data acquisition and model rendering, but also feature screening and selection actually provide prospective variables to quantify morphology and discriminate groups (Valcárcel and Vargas, 2010), particularly of complex-shaped organisms lacking traditional landmarks.

Regardless, the methodology used here to estimate morphological variables from the 3D models, can be applied to understand a wider variety of phenomena such as morphological plasticity, development, and environmental effects on shape and biodiversity. Therefore, the approaches implemented in this study do not only intend to inform taxonomy, but also to provide tools that can support evolutionary, ecological, and biomonitoring aims to characterize and understand form in complex-shaped taxa in forthcoming studies.

4.4 Limitations

Independent lines of evidence that previously delineated taxonomic units in the data set (i.e., morphology, breeding trials, and molecular analyses) robustly supported the identification of three species (Ramírez-Portilla et al., 2022). Although the 3D-based variables assessed here provided enough power to discriminate morphogroups congruent with such species boundaries delineation, it was not able to delimitate the same three groups when unbiased clustering and feature selection were performed. Only two clusters or components were identified and supported by the multivariate analyses (Supplementary Figures S9, S10), even after a set of variables that best discriminated groups using normal mixture models (NMMs) without *a priori* information was employed (Supplementary Table S6). In this regard, the results obtained from the density distributions suggest that the heterogeneity of the phenotypic variation detected by 3D-morphological analyses within some of the species could have confounded the identification of components in the mixture (Supplementary Figure S5 and Supplementary Table S10). This would be consistent with the close ties between intraspecific variability and interspecific similarity that have hampered the widespread use of morphological features to delineate taxonomically intricate taxa (Sites and Marshall, 2004), particularly in speciose groups such as the coral genus *Acropora* where both high morphological intraspecific variability and interspecific similarity, particularly between closely related species, has been reported (Wallace and Willis, 1994; Wallace, 1999).

Alternatively, the scanning quality achieved in the present study could have hindered the potential of species-level delimitation given that important differences in the microstructure could be masked by the technical resolution of the underlying 3D mesh (Gutierrez-Heredia et al., 2016; Reichert et al., 2017). Indeed, features at micromorphological level, such as corallite shape and dimension, have been deemed crucial skeletal characters for discriminating between complex-shaped coral species like those of the genus *Acropora* (Wallace, 1999; Wolstenholme et al., 2003; Ramírez-Portilla et al., 2022). The potential masking of these features and the effect of the sample sizes used for validation, which in several cases were lower than $n = 10$ (Finch and Schneider, 2006), could explain the relatively low prediction accuracy achieved in this study when randomly partitioning the data set in training and testing subsets (Supplementary Tables S9, S10). These results suggest that further refinements of 3D-morphological analyses, such as increased precision and reduced errors, might even enable *a priori* delimitation of phena rather than *a posteriori* confirmation. For instance, the integration of 3D models from structured light scanning or photogrammetric approaches with high-resolution 3D methodologies such as CT scanning could solve some of the present accuracy issues (Laforsch et al., 2008; Naumann et al., 2009; Veal et al., 2010; Gutiérrez-Heredia et al., 2015; Aston et al., 2022). In the meantime, our results support the discriminative value of implementing 3D-based variables either as hypotheses testing or validation approaches rather than discovery ones.

4.5 Future perspectives

Progress in the development of methods to delineate species using morphological data is urgently needed, particularly to improve modelling of phenotypic variation in agreement with evolutionary theory (Cadena and Zapata, 2021). Due to the potential for morphological plasticity of marine taxa such as corals (Todd, 2008), assessing the discriminative power of 3D-

morphological variables to distinguish species throughout the environmental ranges they occupy ought to be explored further, particularly for species with relatively wide distributions where geographical differences can mislead morphology-based species delimitation (Fukami et al., 2004; Forsman et al., 2015). Moreover, the link between 3D-based phenotypic variables and their biological, ecological, and functional significance need to be addressed in future studies that will not only intend to assess interspecific variations in morphology and their taxonomic relevance, but also their potential role in speciation and adaptation, particularly for complex-shaped organisms such as corals (Zawada et al., 2019a; Zawada et al., 2019b; Torres-Pulliza et al., 2020; Aston et al., 2022; Siqueira et al., 2022), which inhabit one of the ecosystems most threatened by climate and anthropogenic disturbances (Hughes et al., 2017; Hughes et al., 2018).

5 Conclusions

Morphological data rendered by 3D scanning approaches in this study showed great potential for discriminating phena among complex-shaped organisms. Curvature features were most prominent in differentiating morphogroups congruent with species boundaries supported by independent evidence. Yet, variable screening and selection proved key to providing sufficient resolution for discriminating closely related species that overlap in ecological and morphological traits. Although our methodology was assessed using coral species as model organisms, the approaches outlined here are in principle applicable to a wide variety of irregular and complex-shaped plant and animal taxa for which 3D data can be readily obtained. However, variables derived from 3D-morphological approaches can complement other lines of evidence but not substitute them when delineating species boundaries within an integrative framework. Ultimately, combining informative quantitative morphological features with other independent lines of evidence will advance our understanding of morphological variation in complex-shaped life forms.

Data availability statement

Data and code can be found in the GitHub repository https://github.com/catalinarp/Coral3Dmorphomeasures. In addition, the analysed triangulated polygon meshes of the studied corals specimens are available at Morphobank Project 4205 (http://morphobank.org/permalink/?P4205).

Author contributions

J-FF, JK, CR-P, and TW conceived the ideas behind the manuscript; AHB, J-FF, SH, CR-P, and FS collected the data; IMB, RB, JK, and CR-P analysed the data; CR-P and IMB led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.955582/full#supplementary-material

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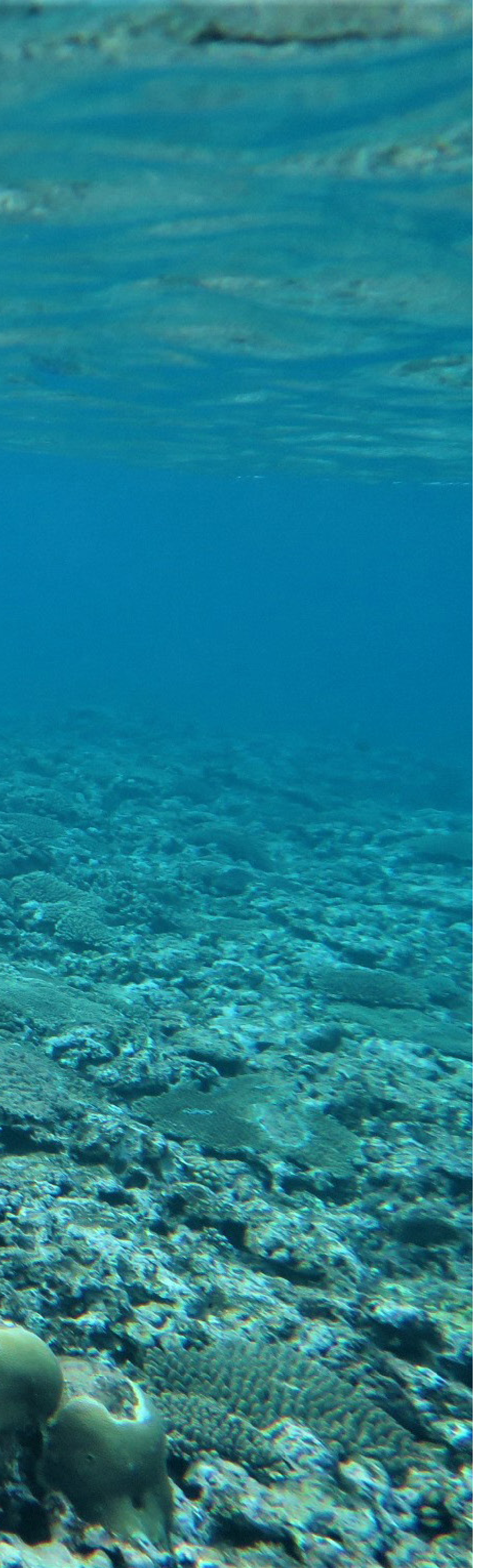
Diving at Bisezaki point
Motobu, Okinawa, Japan
Photo by J.-F. Flot



4 | Chapter IV

4.1 | Discussion

This doctoral thesis addresses the primary endeavors of delineating coral species using tabular representatives from the speciose genus *Acropora* as a case study. For this purpose, the four chapters that integrate this work aimed to review, assess, and refine current species boundaries in a group of tabular corals that embody the main challenges taxonomy faces in this branch of the tree of life. Accordingly, a historical account of coral taxonomy approaches, the evolution of species criteria, and the challenges faced by coral species delimitation set the background of this thesis (**Chapter I**). Then, tabular *Acropora* corals were used by Ramírez-Portilla et al. (2022a) to elucidate the coral species delimitation conundrum by comparing different molecular approaches and assessing their congruence with lines of evidence such as breeding crosses and morphology to delineate species boundaries (**Chapter II**). Finally, the discriminative potential of a 3D-based approach was examined by Ramírez-Portilla et al. (2022b) in the quest for alternative morphological measures to long-standing challenges for differentiating morphogroups and assisting the delineation of coral species boundaries (**Chapter III**). The approaches presented in this thesis lined up to portray a more integrative picture of coral species boundaries by using an array of complementary lines of evidence based on sensitive criteria. Ultimately, the goal was to pave the way for a taxonomic revision of the *Acropora* genus that in the future can provide a solid basis to support conservation in a wide variety of keystone organisms..

4.1.1 | The history of coral taxonomy: a long way treading thin lines

Delineation of species boundaries remains challenging in taxonomically puzzling clades such as corals. Yet, this thesis shows that scleractinian taxonomy has come a long way since Linnaeus referred to a coral specimen using the binomial nomenclature for the first time (Linnaeus, 1758). Previous reviews on the subject have focused either on the historical dimension (e.g., Veron, 2013), the discussion of different cases of study (e.g., Kitahara et al., 2016), or the coral holobiont (i.e., metaorganism integrated by the coral host and an assemblage of bacteria, archaea, viruses, and algal symbionts among other eukaryotes) perspective (e.g., Stat et al., 2012). In contrast, this thesis reviews the evolution of coral taxonomy (**Chapter I**), emphasizing why it may have seemed like a "never-ending" story and how the criteria to delineate coral species boundaries have shifted over the past two and a half centuries (section **1.1.1**).

Coral species delimitation has progressed thanks to the advances in oceanic exploration and the development of analytical approaches for species delimitation (section **1.1.2**). As a result, integrating lines of evidence based on different criteria to delineate species boundaries has evolved the way to move forward in coral taxonomy. However, challenges remain for the advancement of coral species delimitation. The seemingly widespread incongruence between morphological and molecular lines of evidence (a.k.a., the coral species delimitation conundrum; see section **1.1.3**), for instance, has been primarily interpreted as evidence for interspecific hybridization and the assumption that corals frequently integrate complexes of species that can interbreed (i.e., syngameons; Veron, 1995). Yet, in light of coral morphological diversity and the relatively recent divergence of some clades, this notion should not preclude the exploration of alternative hypotheses that may explain the nonmonophyletic gene tree patterns (Márquez et al., 2002a; Flot et al., 2010). Testing other reasonable explanations for polyphyly, such as incomplete lineage sorting (ILS) and specimen misidentification, can be facilitated by the integration of several lines of evidence and novel approaches (e.g., Arrigoni et al., 2020).

Overall, this literature review found that coral species delimitation has been largely dominated by studies focusing on the animal host (Stat et al., 2012). The need to resolve species boundaries of the best-understood entities of the coral holobiont as a pre-requisite to test hypotheses in all types of studies stemming from these organisms can explain such a trend (Voolstra et al., 2021). Still, accounting for all the components of the holobiont can provide additional lines of evidence to gauge coral species boundaries and evaluate patterns of phylosymbiosis (i.e., the correlation between host phylogenetic relatedness and multivariate community similarities of the

associated microbiome) with potential significance at evolutionary level (van Oppen and Blackall, 2019). For instance, patterns of symbiont specificity can help distinguishing between cryptic species or deepen our understanding of the factors that shape coral microbiomes in different species through time (Pollock et al., 2018; Johnston et al., 2022).

Difficulties quantifying morphological traits and assessing intra- and interspecific variation in complex-shaped organisms also permeate coral species delimitation according to the literature (e.g., Kruszyński et al., 2007; Filatov et al., 2013; Marti-Puig et al., 2014; Forsman et al., 2015; Reichert et al., 2017; Zawada et al., 2019a). The coral genus *Acropora* embodies such challenges (section **1.1.4**). The dearth of informative and diagnostic characters for morphology-based taxonomy contrasts with the high morphological diversity displayed by this genus (i.e., >400 nominal species based on morphology). Therefore, revisiting traditional and validating new approaches using the *Acropora* as a case study can aid in overcoming long-standing challenges in species delimitation and, thus, expediting the refinement of coral species boundaries (Wallace and Willis, 1994).

Intricate taxonomy is a common denominator in anthozoans; including scleractinians, octocorals and antipatharians (Quattrini et al., 2018). Scleractinian corals have been the most sampled to date (Quek and Huang, 2022), which has likely rendered more clues regarding the main strengths and limitations of currently available approaches to delineate species boundaries in this group. However, issues such as the dearth of species-level markers and diagnostic characters are pervasive in anthozoans (Quattrini et al., 2019; Erickson et al., 2021; Terrana et al., 2021). Therefore, future work should include comparative information for these related branches of the three of life and thus shed light in the complex evolutionary patterns that may have confused their taxonomy.

4.1.2 | Sensitive approaches challenge apparent incongruence between morphology and molecular lines of evidence despite no universal strategy to delineate coral species

Species from the coral genus *Acropora* epitomize the main challenges currently faced by coral taxonomy (see section **1.1.4**). This thesis aimed to test and refine the morphology-based species boundaries by comparing and assessing the congruence of different species delimitation approaches and criteria in this stony coral genus. In consequence, Ramírez-Portilla et al. (2022a) used three lines of evidence to delineate species boundaries in a group of sympatric tabular *Acropora* corals at Sesoko Island (Okinawa, Japan): morphology, breeding trials, and molecular approaches (**Chapter II**).

Three morphospecies (primary species hypotheses - PSHs) were distinguished first in the field and later confirmed via multivariate morphological analyses of mixed data (i.e., quantitative, qualitative, and categorical). Although the high overall similarity between *Acropora* species (particularly within morphological groups; Wallace, 1999) usually hampers the identification of taxonomically informative traits and suggests the existence of cryptic diversity (e.g., Ladner and Palumbi, 2012), qualitative characters largely contributed to resolve morphospecies in this thesis (Ramírez-Portilla et al., 2022a). Similar to results from other studies of morphological variation in *Acropora*, qualitative characters displayed considerably greater species resolution than continuous characters (Wallace et al., 1991; Wolstenholme et al., 2003). Yet, subjectivity may affect qualitative traits assessment, and qualitative diagnostic character determination is generally unattainable when using finite sample sizes (Wiens and Servedio, 2000). Therefore, performing quantitative character analyses is favored and advantageous for extending the scope of diagnosability and comparability between systems (see **Chapter III** and section **4.1.3**).

By comparison with relevant type material, the morphological PSHs were tentatively identified as *A.* cf. *cytherea*, *A.* aff. *hyacinthus*, and *A.* cf. *bifurcata* (Nemenzo, 1971; Wallace, 1999). While *A. cytherea* and *A. hyacinthus* correspond to accepted nominal species, the last major revision of the genus synonymized *A. bifurcata* with *A. hyacinthus* (Wallace, 1999). Contrastingly, field images and alternative morphology-based taxonomic assessments have recorded *A. bifurcata* as a valid species (Nishihira and Veron, 1995; Veron, 2000). Taxonomic disagreements, as such, can cause difficulties for ensuing species

boundary assessments. For instance, DNA-based studies have indicated that multiple cryptic species maybe be concealed within different sympatric *A. hyacinthus* complexes (Ladner and Palumbi, 2012; Suzuki et al., 2016; Sheets et al., 2018; Nakabayashi et al., 2019). However, Ramírez-Portilla et al. (2022a) found that distinctive phenons could be singled out through morphological analyses of *A. hyacinthus*-like colonies, suggesting that patterns resembling cryptic diversity may correspond to pseudo-cryptic species. Accordingly, close examination of specimens, morphological analysis, identification of taxonomically informative characters, and comparison with type material can shed light on robust coral species hypotheses (Arrigoni et al., 2019; Bonito et al., 2021).

Few species delimitation studies in corals have incorporated reproductive characters and experimental breeding crosses despite interbreeding evidence constitutes one of the ultimate lines to substantiate species boundaries according to the mixiological criterion (Lang, 1984; Willis, 1990; Willis et al., 2006). Studies conducted in this matter have deemed overlapping gamete release times determinant to support the presence or absence of species boundaries in broadcast spawners (even more so than compatibility in breeding experiments), as viability conditions the opportunity for gametes to cross-fertilize in nature (van Oppen et al., 2002; Wolstenholme, 2004; Furukawa et al., 2020; Mitsuki et al., 2021). In breeding experiments by Ramírez-Portilla et al. (2022a), only crosses performed within morphospecies achieved significant fertilization success, despite no temporal differentiation in spawning times (Baird et al., 2022). These results contrast with previous studies, where interspecific crosses between tabular *Acropora* resulted in moderate levels of mean fertilization (48 - 50 %; Willis et al., 1997). Thus, breeding compatibility outcomes supported the PSHs and suggested negligible interspecific hybridizing potential in this study system (Ramírez-Portilla et al., 2022a).

Although growing evidence supports the key role of introgressive hybridization in the evolution of *Acropora* species in the Caribbean (e.g., Vollmer and Palumbi, 2002; Miller and van Oppen, 2003; Fogarty et al., 2012; Palumbi et al., 2012; Nylander-Asplin et al., 2021), the syngameon hypothesis by Veron (1995) has accumulated fewer compelling evidence in other regions. In the Indo-Pacific, the incidence of intermediate forms that cannot be unequivocally gauged and the successful cross-fertilization achieved in a few *in vitro* systems support the occurrence of interspecific gene flow under certain conditions (Isomura et al., 2013, 2016; Kitanobo et al., 2016, 2022). Still, evidence for coral hybridization in nature remains limited (Hobbs et al., 2022), so other processes such as deep coalescence due to short divergence times (i.e., ILS) might explain the widespread incongruence between morphology and traditional tree- and distance-base approaches (Maddison, 1997; Funk and Omland, 2003). Due to the relatively large expected population sizes ($\boldsymbol{N_e}$ ~ 40.000; Prada et al., 2016; Mao et al., 2018 but see Márquez et al., 2002b) and variable generation times (***g***; approx. 5 – 30; Potts, 1984; Rapuano et al., 2023) in rather long-lived organisms such as corals (Bythell et al., 2018), the time (***T***) needed for divergent species from an ancestral population to attain reciprocal monophyly in at least 90% of their loci would be roughly between 2 and 12 myr ($\boldsymbol{T} = 10 * g * N_e$, assuming equal size populations with no gene exchange; Hudson and Coyne, 2002). Given the recent divergence time of tabular *Acropora* species (~2.58 Ma; Wallace, 1999), it is unlikely that reciprocal monophyly will be reached in most of the assessed loci, thus, rendering tree-based approaches unfit to detect species boundaries (Flot et al., 2010). Therefore, Ramírez-Portilla et al. (2022a) tested these notions using different molecular approaches to detect species boundaries.

Standard molecular approaches using conventional markers, such as the mitochondrial putative control region (AcroCR), displayed patterns of interspecific overlapping genetic distances and morphospecies scattered throughout the gene trees (Ramírez-Portilla et al., 2022a; similar to previous studies see van Oppen et al., 2001; Wolstenholme, 2004; Suzuki et al., 2016; Fukami et al., 2019). Conflicting patterns between morphological traits and standard molecular approaches based on genetic distances or species-level monophyly, as such, may result from the misidentification of the specimens, incomplete lineage sorting, or hybridization (see section **1.1.3**). Yet, considerable overlap in spawning time (e.g., Baird et al.,

2021, 2022) and high rates of interspecific breeding *in vitro* in the genus *Acropora* have led to the widespread assumption that interspecific hybridization is liable for this seeming conundrum (see section **1.1.4**).

However, contrary to what has been observed in other coral study systems (see van Oppen et al., 2000; Vollmer and Palumbi, 2002; Miller and van Oppen, 2003), no evidence suggested hybridization potential between these *Acropora* species (Ramírez-Portilla et al., 2022a). Robust results were not only obtained using HTS-derived approaches (i.e., target enrichment followed by genomic sequencing) as in recent studies (Erickson et al., 2021; Oury et al., 2023), but also traditional ones (i.e., PCR amplification followed by Sanger sequencing). Indeed, results obtained using genetic clustering, allele sharing, and coalescent-based multilocus approaches supported the ILS hypothesis, thus, challenging the generally accepted idea that morphospecies of *Acropora* cannot be distinguished using molecular approaches because of hybridization (see Miller and van Oppen, 2003).

By disregarding the reciprocal monophyly criterion or a genetic barcode gap detection, methods used by Ramírez-Portilla et al. (2022a) were sensitive enough to delineate closely-related species boundaries. The availability of a multilocus HTS-derived data set and the multispecies coalescent (MSC) model, for instance, enabled species tree estimation while accounting for unresolved lineage sorting (Mirarab and Warnow, 2015; Rabiee et al., 2019). Likewise, mutual allelic exclusivity implementation through haplowebs and conspecificity matrices allowed more sensitive species delineation because it is more likely that two species stop sharing sequences earlier while diverging than reaching reciprocal monophyly at most of their loci (i.e., mutual exclusivity can be reached before or at the same time as reciprocal monophyly; Doyle, 1995; Flot et al., 2010). Hence, alternative strategies overcame potential limitations due to previously unwarranted expectations likely emerging from deep coalescence. In this way, Ramírez-Portilla et al. (2022a) demonstrated the feasibility of improving confidence in coral species boundaries by illustrating that *Acropora* species are reproductively isolated, independently evolving, and morphologically distinct.

4.1.3 | One type may not fit all: evaluating novel approaches for coral species delimitation

Most complex-shaped organisms such as corals and other marine invertebrates lack characteristic features, which hinders the robust delineation of phenons or phenotypically distinct groups (Kaandorp and Kübler, 2001). Yet, this thesis aimed to evaluate the congruence between independent assessments of species boundaries. Hence, the main objective of Ramírez-Portilla et al. (2022b) was to gauge the potential of novel approaches stemming from 3D-morphological analyses to delineate phenons congruent with *a priori* delimited species or discriminate *a posteriori* morphogroups among specimens of complex-shaped and taxonomically intricate coral taxa (**Chapter III**).

Ramírez-Portilla et al. (2022b) estimated three variable types from triangulated polygon meshes and medial axis skeletons derived from the 3D models of coral specimens from three closely-related species of *Acropora* previously delimited using independent lines of evidence (Ramírez-Portilla et al., 2022a): complexity, curvature, and branching. Univariate and multivariate analyses of phenotypic variation of these 3D variables allowed the detection of significant differences in central tendencies between *a priori*-defined species. In particular, a set of curvature features achieved enough resolution to capture the morphological differences between the specimens of the three complex-shaped coral taxa (Ramírez-Portilla et al., 2022b). Such features seem to adequately seize geometrically complex forms, such as corals, which are only represented satisfactorily in three dimensions (Kaandorp and Kübler, 2001; Courtney et al., 2007). Thus, curvature measures emerged as promising alternative descriptors of shape.

In lay terms, the curvature (k) is the extent to which a surface departs from a plane, in which the sign indicates either its convex ($k > 0$) or concave ($k < 0$) nature (Hyde et al., 1997). The biological significance of curvature stems from and shapes the behavior of organisms at multiple scales (e.g., molecules, cells, organs, systems; Schamberger et al., 2023). Studies in several organisms support curvature's potential for improving our understanding of the link between morphology and other traits (e.g., Walker, 2000; Basu et al., 2007; Moulia and Fournier, 2009; Porter et al.,

2009). In coral reefs, curvature measures have been used to characterize seascape surfaces and assess their potential as drivers of ecological patterns in marine organisms (e.g., Wilson et al., 2007; Pittman et al., 2009; Pittman and Brown, 2011; Burns et al., 2015; Robert et al., 2015; Fukunaga and Burns, 2020). Yet, curvature estimation in coral shape research has received much less attention despite previous studies have pointed to its prospective importance. For instance, growth modeling has shown that curvature governs the amount of contact between a coral and its environment (Kaandorp and Sloot, 2001). Given that such features determine the access of corals to resources in the water column, curvature's role stands key in the survival of sessile organisms and explains why higher growth rates in corals generally occur at convex areas of high curvature (Soong and Lang, 1992; Merks et al., 2002). Yet, this study is among the first approximations to measure coral curvature and understand its intra- and interspecific variation (Ramírez-Portilla et al., 2022b). Future studies should focus on strengthening the link between morphological variables of taxonomic significance as curvature and the response of coral species to anthropogenic-driven change (Zawada et al., 2019b).

Statistical analyses by Ramírez-Portilla et al. (2022b) revealed considerable overlapping of species morphospaces, endorsing what has been formerly reported in *Acropora*; high intraspecific variability coupled with interspecific similarity (see Wallace and Willis, 1994; Wallace, 1999). Indeed, morphogroups delineated without *a priori* information were not congruent with species boundaries assessed using other lines of evidence (Ramírez-Portilla et al., 2022a). Moreover, quantitative variables derived from skeleton measurements displayed lower species resolution than qualitative characters for *Acropora* species as in previous studies (Wallace et al., 1991; Wolstenholme et al., 2003; Ramírez-Portilla et al., 2022a). These results emphasize the difficulties in assessing relevant features that enable detecting morphological gaps and nonoverlapping patterns used to hint at species boundaries using morphological information (Sites and Marshall, 2004; Zapata and Jiménez, 2012).

Evidence for delineating coral species boundaries using morphology has been largely obtained from hierarchical clustering analyses and differences in central tendencies (e.g., Wolstenholme et al., 2003; Filatov et al., 2013; Reichert et al., 2017; Ramírez-Portilla et al., 2022a). However, such widespread approaches tend to force data into groups, either disregarding the nature of variation at a specific and infraspecific level or assuming that statistic mean differences between samples can sustain the delineation of phenons (Zapata and Jiménez, 2012). Consequently, neither of the previous approaches holds robustly in the light of evolutionary theory (Cadena et al., 2018). In contrast, discontinuities in the morphospace defined by quantitative traits suggest the existence of barriers or evolutionary forces preventing homogenization under the assumptions of polygenic phenotypic variation and random mating (Wiens and Servedio, 2000; Sites and Marshall, 2004; Zapata and Jiménez, 2012). In this scenario, quantitative characters would reasonably tend to be normally distributed, and detection of distinct distributions or components in a sample could lead to draw species boundaries (Cadena et al., 2018). The study by Ramírez-Portilla et al. (2022b) constitutes the first implementation of such an approach (i.e., normal mixture models - NMMs) to assist in coral species delineation. Two morphogroups were identified by those analyses, contrasting with the three robustly supported species hypotheses in the data set (Ramírez-Portilla et al., 2022a). Because identifying the number of components in the underlying distributions is the most methodologically challenging problem associated with mixture models (McLachlan et al., 2019), the heterogeneous intraspecific variation of characters seems to hamper the delineation of phenons in this system . Indeed, more than one component was identified within each morphospecies' distribution, suggesting that intraspecific variation may interfere with denoising species-specific signals in the samples (Ramírez-Portilla et al., 2022b). Moreover, due to the recent application of NMMs in species delimitation, limitations arise from the scarce knowledge of their implementation, particularly regarding the selection of variables for this type of analysis (Cadena et al., 2018). However, forthcoming studies can benefit from active ongoing research on this subject (Scrucca, 2010; Scrucca et al., 2016; Scrucca and Raftery, 2018; McLachlan et al., 2019).

The selection of informative variables by Ramírez-Portilla et al. (2022b) allowed the identification of a prospective combination of characters that allowed clear *a posteriori* discrimination of *Acropora* phena consistent with other lines of evidence (Ramírez-Portilla et al., 2022a). In this context, 3D-variable screening provided enough resolution for discriminating closely related species and opened a promising avenue for tackling long-standing challenges in species delimitation of complex-shaped taxa such as corals (Ramírez-Portilla et al., 2022b). Yet, comprehensive research is needed to understand the variation of 3D-morphological variables in coral species across geographic and environmental ranges. Also, given that feature selection largely relies on the overall morphology of the studied organisms to avoid issues that stem from the exclusion of important characters during dimensionality reduction (Cadena et al., 2018), "one type" of variables "may not fit all" cases of study. Thus, comparative studies in other coral growth forms, such as massive and encrusting, are needed to evaluate the informative value of 3D variables assessed by Ramírez-Portilla et al. (2022b) in those systems. Ultimately, these results advocate for the integrative use of variables derived from 3D-morphological approaches with other lines of evidence both to delineate species boundaries and deepen our understanding of morphological variation in complex-shaped organisms.

4.2 | Future avenues and perspectives

Understanding how advancing taxonomy can support conservation has become paramount following fast-paced global change and consequent biodiversity losses (Bellard et al., 2012). For threatened ecosystems such as coral reefs, the link between taxonomy, functional ecology, and ecosystem services is being reshaped to reflect the challenge of facing the Anthropocene while acknowledging different ecological roles and susceptibilities of coral species to growing stressors (Woodhead et al., 2019; Zawada et al., 2019b). In this context, the taxonomy and conservation of tabular *Acropora* species have become a pressing need due to their high vulnerability, low functional redundancy, and role in maintaining ecosystem services (Alvarez-Filip et al., 2013; Ortiz et al., 2021).

However, beyond the general notion that biodiversity correlates with services provided by ecosystems, the motivation behind taxonomic research for supporting their conservation remains concealed to a large extent (Mace et al., 2012). Lessons learned in agroecosystems have shown that the economic pay-off behind the ecosystem-services argument has led to conservation strategies targeting species that provide the best short-term cost-benefit ratio (Schwartz et al., 2000). Yet, such approaches generally thwart the conservation of rare threatened species that usually do not respond to simple management actions (Kleijn et al., 2015). Based on the premise that a few abundant species can provide the majority of functions, conservation approaches stemming from an ecosystem services perspective suggest that relatively little biodiversity could support function (Ridder, 2008). Biodiversity, however, is crucial to sustaining ecosystem functions in the long term (Lemanski et al., 2022). Therefore, motivation for improving taxonomy that supports conservation initiatives in reef ecosystems will require more than just ecosystem service-based arguments if we are to preserve coral reef biodiversity over time.

Accurate delineation of species boundaries, underpins our understanding of evolutionary trends in response to global change and supports biodiversity estimates, both of which are the basis of research directed to ecosystem management (Agapow et al., 2004). Studies targeting potential cryptic species, for instance, have shown that failure to identify coral species boundaries leads to unwarranted conclusions about susceptibility to stressors and distribution ranges, thus, underestimating potential threats for these species (Boulay et al., 2014; Dziedzic et al., 2019; Nakabayashi et al., 2019; Gómez-Corrales and Prada, 2020). However, despite the consequences of poor taxonomy in conservation have been discussed extensively (Gaston and Rodrigues, 2003; Isaac et al., 2004; Mace, 2004; Bortolus, 2008; Vogel Ely et al., 2017), effectively translating taxonomic knowledge to a biodiversity currency that conservation and management can use remains a path to be walked (Gaston and Mound, 1993; Dubois, 2003; Hortal et al., 2015).

This thesis shows how current efforts to rebuild

coral taxonomy can significantly benefit from the lessons gained throughout its "brief" two-century history (see **Chapter I** and Fig. 1). Revisiting and actively integrating type material, for instance, should be increasingly prioritized as it is the only way to effectively link nomenclature with taxonomy and elucidate taxonomic quandaries from the root (Bonito et al., 2021). Indeed, pairing robust taxonomic hypotheses with newly collected specimens acquires particular relevance in light of the genomic revolution that coral research is experiencing (Coates et al., 2018; Voolstra et al., 2021). Therefore, to move forward with novel approaches, it is necessary to take a few steps back and review the standing species hypotheses in light of the data at hand (Pante et al., 2015; Ramírez-Portilla et al., 2022a).

Development and validation of novel approaches for coral species delimitation will require diverse study systems that provide the training ground for taxonomical practices (e.g., Ramírez-Portilla et al., 2022a, 2022b). However, selecting such systems call for biological, ecological, evolutionary and physiological insights to promote using miscellaneous lines of evidence that supply a comprehensive perspective of species boundaries (e.g., Baird et al., 2021, 2022). In that context, a better understanding of corals' natural history is needed if the gap between delimiting species and speciation research is to be bridged. For instance, advances in phylogeographic models and fields like seascape genomics may provide alternative tools to study coral species boundaries, their diversity patterns and the processes driving them (Riginos et al., 2016; Bongaerts et al., 2021; Prata et al., 2022). Incorporating thorough knowledge about corals will be critical for accurate species delimitation and understanding resilience and global responses of reef-building ecosystems to growing stressors in future studies (see Richards et al., 2016; Gómez-Corrales and Prada, 2020; Burgess et al., 2021). Expanding the geographical coverage and integrating available sources of information, for instance, is crucial to avoid biased conclusions about coral species boundaries worldwide (Naciri and Linder, 2015). In the end, providing robust species hypotheses will support coral-based research conclusions in every discipline that counts on the accuracy of taxonomical assumptions. Moreover, establishing the incidence of evolutionary processes in nature, such as coral hybridization, will impact conservation and management endeavors, particularly in corals and taxa severely threatened by climate and anthropogenic disturbances (Richards and Hobbs, 2015; Mao et al., 2018; Hobbs et al., 2022).

4.3 | Concluding remarks

> *"Before anyone engaged in the study of living creatures attempts to classify or arrange their material, it is essential that they should first observe, to the best of their ability, the life-processes of those creatures the affinities of which they would determine"*
>
> (Wood-Jones, 1907)

It is of the utmost importance to acknowledge that the advancement of taxonomy in any branch of the tree of life heavily relies on having a comprehensive picture of the target study systems. In this context, this thesis brings insights into the advances and challenges of species delimitation in corals. The evolution of coral taxonomy through the past two and a half centuries showcased how the foundation of most of today's successful approaches to delineate coral species boundaries evolved from basic knowledge of ecology, biology, physiology and the evolution of these organisms. Thus, the need to preserve prior knowledge in the face of implementing novel approaches is clear. Undoubtedly, there is still so much to learn about the complex process of speciation that we can't lose sight of the fact that species delimitation provides taxonomic hypotheses, and as such, they are subjected to the possibility of being proven false. Hence, the criteria used for species delimitation need to be openly stated in each study if the taxonomic feedback system is to be efficient.

The challenge embodied by the taxonomically intricate *Acropora* coral genus facilitated demonstrating these points. The tabular *Acropora* corals, in particular, display various characteristics of species that have diversified recently or that share a significant number of traits, making it plausible that processes such as gene flow and incomplete lineage sorting may be at interplay in this study case. As suspected from data collected in other systems, the results of mainstream molecular approaches and markers were at odds

with other lines of evidence. In contrast, methods that circumvent potential limitations emerging from deep coalescence robustly resolved species boundaries between the *Acropora* in the present study case. Therefore, contrasting with the seeming conundrum observed in other study systems, no evidence suggested any potential for hybridization between these species.

Additionally, careful screening and informative feature selection allowed to outline characters, approaches, and criteria with potential discriminative power at the species level. It also illustrated how novel approaches, such as 3D quantitative morphology, do not always outperform traditional methods to delineate specions. Overall, using approaches sensitive enough to capture morphological, reproductive and molecular differentiation allows delineating boundaries between closely related and hard-to-distinguish species. Ultimately, comparing independent lines of evidence improved confidence in coral species boundaries and paved the way for a future taxonomic revision of scleractinian corals

4.4 | References

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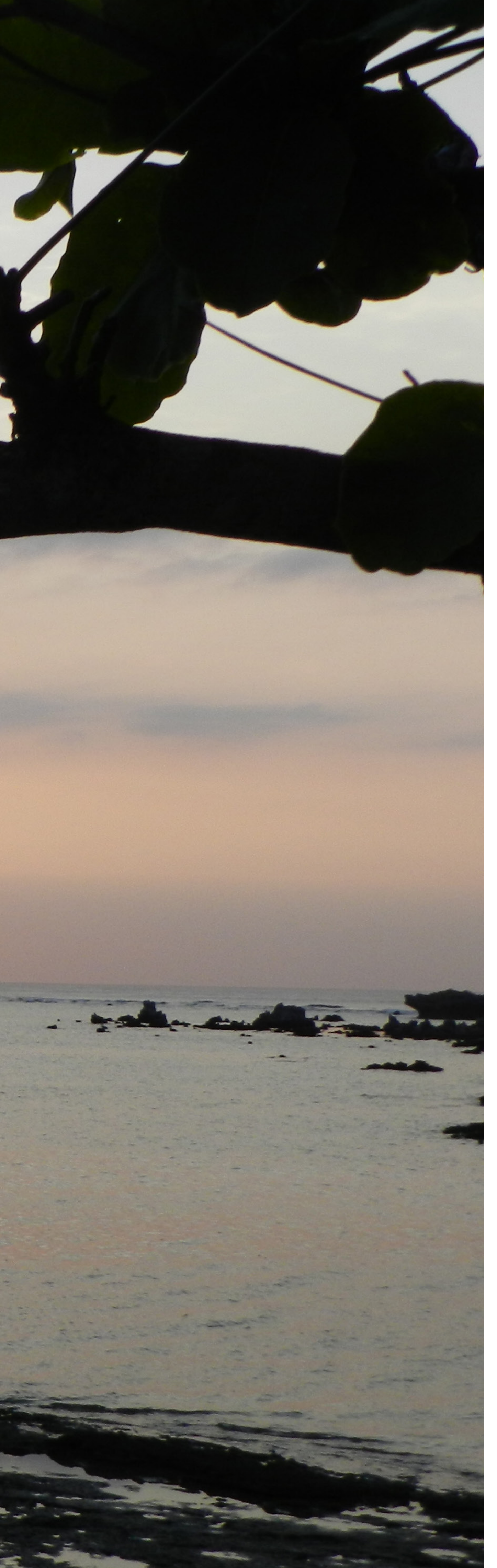
Sunset from Bisezaki point
Motobu, Okinawa, Japan
Photo by C. Ramírez-Portilla



Appendix

Chapter II: The tabular *Acropora* as case study for coral species delimitation

Supplementary material

Title:

Solving the Coral Species Delimitation Conundrum

Authors:

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This file includes:

- Figures S1 to S5
- Tables S1 to S10
- Supplementary References

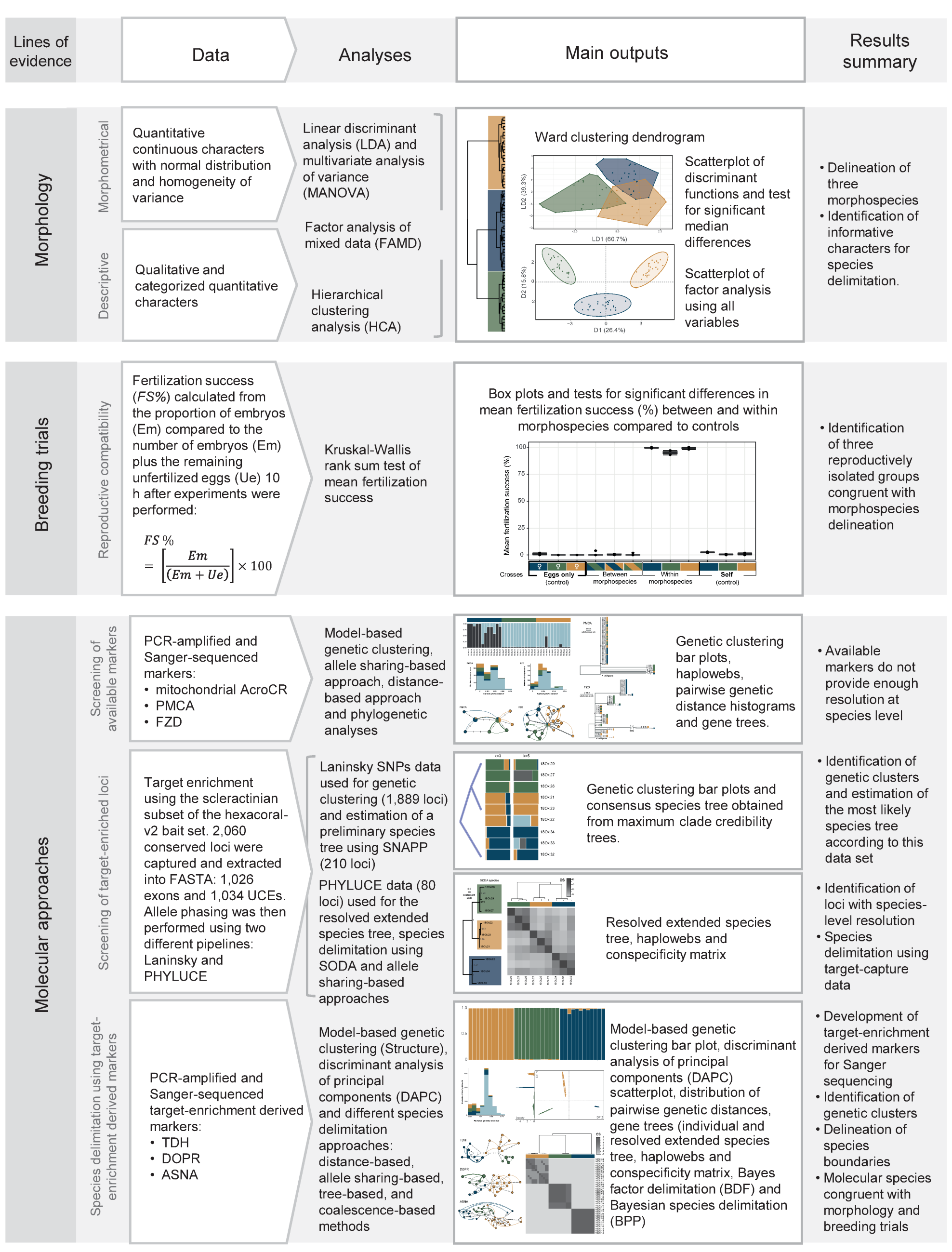
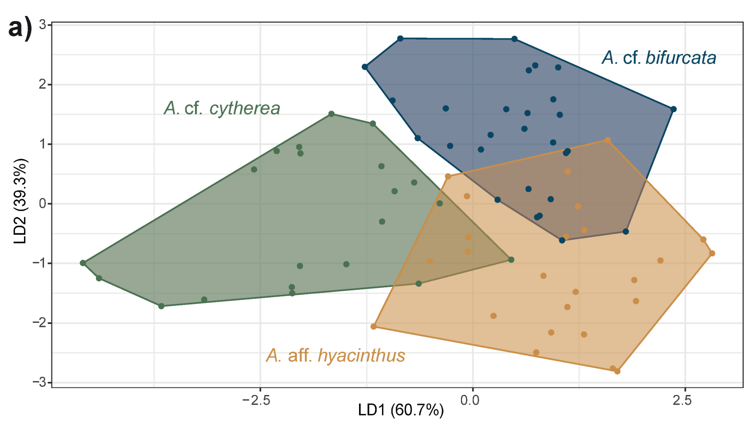


FIGURE S1. Multiple lines of evidence used to delineate species boundaries in the tabular *Acropora* from Sesoko Island (Okinawa, Japan). A summarized workflow of the lines of evidence used in this study is presented. The data used, the analyses, the main outputs and a brief summary of the results are shown.



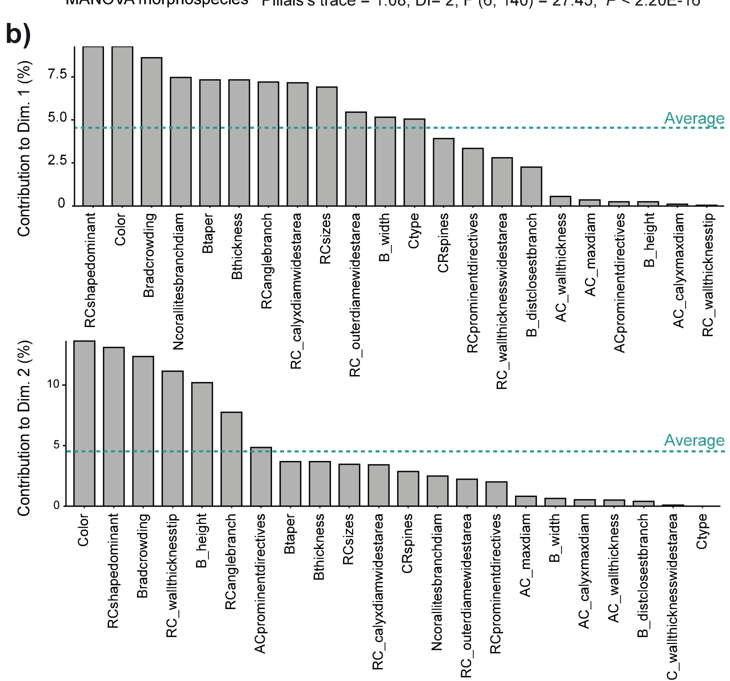


FIGURE S2. Multivariate analyses of morphological characters. a) Linear discriminant analysis (LDA) of continuous variables exhibiting 81.08% accuracy in discriminating between morphospecies (n= 74): B_width (r = 0.49, P < 0.001), B_height (r = 0.51, P<0.001) and B_distclosestbranch (r = 0.19, P < 0.001). The result of a one-way MANOVA test for these variables is also displayed. Additionally, statistically significant differences between species were found when using post-hoc univariate ANOVA tests for each dependent variable (n= 74, P < 0.001 for each, see details in Dataset S1). b) Individual contribution (%) of each character to the mapping dimensions 1 (left) and 2 (right) of the FAMD. Each variable code corresponds to those described in Tables S1 and S2. The dashed lines indicate the average contribution value (%) for each dimension.

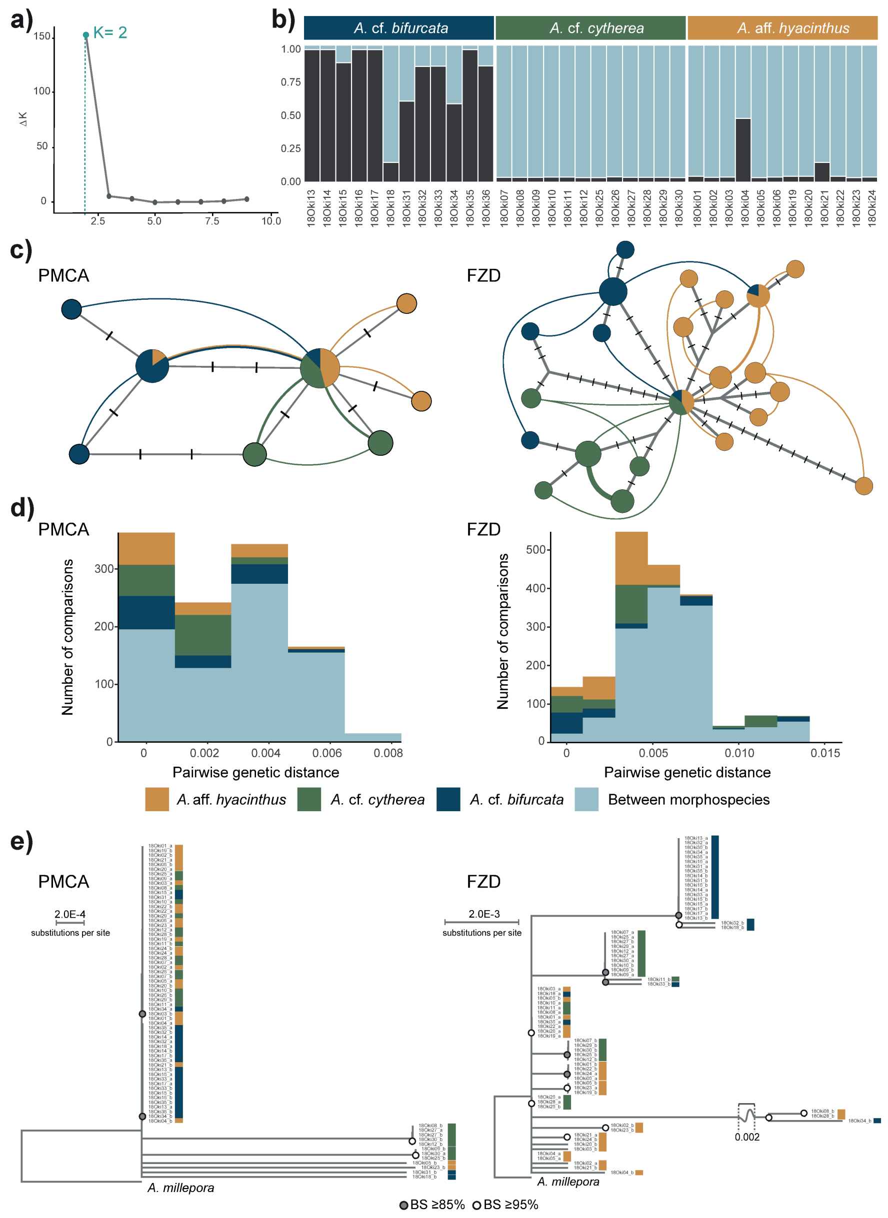


FIGURE S3. Preliminary screening of PMCA and FZD markers. a) Evanno ΔK plot suggesting the most likely K value (dashed line) for model-based genetic clustering. b) STRUCTURE plot obtained by assigning the probability of individual membership with K=2. b) Allele sharing-based haplowebs with shades corresponding to the groups delineated by morphology and breeding trials. c) Histograms of pairwise comparison of genetic distances within and between morphospecies. d) Ultrafast bootstrap trees for each phased marker with bootstrap support (BS) of 85-94% indicated by grey and BS ≥ 95% by white circles at the corresponding nodes. Alleles for each individual are differentiated by the suffixes "_a" and "_b".

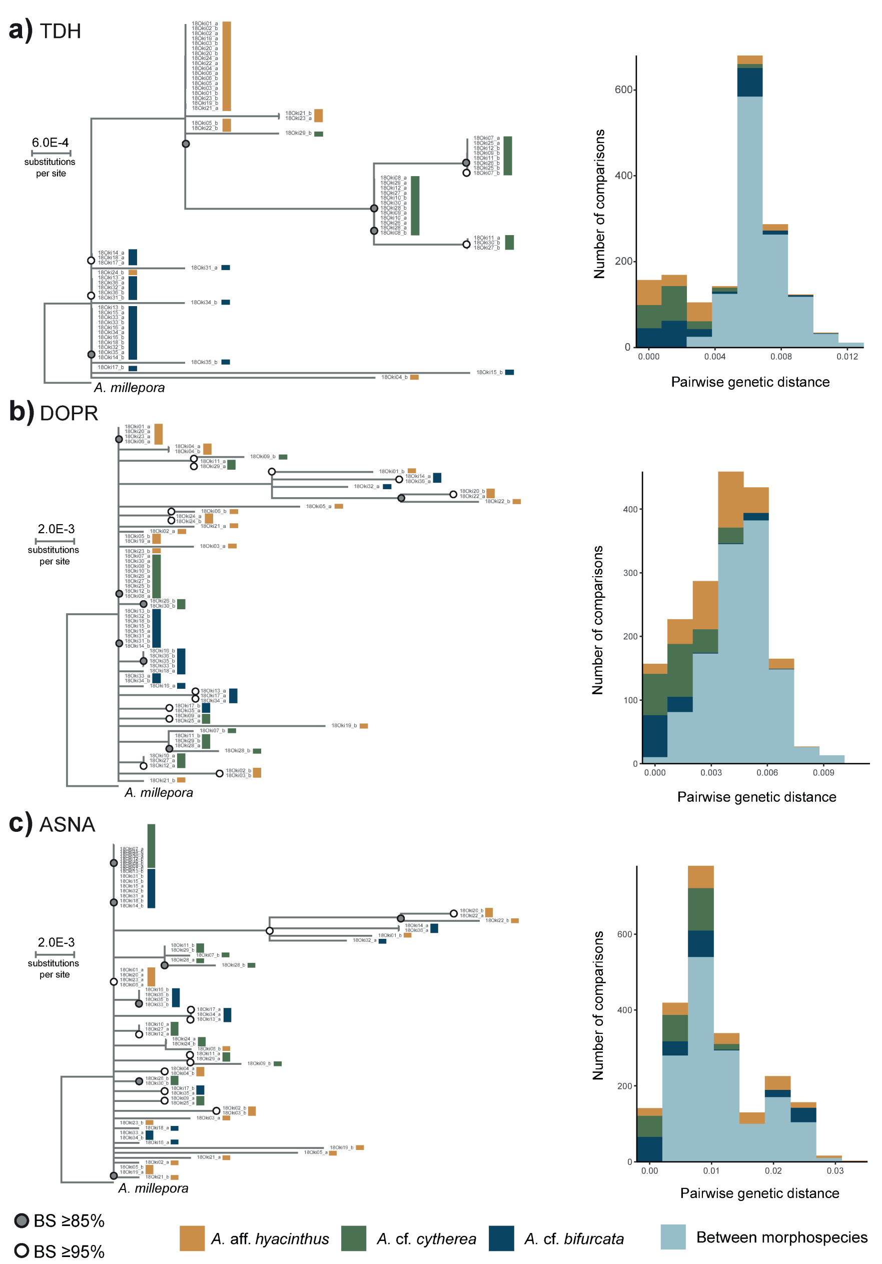


FIGURE S4. Phylogenetic trees and genetic distance histograms for each marker derived from target-capture sequencing. Ultrafast bootstrap trees and pairwise genetic distances comparison within and between morphospecies for markers defined from target-capture sequencing: a) TDH, b) DOPR and c) ASNA. In the trees, nodes with less than 85% of support were collapsed whereas bootstrap support (BS) of 85 to 94% is indicated by grey and 95% by white circles at the corresponding nodes. Alleles of each individual are differentiated by the suffixes "_a" and "_b".

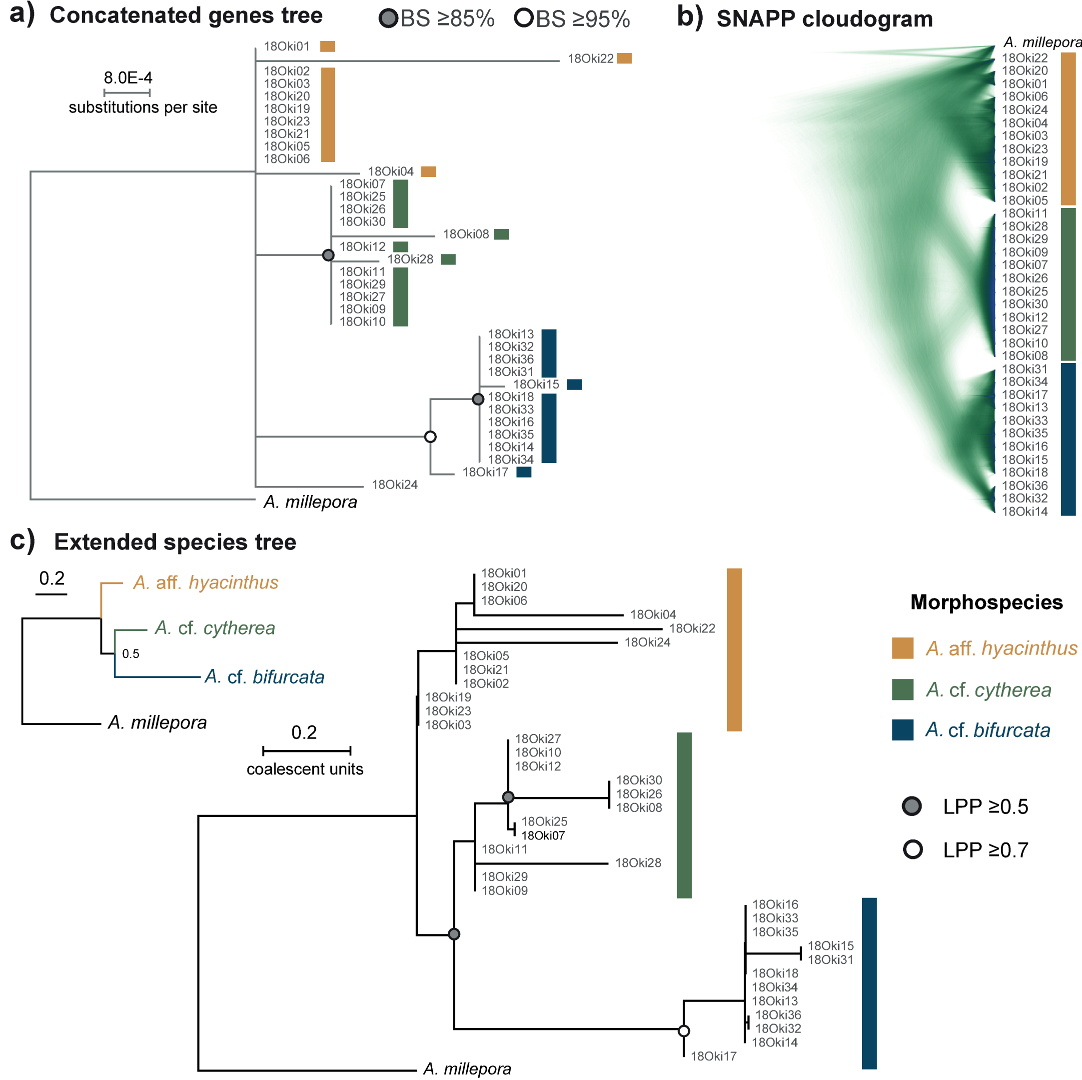


FIGURE S5. Concatenated gene and species trees for the three target enrichment-derived markers. Phylogenetic trees of three target capture derived loci (TDH, DOPR and ASNA) shaded according to the primary species hypotheses (PSHs) regarding to morphospecies. a) Ultrafast bootstrap tree for the concatenated IUPAC consensus sequences of the three markers. Nodes with less than 85% of bootstrap support (BS) were collapsed. b) SNAPP cloudogram using SNP information extracted from the target enrichment defined markers, where each individual was allowed to be a terminal tip (i.e. without constraining individuals into species). c) ASTRAL extended species trees obtained by mapping alleles to individuals, where both morphospecies were monophyletically constrained (inset) and left unconstrained (main tree, resolved extended species trees). Nodes with less than 10% of local posterior probability (LPP) were collapsed.

TABLE S1. Qualitative (QL) and quantitative (QN) characters used for morphological taxonomic assessment.

Type	No.	Code	Character	(Coding) States / description
QL	1	Color	Colony color in the field	(1) yellow-brown; (2) darker-brown; (3) orange-brown
	2	Bthickness	Relative contribution of corallites to B thickness	(1) axial-dominated; (2) 50/50; (3) radial-dominated
	3	Btaper	Branch taper	(1) tapering; (2) terete
	4	Bradcrowding	Radial crowding (density) along the B	(1) radials do not touch; (2) some radials touch; (3) radials touching
	5	ACshape	Axial corallite dominant shape	(1) tubular; (2) conical; (3) barrel
	6	ACprimaryseprelradius	Relation of AC primary septum to R	(1) <1/4; (2) 1/4 to 3/4; (3) >3/4 R
	7	ACprominentdirectives	Number AC prominent directives	(0) 0; (1) 1; (2) 2
	8	RCshapedominant	RC dominant shape	(1) rounded lip; (2) flaring lip; (3) straight lip
	9	RCsizes	RC sizes	(1) one size; (2) two sizes; (3) mixed; (4) increasing down branch
	10	RCprimaryseprelradius	Relation of RC primary septum to R	(1) <1/4; (2) 1/4 to 3/4; (3) >3/4 R
	11	RCprominentdirectives	Number of RC prominent directives	(0) 0; (1) 1; (2) 2
	12	RCanglebranch	Approximate angle of the RC to the B	(1) 0-30º; (2) 30-60º; (3) 60-90º; (4) 90º
	13	CRtype	Coenosteum type on RC	(1) costate; (2) reticulate; (3) spinous
	14	CRspines	Coenosteum spines on RC	(0) none; (1) simple; (2) forked; (3) elaborate
	15	Ctype	Coenosteum type between RC	(1) costate; (2) reticulate; (3) spinous
	16	Cspines	Coenosteum spines between RC	(0) none; (1) simple; (2) forked; (3) elaborate

	17	B_width	Branch width	Diameter at the base of the B
	18	B_height	Branch height	Distance from tip to base of the B
	19	B_distclosestbranch	Distance to the closest branch	Distance from outer wall of AC to AC outer wall in the nearest B
	20	AC_calyxmaxdiam	Maximum diameter of axial calyx	Maximum distance between inner walls of the AC
	21	AC_maxdiam	Maximum diameter of axial corallite	Maximum distance between outer walls of the AC
QN	22	AC_wallthickness	Axial wall thickness	Difference between maximum diameters of the AC and calyx dived by two (21-20)/2
	23	Ncorallitesbranchdiam	Number of corallites per B	RC per branch diameter at point where B stops tapering
	24	RC_calyxdiamwidestarea	Radial calyx diameter in the widest area	Maximum distance between inner walls of the RC
	25	RC_outerdiamewidestarea	RC diameter in the widest area	Maximum distance between outer walls of the RC
	26	RC_wallthicknesswidestarea	RC wall thickness in the widest area	Wall width at the widest area of the RC
	27	RC_wallthicknesstip	RC wall thickness at the outer tip	Wall width at the outer tip of the RC

Color (No. 1) was assessed from photographs taken from each coral colony. Descriptive characters (2 – 16) were recorded from overall observation of skeletal fragments. Morphometric characters (17 – 19) were measured directly from the branches using Vernier callipers. Corallite features (20 – 27) were obtained using a stereo microscope and an ocular graticule (except for 23 that was counted from above). Character code: branch (B), axial corallite (AC), radial corallite (RC), radius (R).

TABLE S2. Transformation of quantitative morphometric variables.

Type	Code	N (*P*)	H (*P*)	Transformation
QN	B_width	0.0952	0.2637	N/A
	B_height	0.1766	0.1111	N/A
	B_distclosestbranch	0.0755	0.9618	N/A
CT	AC_calyxmaxdiam	<0.001	0.5962	Discretization: 3 L, B= [0.60, 0.78, 0.85, 1.04]
	AC_maxdiam	0.0052	0.5484	Discretization: 4 L, B= [1.34, 1.5, 1.68, 1.85, 2.01]
	AC_wallthickness	0.0002	0.2338	Discretization: 3 L, B= [0.15, 0.20, 0.23, 0.28]
	Ncorallitesbranchdiam	0.0011	0.0230	Discretization: 3 L, B= [5, 7, 9, 12]
	RC_calyxdiamwidestarea	<0.001	0.0214	Discretization: 3 L, B= [0.60, 0.70, 0.85, 1.04]
	RC_outerdiamewidestarea	0.0023	0.2130	Discretization: 3 L, B= [1.04, 1.25, 1.50, 1.79]
	RC_wallthicknesswidestarea	<0.001	0.5247	Discretization: 3 L, B= [0.11, 0.14, 0.18, 0.22]
	RC_wallthicknesstip	<0.001	0.0079	Discretization: 3 L, B= [0.22, 0.28, 0.35, 0.45]

A Shapiro-Wilk test for normality (N) and a Levene test for homogeneity of variances (H) was performed for each quantitative variable with a significance level (α) of 0.05. Quantitative variables that exhibited normal distribution and homoscedasticity ($P>\alpha$) were analyzed as continuous numeric variables (QN). The variables that did not conform to these assumptions (even after applying the optimal transformation using the bestNormalize R package v1.5.0) were discretized into categorical variables according to their distribution (arules R package v1.6-5), and analyzed along with the qualitative characters (CT). Number of levels (L) and breaks (B) are shown for each of those variables.

TABLE S3. Summary of techniques, loci and methods used in the different stages of the molecular analyses performed in this study.

Stage	Molecular technique	No. loci/markers [n= samples]	Pre-processing		Downstream analyses
Preliminary screening of available molecular markers	PCR-based amplification followed by Sanger sequencing	Three genetic markers (AcroCR, PMCA, FZD) [*n*= 36]	Chromatograms edition, sequence alignment and phasing		Genetic clustering, genetic distances and gene trees
Screening of target-enriched loci	Target enrichment and high-throughput sequencing of conserved elements (exons and UCEs) captured using the hexacoral-v2 bait set	2060 loci (1026 exons, 1034 UCEs) [*n*= 9]	Reads de-multiplexing and trimming, contigs assembly and probe matching	Phasing Laninsky pipeline Phasing PHYLUCE pipeline	Genetic clustering (1889 loci), SNAPP species tree (210 loci) Allele sharing-based approaches and extended species trees (79 loci)
Implementation of target-enrichment derived markers in molecular species delimitation	PCR-based amplification followed by Sanger sequencing	Three genetic markers (TDH, DOPR, ASNA) [*n*= 36]	Chromatograms edition, sequence alignment and phasing		Genetic clustering, genetic distances, gene trees, species trees, coalescent and allele sharing-based approaches

Detailed information about the techniques, the number of loci, the number of individual samples, and the general pre-processing steps and downstream analyses used at each stage of the molecular approaches used in this study.

TABLE S4. Samples included in the target enrichment sequencing.

Sample ID / Target enrichment ID	SRA accession ID	#C	Mean cov	# Loci (total) UCE / exon	Mean length (bp) UCE / exon
18Oki21 / Acropora_CFhyacinthus1C282	SAMN16242367	17611	22.5	1278 675 /603	1019.7 / 1091.3
18Oki22 / Acropora_CFhyacinthus1C283	SAMN16242368	14902	28.8	1322 680 / 642	1085.9 / 1119.5
18Oki23 / Acropora_CFhyacinthus1C284	SAMN16242369	9907	22.8	1419 717 / 702	895.9 / 949.2
18Oki26 / Acropora_CFcytherea5C285	SAMN16242370	6718	11.3	1686 873 / 813	602.4 / 599.8
18Oki27 / Acropora_CFcytherea5C286	SAMN16242371	8530	21.3	1533 792 / 741	812.1 / 838.6
18Oki29 / Acropora_CFcytherea5C287	SAMN16242372	13314	19.6	1442 731 / 711	1001.4 / 1057.3
18Oki32 / Acropora_CFbifurcataC288	SAMN16242373	23124	38.8	1400 698 / 702	1131.2 / 1183.7
18Oki33 / Acropora_CFbifurcataC289	SAMN16242374	11723	16.2	1465 742 / 723	838.0 / 847.4
18Oki34 / Acropora_CFbifurcataC290	SAMN16242375	15105	23.2	1301 676 / 625	1068.9 / 1132.0

Summary of pre-processing statistics of the contigs assembled for the subset of tabular *Acropora* samples. For these samples, target enrichment sequencing was performed using a re-designed set of baits for Hexacorallia that included loci flanking both UCEs and exons (Cowman et al. 2020). Using this target capture approach, 2,060 loci (1,026 exons and 1,034 UCEs) were recovered for the nine samples. Sequence Read Archive (SRA) accession numbers for the raw data are also shown and are gathered under the Bioproject PRJNA665126. Number of contigs (#C), Mean coverage (Mean cov).

TABLE S5. Primers and conditions for PCR-based amplification and Sanger sequencing.

Loci (GenBank IDs)	PCR primers (5' - 3')	PCR conditions	Sequencing primers (5' - 3')	Product length (bp)
AcroCR (MT945838 - MT945873)	AcroCR-F[a]: GCCCCTCAAGAGGGTTTCTA AcroCR-R[a]: CTAGACAGGGCCAAGGAGAAG	Ta: 55º 55 cycles	Same as for PCR amplification	1265 – 1352
PMCA (MT945609 - MT945656)	PMCA-F[b]: AAGGAATTGGTGGCTTTCCT PMCA-R[b]: CACAGACGACCATCTTTCCA	Ta: 53º 50 cycles	PMCA-Fint[b]: GAATTGGTGGCTTTCCTGAG PMCA-Rint[b] : CGACCATCTTTCCACTACCTTC	545
FZD (MT945657 - MT945718)	5491-F[b]: TATGGCTGCGACAATTTGGT 5491-R[b]: GCTAGCGTTTCGAGTTCCAC	Ta: 55º 50 cycles	5491-Fint[b]: CCTTGAGTTGGTTCCTTGCT 5491-Rint[b]: TCGAGTTCCACCGTTCTTCT	639
	FZD-F[b]: CCTTGAGTTGGTTCCTTGCT FZD-R[b]: CGCCTAGACAGCAGCTAAAA		Same as for PCR amplification[c]	994 – 1006[d]
TDH (MT945719 - MT945777)	TDH-F[b]: TTTTTCTTTCACTTTTGGCTGT TDH-R[b]: ATCTCTGCTGCAATCCCAAT	Ta: 53º 50 cycles	Same as for PCR amplification[c]	736 – 744
DOPR (MT945778 - MT945837)	DOPR-F[b]: AGGGTCAGGTTTTTGGGAAT DOPR-R[b]: GAGTTTTGACCGTCAGTTGG	Ta: 53º 50 cycles	Same as for PCR amplification[c]	747 – 760
ASNA (MT945874 - MT945940)	ASNA-F[b]: CTGTGTGCTGGCGAAAAA ASNA-R[b]: GAAAGGCCCCTCTATTTTCA	Ta: 53º 50 cycles	Same as for PCR amplification[c]	748 – 763

[a] Primers designed and tested in-house.

[b] Primers from previous studies (Ladner and Palumbi 2012).

[c] Samples that proved difficult to amplify were re-amplified using M13-tailed PCR primers then sequenced using M13 primers M13F (TGTAAAACGACGGCCAGT) and M13R (CAGGAAACAGCTATGAC).

[d] Product length was extended by assembling contigs using sequences obtained with previously reported primers (Ladner and Palumbi 2012), and sequences obtained using primers designed in-house.

General PCR conditions: start 1 sec 95°C; 1 min 95°C; [30 sec 95°C; 30 sec Tº annealing (Ta); 2 min 72°C]x Number of cycles; 10 min 10°C. GenBank accession numbers (GenBank IDs) for the sequences obtained with each marker are also shown. Different internal primers (int) were used for sequencing in some cases.

TABLE S6. megaBLAST matches for the selected target capture loci and allelic exclusivity screening.

ID dataset	Accession numbers	Description	Code	FFRs gaps as 5th char.	FFRs masked gaps
Exon99029792	XM_029335609	*A. millepora* L-threonine 3-dehydrogenase	TDH	4	3
UCE111109	XM_015902484	*A. digitifera* dopamine receptor 2-like	DOPR	6	3
Exon2711	XM_029333081	*A. millepora* ATPase ASNA1 homolog	ASNA	7	4

The closest megaBLAST hit (accession numbers) is displayed for each of the loci derived from target enrichment sequencing along with a short description that was used to recode them accordingly throughout the text. The number of putative species or fields for recombination (FFRs) they delineated when used in the allele sharing-based approach based (both using gaps as a 5th character or masking them using HaplowebMaker; Spöri and Flot 2020) was used as proxy of their variability and resolution at species-level when compared to the primary species hypotheses (PSHs) derived from morphology.

TABLE S7. *Acropora* genome assemblies used for PCR primer design.

Species	Assembly version	Reference (source)
A. digitifera (Dana, 1846)	Adig_1.1	Shinzato et al. 2011 (https://www.ncbi.nlm.nih.gov/genome/10529)
A. millepora (Ehrenberg, 1834)	amil_sf_1.1	Ying et al. 2019 (https://www.ncbi.nlm.nih.gov/genome/2652)
A. hyacinthus (Dana, 1846)	Acropora_hyacinthus.discovar_002	Liew et al. 2016, ReFuGe 2020 Consortium 2015 (http://ahya.reefgenomics.org/)
A. palmata (Lamarck, 1816)	Apalm_assembly_v1.0	Kitchen et al. 2019 (requested at: http://baumslab.org/research/data/)
A. cervicornis (Lamarck, 1816)	Acerv_assembly_v1.0	

Acropora genome assemblies used to map nuclear loci in order to design primers that maximized target product length for each region.

TABLE S8. Results of the haploweb allele sharing-based approach to delineate species.

Markers	Feature	Total	Morphospecies		
			A. aff. *hyacinthus*	*A.* cf. *bifurcata*	*A.* cf. *cytherea*
TDH	FFRs	3	1	1	1
	Exclusive alleles	20	8	8	4
	Shared alleles	0	0	0	0
DOPR	FFRs	6	4	1	1
	Exclusive alleles	23	14	4	5
	Shared alleles	0	0	0	0
ASNA	FFRs	9	6	1	2
	Exclusive alleles	35	16	10	9
	Shared alleles	0	0	0	0

Haplowebs delineate putative species according to the fields for recombination (FFRs), or common allele pools that can be identified. The absence of shared alleles between morphospecies supports the primary species hypotheses (PSHs) based on morphology, using the criterion of allelic exclusivity as indirect evidence for reproductive isolation.

TABLE S9. Testing alternative species models with the SNAPP coalescence-based approach.

Model	Model description	No. of species	MLE	BF	Rank
1	A single species of tabular *Acropora*	1	-1233.87	-366.13	3
2	Current taxonomy: *A. cytherea* and *A. hyacinthus* (lump *A.* aff. *hyacinthus* + *A.* cf. *bifurcata)*	2	-1050.80	---	2
3	Morphology + breeding trials + genetic clustering: *A.* cf. *cytherea, A.* aff. *hyacinthus* + *A.* cf. *bifurcata*	3	-880.96	339.68**	1

The most likely species models were ranked according to the Bayes factor delimitation (BFD) by calculating the average marginal likelihood estimates (MLE) of five SNAPP-BEAST runs to perform pairwise comparisons between the current taxonomy (model 2) and the alternative species models using Bayes factors (Kass and Raftery 1995):

BF= 2 * [MLEx – MLE1] (see Grummer et al. 2014; Herrera and Shank 2016)

A positive BF value indicates support in favor of the alternative model, while a negative value indicates support of the current taxonomy (model 1) over the alternative one. BF values >10 (**) provide decisive support to distinguish between species models.

TABLE S10. Testing scenarios with different parameter prior distributions in Bayesian species delimitation using BPP.

ID	Scenario	Parameters	rjMCMC algorithm [parameters]	Most likely number of species [PP]	Best tree topology [PP]
1	Large ancestral population size and deep divergence of species	$\theta \sim$ IG(3, 0.2) mean = 0.1 $\tau_0 \sim$ IG(3, 0.2) mean = 0.1	0 [ε=2]	3 [1.0]	(B, (A, C)) [0.712165]
			1 [α= 2, *m*= 1]	3 [1.0]	(B, (A, C)) [0.630295]
2	Small ancestral population size and shallow divergence of species	$\theta \sim$ IG(3, 0.002) mean = 0.001 $\tau_0 \sim$ IG(3, 0.002) mean = 0.001	0 [ε=2]	3 [1.0]	(A, (B, C)) [0.768815]
			1 [α= 2, *m*= 1]	3 [1.0]	(A, (B, C)) [0.830950]
3	Large ancestral population size and shallow divergence of species[a]	$\theta \sim$ IG(3, 0.2) mean = 0.1 $\tau_0 \sim$ IG(3, 0.002) mean = 0.001	0 [ε=2]	3 [1.0]	(B, (A, C)) [0.435920]
			1 [α= 2, *m*= 1]	3 [1.0]	(B, (A, C)) [0.471725]

[a] Conservative combination of priors (large values for θ and small values for τ_0) that should favor models with a lower number of species (Leaché and Fujita 2010; McFadden et al. 2017).

Scenarios for testing the influence of three diffuse prior combinations (value 3 for the shape parameter), with inverse gamma distribution [IG(α, ß)] for population size (θ) and divergence time at the root of the species tree (τ_0) using Bayesian Phylogenetics and Phylogeography (BPP) (Yang 2015) for species delimitation. Posterior probabilities [PP] for the most likely number of species and the best tree topologies out of five runs are shown. Species in the topologies correspond to A) *A.* aff. *hyacinthus*, B) *A.* cf. *bifurcata*, C) *A.* cf. *cytherea.*

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Supplementary Material

1 Supplementary Materials and Methods

1.1 Data acquisition, model rendering and processing

The coral fragments were placed at 30° in a manual rotating plate with the help of a rubber wedge, to ensure that the corals were comprehensively documented and to decrease the effect of self-shading (Supplementary Figure 1). In this position, both the top (towards where the branches grow) and bottom faces (underneath the table-like shape), were registered over two full-plate rotations and at least three different angles from the rotating plate (0°, 45°, and 90°). In a few cases where the preliminary model (obtained using real-time fusion) displayed missing data (holes) in some complex areas of the coral morphology, additional angles (approx. 25° and 65°) and full-plate rotations were added to improve the quality of the rendered 3D model.

Following Reichert et al. (2016; 2017), the Artec Studio software was used to render and clean up the 3D models. First, fine serial registration was performed using a final mesh size of 0.2 mm. Global registration followed using a minimal distance of 10 and 100 iterations based on the geometry of the objects. Finally, sharp fusion was performed with 0.2 mm resolution, and holes were also filled by radius (max. hole radius=5). The small objects filter was applied to get rid of any artefacts generated by shading or reflection (mode: Leave_biggest_objects). The algorithm of this filter identifies all the outliers obtained after the fusion as all the objects that are not connected to the main object (in this case the coral fragment) and then it removes them, keeping only the object that includes most of the polygons. Additional editing was performed using the rectangular selection eraser when needed.

1.2 Polygon mesh-based estimations

1.2.1 Complexity

The function *vtkMassProperties* estimates the volume (*V*) with the aid of a discrete divergence theorem algorithm, and surface area (*SA*) by taking the sum of each face area of the polygon mesh (Alyassin et al., 1994). Two quantitative measures were extracted directly from *SA* and *V*, the surface-area-to-volume (*S/V*) ratio and the sphericity (*φ*). Sphericity is a measure to estimate the compactness of an object since it describes the ratio between the surface area of a sphere – the minimum surface area – with a volume equal to *V* to the actual value of *SA* (see Equation 1; Wadell, 1935):

$$\boldsymbol{\varphi} = \frac{\boldsymbol{\pi}^{\frac{1}{3}}(\boldsymbol{6V})^{\frac{2}{3}}}{\boldsymbol{SA}^{-1}} \quad (1)$$

1.2.2 Curvature

Gaussian curvature (*K*) and mean curvature (*H*) are two common characteristics of surface curvature. The Gaussian curvature of a point (*p*) on a surface is defined as the product of the two principal curvatures at that location:

$$K(p) = k1(p)\, k2(p) \quad (2)$$

where $k1$ is the maximum curvature and $k2$ is the minimum curvature at p. The average of the two principal curvatures is called the mean curvature:

$$H(p) = \frac{1}{2}(k1(p) + k2(p)) \tag{3}$$

However, for obtaining curvature values of a polygon mesh a discrete scheme is required. These schemes do not often derive surface curvatures from the principal curvatures, but rather look at the angle deficit. From the Gauss-Bonnet theorem, a discrete approximation of the Gaussian curvature for vertices in a triangle mesh was derived (Meyer et al., 2003). In this scheme, the curvature for vertex (v) can be obtained as follows:

$$K(v) = \frac{1}{A_{mix}(v)}\left(2\pi - \sum_{f \in F} \alpha f\right) \tag{4}$$

where f are the faces that have v as one of their vertices, α_f is the angle of face f at v, A_{mix} is the surface area associated to the vertex, which is either based on based on the surface area or the Voronoi region of v of each face depending on whether faces are obtuse or non-obtuse, respectively. In addition, Meyer et al. (2003) also proposed a scheme for the mean curvature of triangle meshes. Here, a mean curvature normal operator is derived as follows:

$$\mathbf{H}(v) = \frac{1}{2A_{mix}(v)} \sum_{e \in E} (cot\ \alpha_e + cot\ \beta_e)\ e \tag{5}$$

where E are the edges of v to its neighboring vertices v and α_e and β_e are the opposite angles that correspond to e. The actual mean curvature (H) is equal to:

$$H(v) = \pm\frac{1}{2}\ \|\mathbf{H}(v)\| \tag{6}$$

where the negative sign is added when $\mathbf{H}(v)$ does not follow the direction of the outward normal vector of the surface at v (Mesmoudi et al., 2010). Normal vectors were derived with *vtkTriangleMeshPointNormals*. Finally, the principal curvatures were obtained from K and H by using the definitions described at the start of this section and stated by the following equations:

$$k1(v) = H(v) + \sqrt{H(v)^2 - K(v)} \tag{7}$$

$$k2(v) = H(v) - \sqrt{H(v)^2 - K(v)} \tag{8}$$

In the rare case that H^2–K is negative, due to errors in the estimation, $k1$ and $k2$ were both set to H.

1.2.3 Medial axis skeleton-derived measures

The voxel thinning was performed with *itkBinaryImageThinningFilter3D* (Homann, 2007), an implementation of the algorithm by Lee et al. (1994) that results in single-voxel thin skeletons. The voxel skeletons were then transformed into graphs (G). To do so, each voxel of the voxel skeleton was

translated to a vertex (v) with coordinates corresponding to the location that the voxel represents and connected by edges using the method described in Reinders et al. (2000). In a graph, neighboring vertices are those connected to each other by edges and the degree of a vertex ($dG(v)$) corresponds to the number of neighboring vertices. All vertices with degrees greater than two ($dG(v)>2$) were considered junction vertices and the vertices with degrees equal to one ($dG(v)=1$) were considered endpoint vertices. A branch (b) was considered to be the set of neighboring vertices with $dG(v)=2$ and the two connected vertices with $dG(v)\neq2$. All the branches that contained an endpoint vertex were considered terminal branches (b_T), except for the branch with the highest average thickness that was considered the root branch (b_R). Terminal branches with less than four vertices were removed from the skeleton as they were likely spurious.

1.3 Global and average values variable assessment

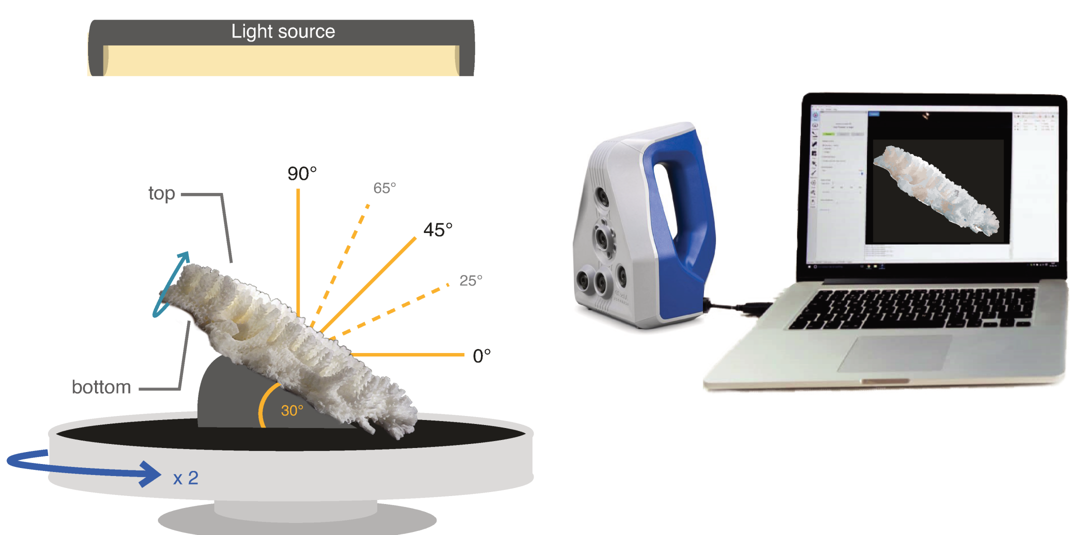
To evaluate the performance of individual features, we first tested the assumptions of normality (q-q plots, ggpubr R package v0.4.0; Kassambara, 2020; Shapiro-Wilk test, stats R package v4.1.0; R Core Team, 2018) and homoscedasticity (Levene's test; car R package 3.0-11; Fox and Weisberg, 2019) in the estimated univariate measures. All variables that deviated from the assumptions were transformed (TR) using the most frequently suggested option after five iterations of the *bestNormalize* function (bestNormalize R package v1.8.1; Peterson and Cavanaugh, 2020). Multivariate normality was then assessed using Mardia's, Henze-Zirkler's, Royston's, energy and Doornik-Hansen's tests (MVN R package v5.9; Korkmaz et al., 2014). Variables that did not meet assumptions of normality and homoscedasticity after transformation, were excluded from downstream analyses.

1.4 Discriminant analysis based on Gaussian mixture modelling

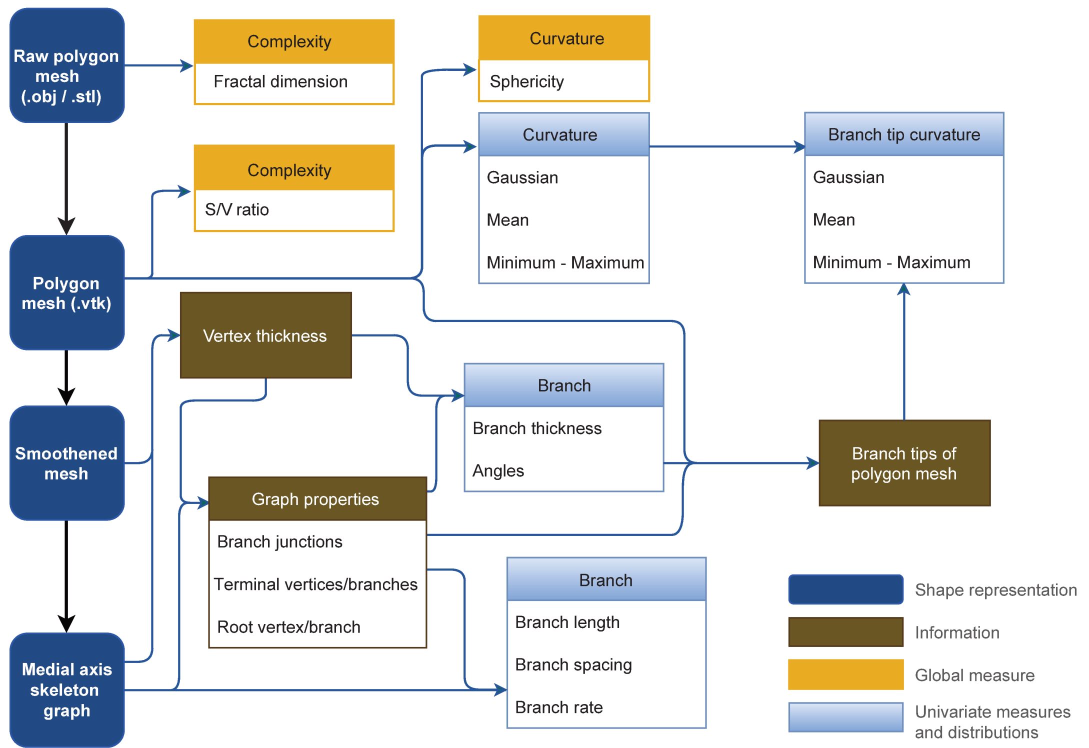
A discriminant analysis based on Gaussian mixture modelling was performed using two different approaches: eigenvalue decomposition discriminant analysis (EDDA), which assumes that the density for each class can be described by a single Gaussian component, and MclustDA, which uses a finite mixture of Gaussian distributions within each class (mclust v5.4.7; Scrucca et al., 2016). The discriminant analysis based on Gaussian finite mixture modelling fitted "EEE" as the mixture model selected according to the Bayesian information criterion (BIC). Following this, the three species distributions were better described by ellipsoidal Gaussian components with equal volume, shape, and orientation (Supplementary Table S10). However, the accuracy of the fitted models to predict the testing subset in this case was only 54.17% (10x cross-validation, error= 0.20, standard error= 0.05) and the prediction accuracy decreased when a finite mixture of Gaussian distributions was used within each species class (50%, 10x cross-validation, error= 0.38, standard error= 0.05). This last approach suggested different mixture models and numbers of components within each species or category: a single component or group for species A (G=1, XXI model= diagonal multivariate normal) and two or more for species B (G=4, EEE model= ellipsoidal, equal volume, shape, and orientation) and C (G=2, EEI= diagonal, equal volume and shape); which suggests higher variation and heterogeneity within these last two groups (Supplementary Table 10).

2 Supplementary Figures and Tables

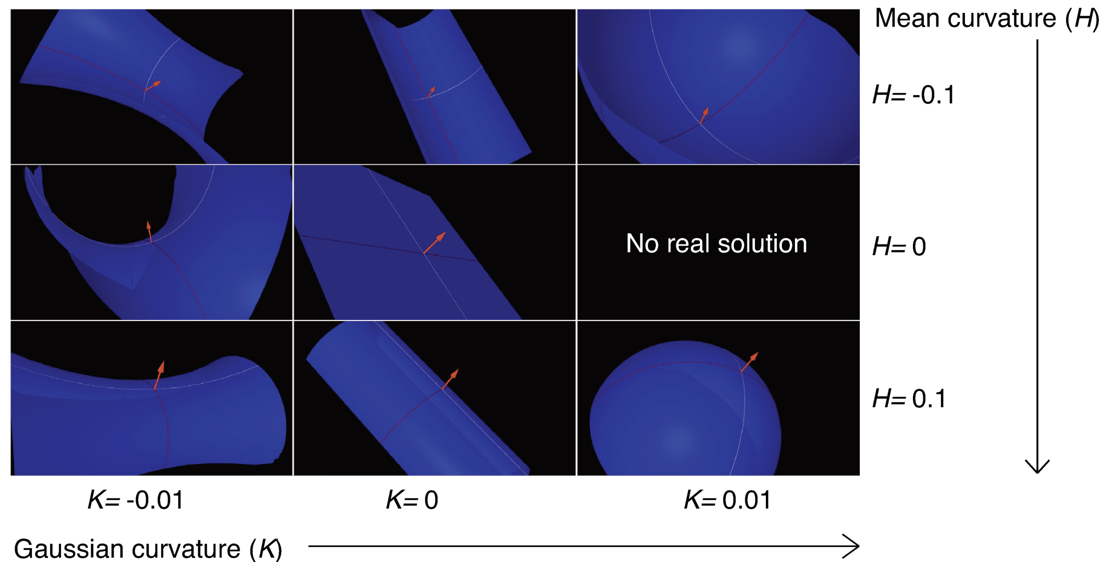
2.1 Supplementary Figures



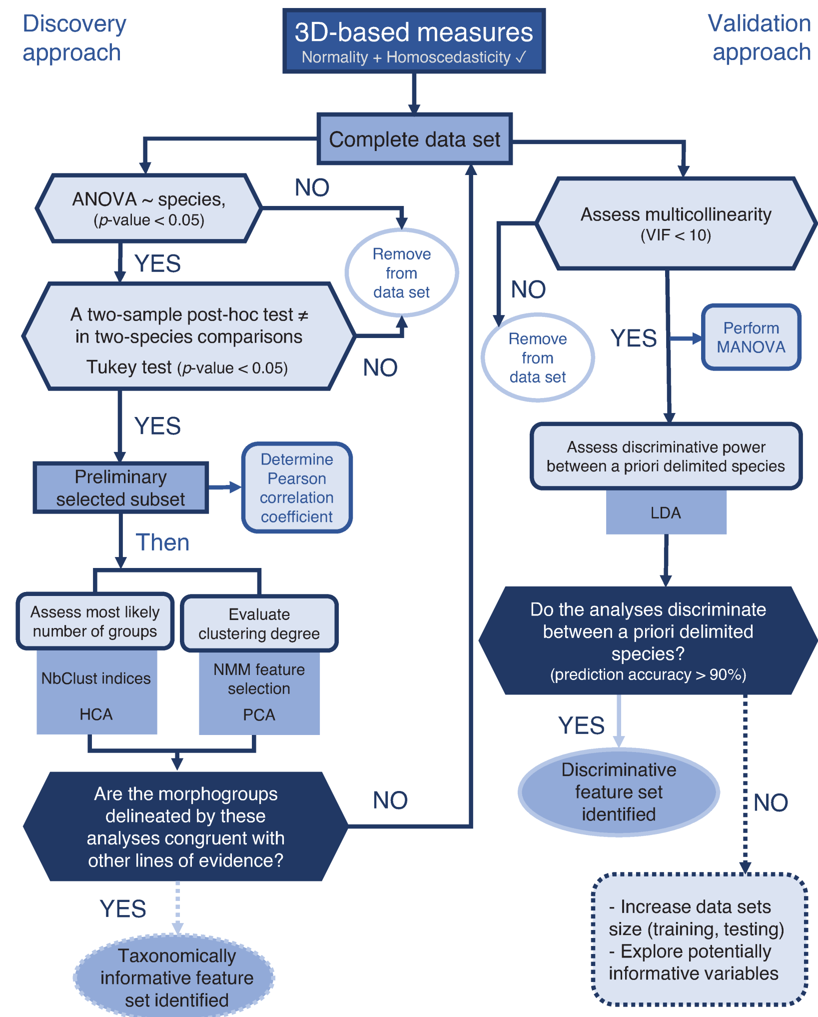
Supplementary Figure S1. Obtaining 3D data from complex-shaped organisms. Schematic representation of how the 3D scanning of the coral specimens was performed, highlighting the primary angles used to best capture the skeleton details (solid lines) and the additional perspectives used when complex branching patterns hampered accurate 3D model rendering (dashed lines). Two full-plate rotations were registered for each one of the angles and both the top and bottom faces of the coral fragments.



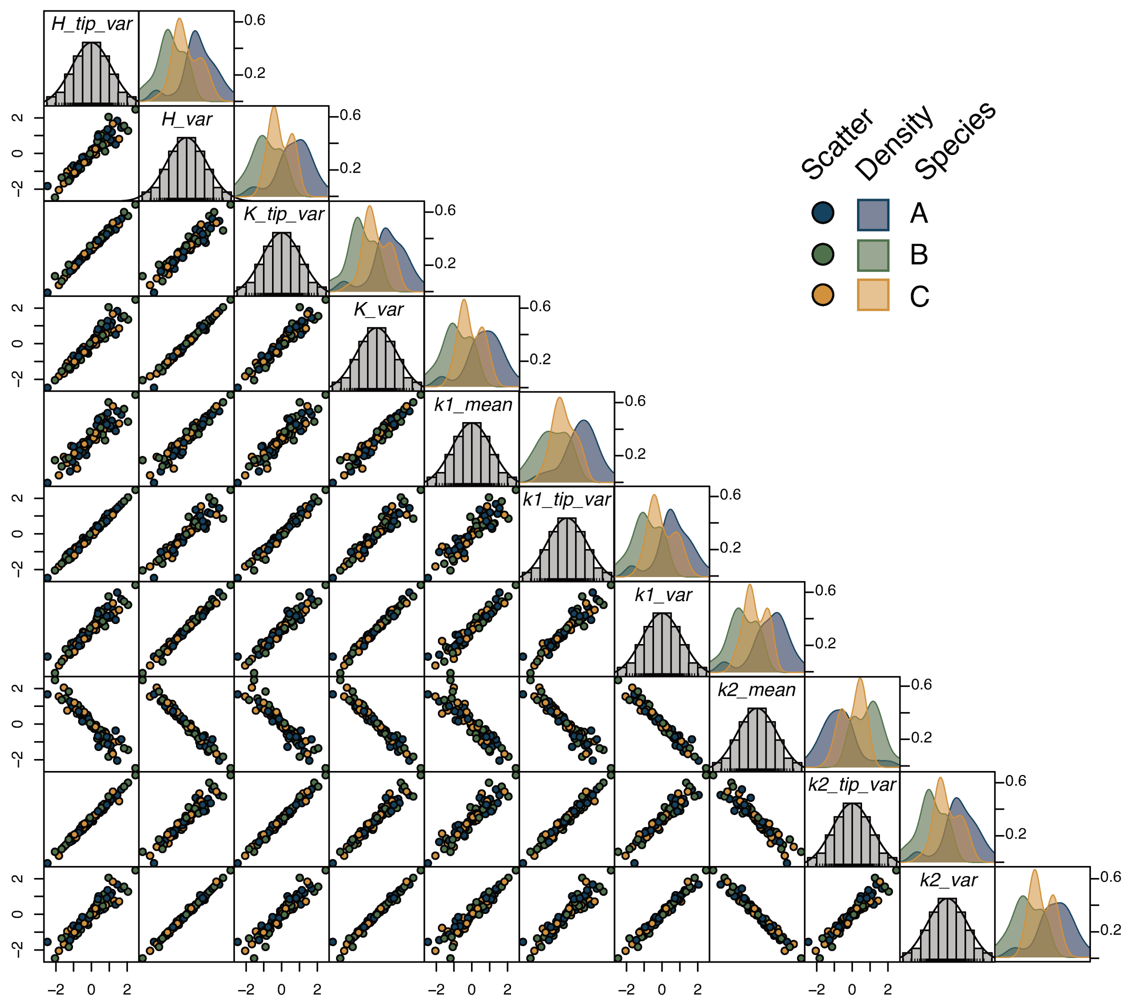
Supplementary Figure S2. Workflow for 3D-based measures estimation. Color-coded representation summarizing the pipeline used to estimate different types of 3D-based measures by extracting and combining information from different shape representations rendered from the light-structured scanning performed of the coral specimens.



Supplementary Figure S3. Expected shape for different surface curvature combinations. The surfaces for different combinations of *H* (mean curvature) and *K* (Gaussian curvature) are displayed. Orange arrows represent the direction of the outward normal (length=2.5). The red and white lines over the surfaces represent the direction of *k1* (maximum curvature) and *k2* (minimum curvature) respectively.

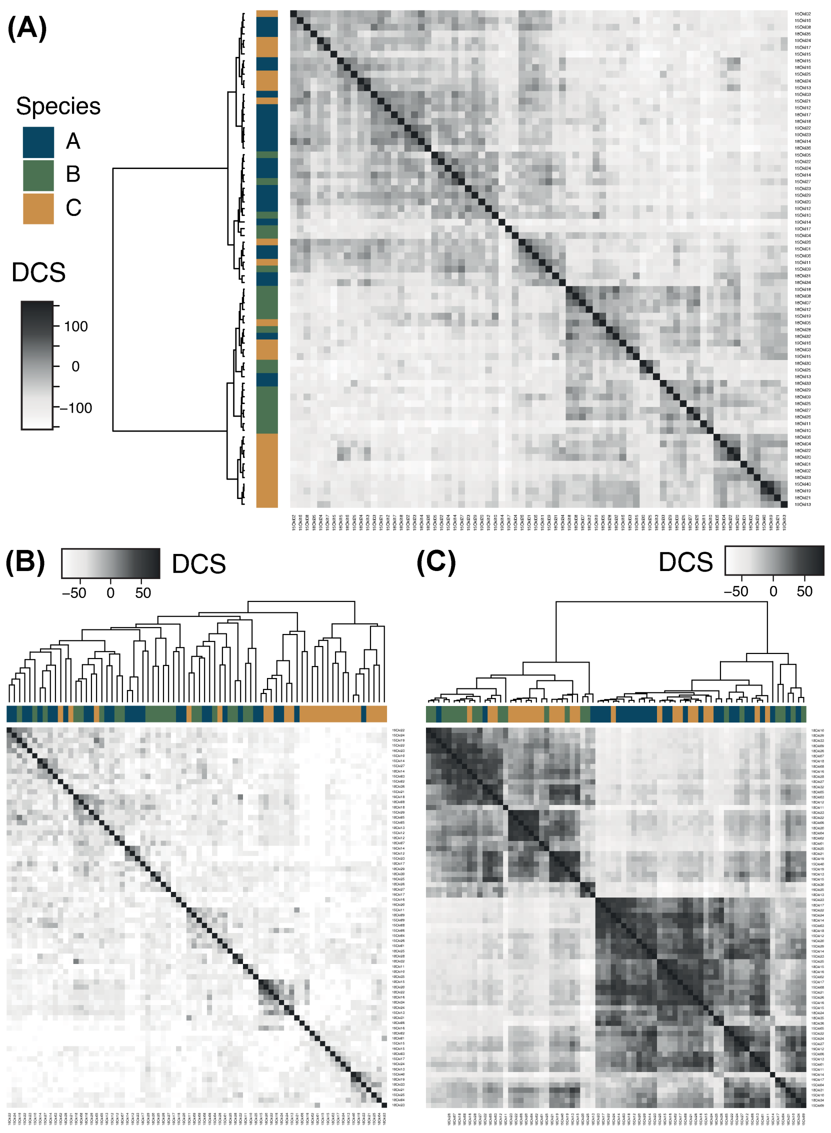


Supplementary Figure S4. Flow chart of 3D-based measures feature screening and selection. To perform feature screening for a prospective combination of representative characters that support interspecific discrimination, 3D-based measures that met the assumptions of normality and homoscedasticity were screened for significant interspecific differences (preliminary selected subset), and clustering was further assessed in this subset to evaluate if morphogroups were congruent with species delineated using other lines of evidence (identify variables useful for discovery approaches) or whether the 3D-morphological analyses enable to discriminate between a priori delimited species (perform a validation approach). Dashed lines and portions within the flow chart represent potential outcomes that were not obtained in this study. Abbreviations: Hierarchical clustering analysis (HCA), normal mixture model (NMM), principal component analysis (PCA), variance inflation factor (VIF), multivariate analysis of variance (MANOVA), linear discriminant analysis (LDA).

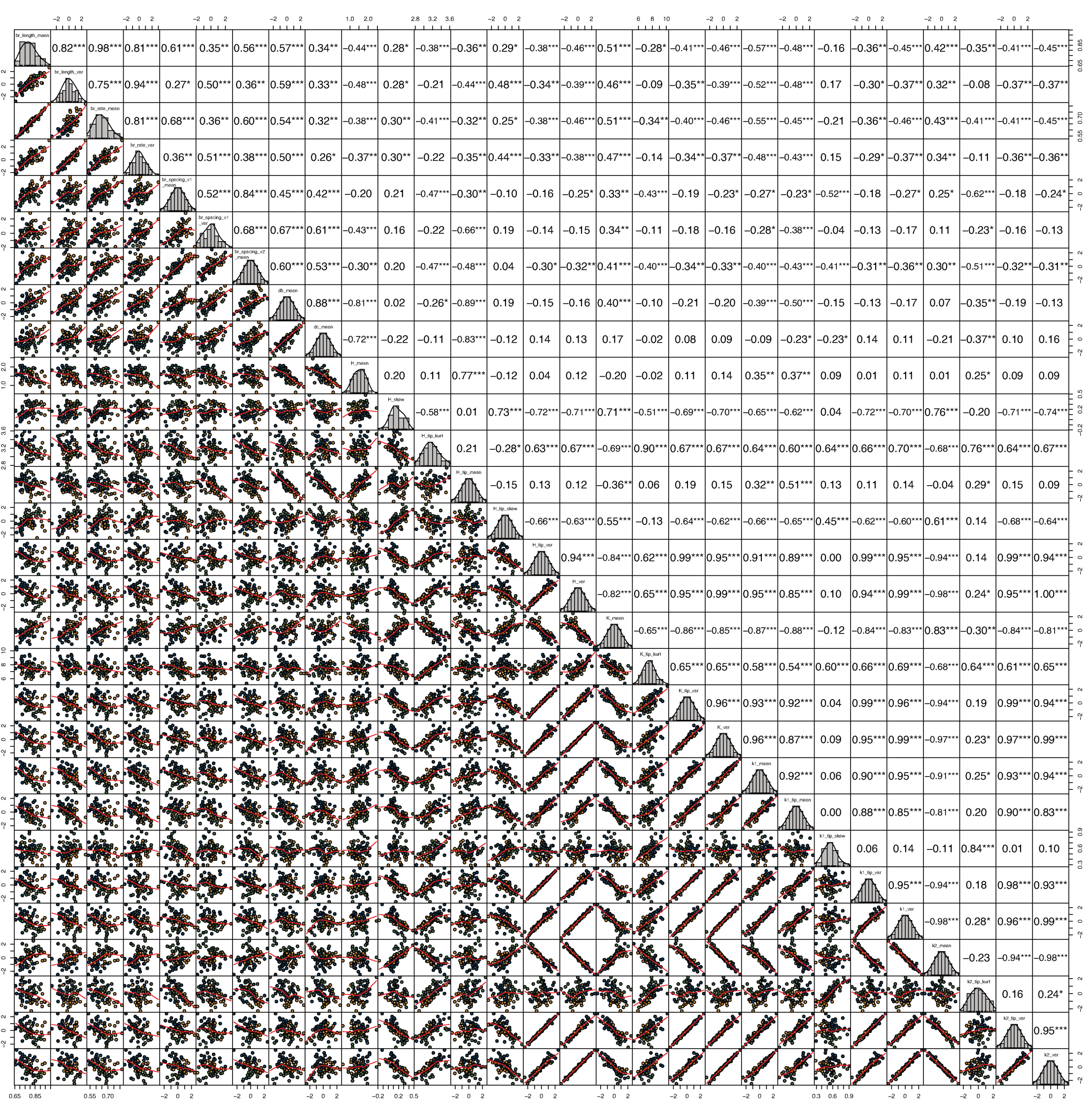


SUPPLEMENTARY FIGURE S5. Morphospaces depiction using variables with significant differences. Bivariate scatter plots (lower triangle) and univariate density distributions (upper diagonal line) of the ten variables that presented significant interespecific differences were used to assess the degree of overlapping between morphospaces of the three different species and the intra-specific variation. The histograms for each variable are also depicted in the main diagonal.

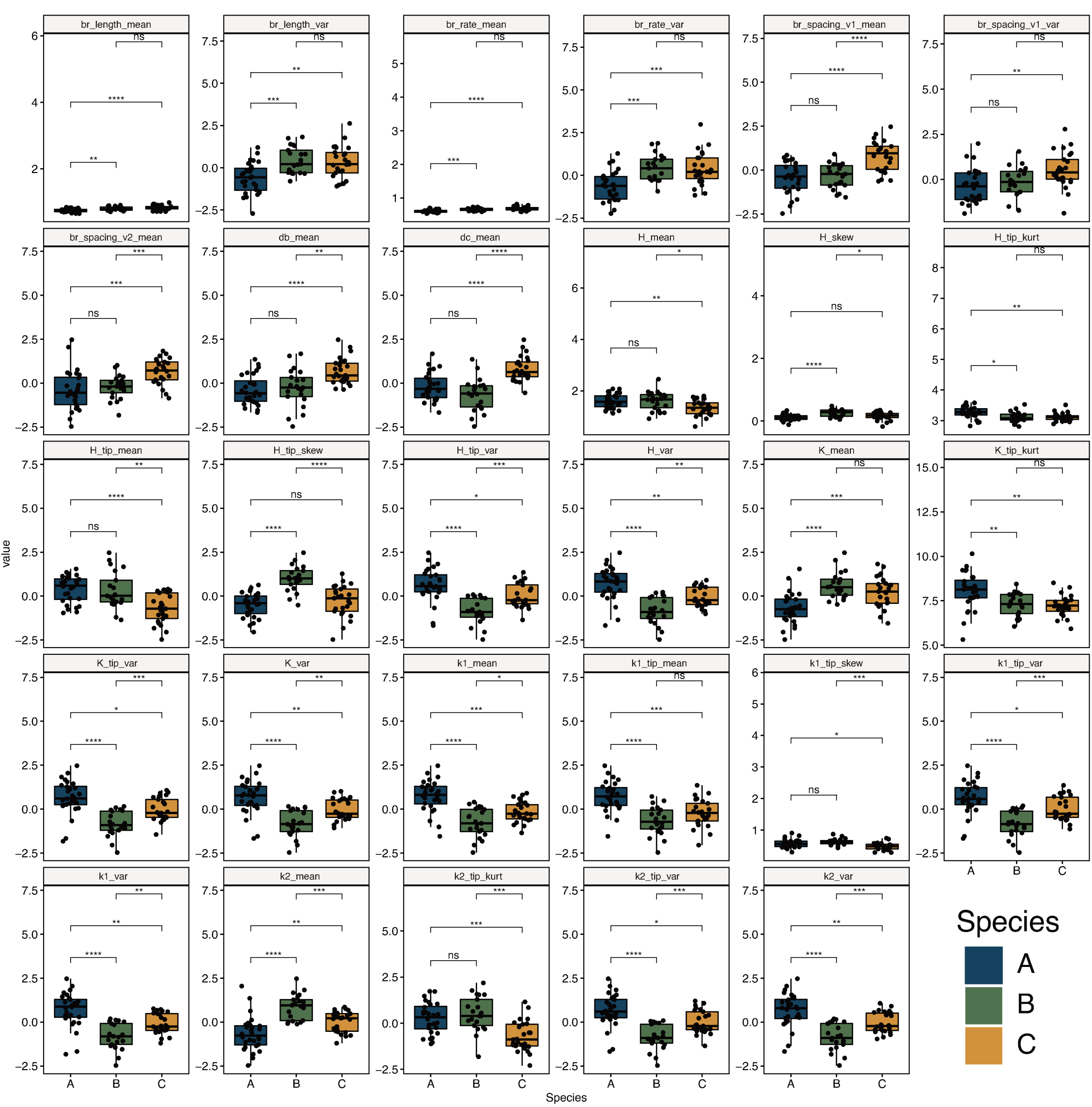
SUPPLEMENTARY FIGURE S6. Assessment of variables using Kernel density. Gaussian kernel densities (KD) were estimated for seven branch-derived variables variables (first two rows) and eight curvature variables (last two rows). The corresponding probability density function profiles (pdf) are depicted for each of the three species.



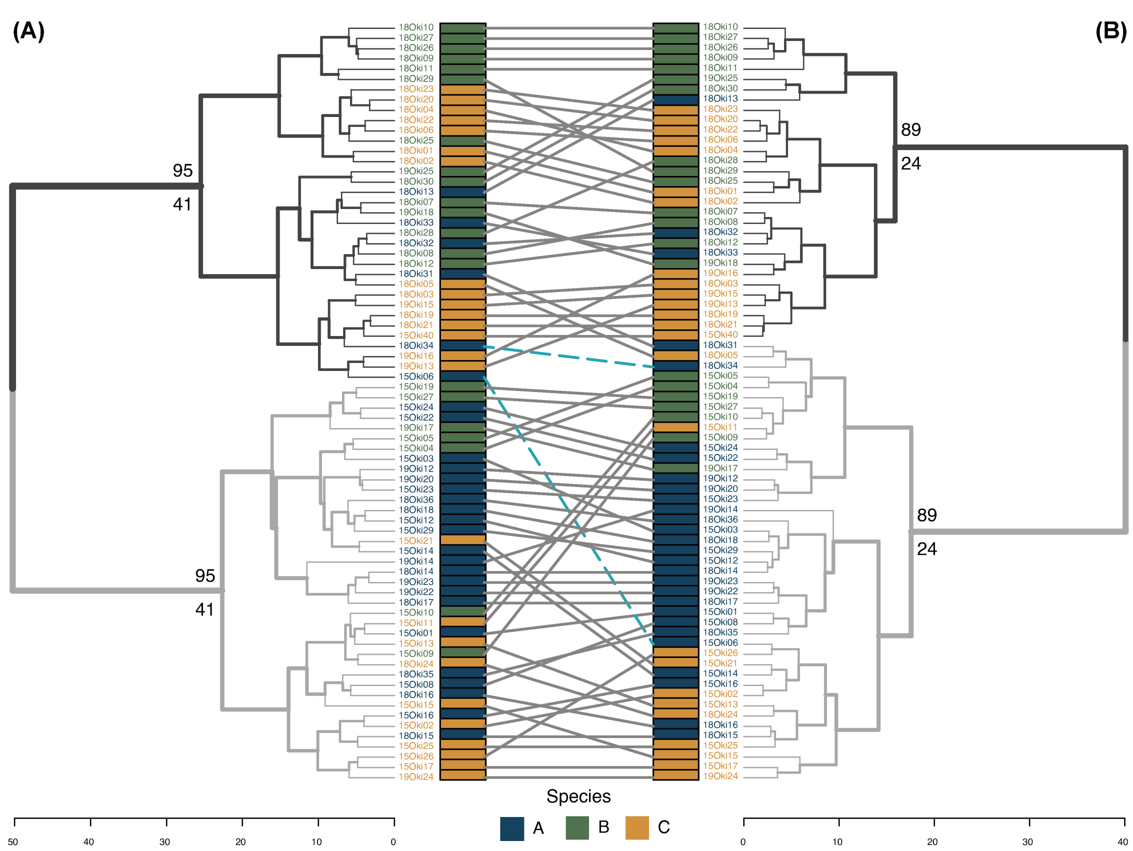
Supplementary Figure S7. Assessment of significant interspecific differences in distributions. Heatmaps depicting recoded and summarized p-values obtained from two-sample Mann-Whitney U tests for different sets of variables: (A) all variables, (B) branch-derived variables, and (C) curvature variables. Samples were re-organized using hierarchical clustering according to similarity in pairwise comparisons, calculated as the distribution comparison score (DCS) or the cumulative value of similarity according to the p-value significance cut-off (α= 0.05).



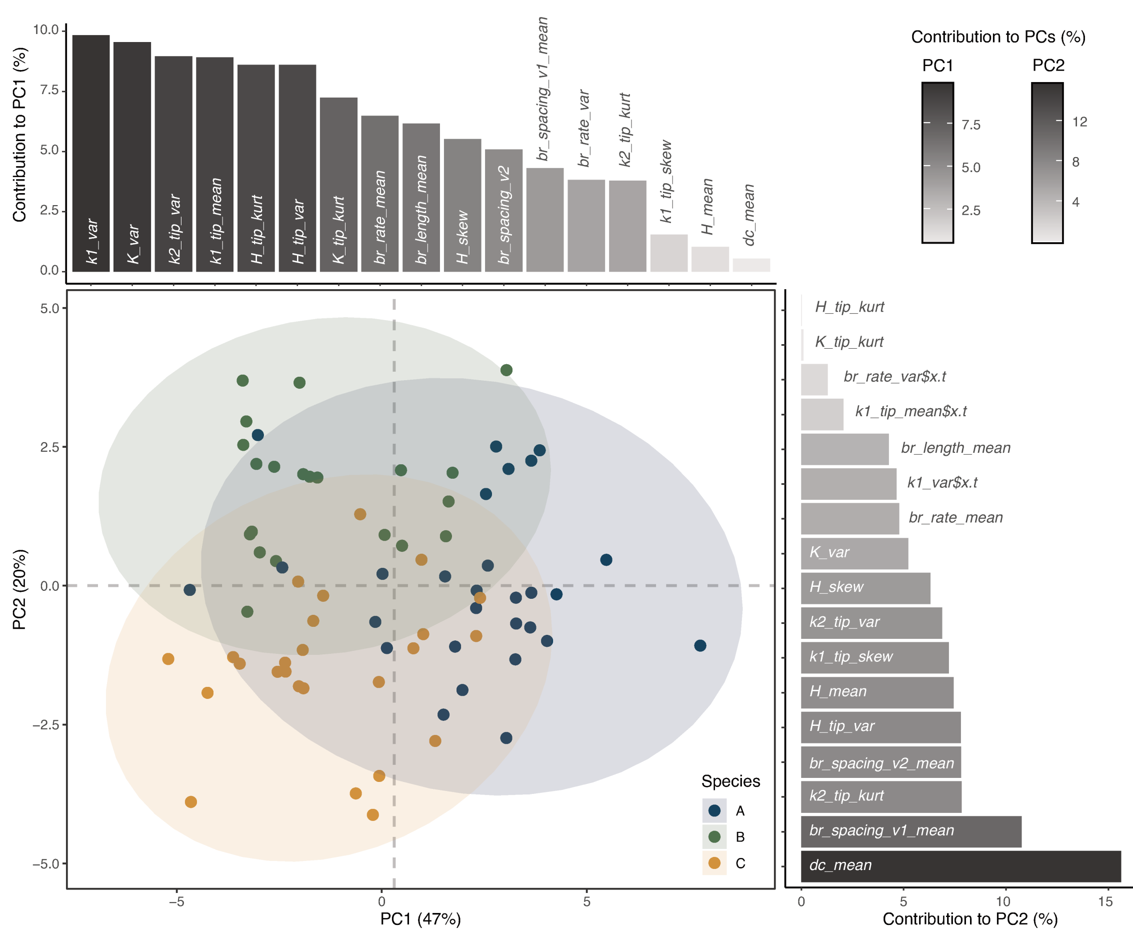
Supplementary Figure S8. Correlation assessment between preliminary selected variables. Correlation plot depicting the pairwise relationships between all the preliminary selected variables. The panels in the diagonal depict histograms of each feature. The graphs in the upper triangle depict Pearson's r correlation coefficients (ρ) and the corresponding asterisks indicate their significance (p-value: * ≤ 0.05, ** ≤ 0.01, *** ≤0.001). The graphs below the diagonal show the scatter plots for each pairwise comparison.



Supplementary Figure S9. Significant interespecific differences in preliminary selected variables. ox plots highlighting significant interespecific differences in the preliminary selected variables. The corresponding significance is displayed by asterisks according to the results of the test (*p*-value: ns = not significant, * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$0.001, **** $\leq$ 0.0001). This subset of 29 variables exhibited significant differences in the ANOVA and in at least two-species comparisons of the two-sample post-hoc Tukey test.



Supplementary Figure S10. Tanglegram of exploratory clustering analyses. Comparison of hierarchical clustering analyses (HCA) using Euclidean distance and Ward linkage for the complete set of variables (A, agglomerative coefficient= 0.8978) and the preliminary selected set (B, agglomerative coefficient= 0.9190). Values on the nodes correspond to the percentage (%) of 1000 bootstrap replications from multiscale re-sampling for approximately unbiased (AU) probability values (top), and bootstrap probability (BP) values (bottom) for the most likely number of groups indicated by the different branch colors (k=2, black and grey). Lines connecting the dendrograms' leaves indicate the topological differences between them, with grey solid lines displaying specimens with positions within the same cluster in A and B, and turquoise dashed lines depicting specimens that belong to different clusters in each dendrogram. Line-width of the branches highlights the height of each dendrogram at a certain point.



SUPPLEMENTARY FIGURE S11. Exploratory principal component analysis. Biplot of the first two principal components of a principal component analysis (PCA) using a subset of the preliminarily selected variables after performing unbiased variable and selection through Gaussian model-based clustering and the greedy algorithm both (forward and backward directions) according to the Bayesian information criterion (BIC; n= 17). The contribution (%) of each of the 17 variables to the principal components (PCs) is depicted along the axes along with the corresponding color scale.

2.2 Supplementary Tables

2.2.1 Supplementary Table S1. Information of specimens used in this study.

Sample ID, year of collection and taxonomy of specimens used for this study are displayed. Open nomenclature qualifiers are used according to the field identification and taxonomic identity of the *Acropora* morphospecies described by Ramírez-Portilla et al. (2022). Briefly, the open nomenclature "aff." is used to indicate that the specimens from species "C" have affinities with *Acropora hyacinthus* but belong most likely to a distinct species. In the case of species "A" and "B" evidence suggest these specimens are similar to *A. bifurcata* and *A. cytherea* respectively, but further information is required to confirm these assessments. For simplicity, species ID are used for this study.

Sample ID	Collection year	Genus	Open nomenclature	Species	Species ID
15Oki01	2015	*Acropora*	cf.	*bifurcata*	A
15Oki02	2015	*Acropora*	aff.	*hyacinthus*	C
15Oki03	2015	*Acropora*	cf.	*bifurcata*	A
15Oki04	2015	*Acropora*	cf.	*cytherea*	B
15Oki05	2015	*Acropora*	cf.	*cytherea*	B
15Oki06	2015	*Acropora*	cf.	*bifurcata*	A
15Oki08	2015	*Acropora*	cf.	*bifurcata*	A
15Oki09	2015	*Acropora*	cf.	*cytherea*	B
15Oki10	2015	*Acropora*	cf.	*cytherea*	B
15Oki11	2015	*Acropora*	aff.	*hyacinthus*	C
15Oki12	2015	*Acropora*	cf.	*bifurcata*	A
15Oki13	2015	*Acropora*	aff.	*hyacinthus*	C
15Oki14	2015	*Acropora*	cf.	*bifurcata*	A
15Oki15	2015	*Acropora*	aff.	*hyacinthus*	C
15Oki16	2015	*Acropora*	cf.	*bifurcata*	A
15Oki17	2015	*Acropora*	aff.	*hyacinthus*	C
15Oki19	2015	*Acropora*	cf.	*cytherea*	B
15Oki21	2015	*Acropora*	aff.	*hyacinthus*	C
15Oki22	2015	*Acropora*	cf.	*bifurcata*	A
15Oki23	2015	*Acropora*	cf.	*bifurcata*	A
15Oki24	2015	*Acropora*	cf.	*bifurcata*	A
15Oki25	2015	*Acropora*	aff.	*hyacinthus*	C
15Oki26	2015	*Acropora*	aff.	*hyacinthus*	C
15Oki27	2015	*Acropora*	cf.	*cytherea*	B
15Oki29	2015	*Acropora*	cf.	*bifurcata*	A
15Oki40	2015	*Acropora*	aff.	*hyacinthus*	C
18Oki01	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki02	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki03	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki04	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki05	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki06	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki07	2018	*Acropora*	cf.	*cytherea*	B

18Oki08	2018	*Acropora*	cf.	*cytherea*	B
18Oki09	2018	*Acropora*	cf.	*cytherea*	B
18Oki10	2018	*Acropora*	cf.	*cytherea*	B
18Oki11	2018	*Acropora*	cf.	*cytherea*	B
18Oki12	2018	*Acropora*	cf.	*cytherea*	B
18Oki13	2018	*Acropora*	cf.	*bifurcata*	A
18Oki14	2018	*Acropora*	cf.	*bifurcata*	A
18Oki15	2018	*Acropora*	cf.	*bifurcata*	A
18Oki16	2018	*Acropora*	cf.	*bifurcata*	A
18Oki17	2018	*Acropora*	cf.	*bifurcata*	A
18Oki18	2018	*Acropora*	cf.	*bifurcata*	A
18Oki19	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki20	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki21	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki22	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki23	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki24	2018	*Acropora*	aff.	*hyacinthus*	C
18Oki25	2018	*Acropora*	cf.	*cytherea*	B
18Oki26	2018	*Acropora*	cf.	*cytherea*	B
18Oki27	2018	*Acropora*	cf.	*cytherea*	B
18Oki28	2018	*Acropora*	cf.	*cytherea*	B
18Oki29	2018	*Acropora*	cf.	*cytherea*	B
18Oki30	2018	*Acropora*	cf.	*cytherea*	B
18Oki31	2018	*Acropora*	cf.	*bifurcata*	A
18Oki32	2018	*Acropora*	cf.	*bifurcata*	A
18Oki33	2018	*Acropora*	cf.	*bifurcata*	A
18Oki34	2018	*Acropora*	cf.	*bifurcata*	A
18Oki35	2018	*Acropora*	cf.	*bifurcata*	A
18Oki36	2018	*Acropora*	cf.	*bifurcata*	A
19Oki12	2019	*Acropora*	cf.	*bifurcata*	A
19Oki13	2019	*Acropora*	aff.	*hyacinthus*	C
19Oki14	2019	*Acropora*	cf.	*bifurcata*	A
19Oki15	2019	*Acropora*	aff.	*hyacinthus*	C
19Oki16	2019	*Acropora*	aff.	*hyacinthus*	C
19Oki17	2019	*Acropora*	cf.	*cytherea*	B
19Oki18	2019	*Acropora*	cf.	*cytherea*	B
19Oki20	2019	*Acropora*	cf.	*bifurcata*	A
19Oki22	2019	*Acropora*	cf.	*bifurcata*	A
19Oki23	2019	*Acropora*	cf.	*bifurcata*	A
19Oki24	2019	*Acropora*	aff.	*hyacinthus*	C
19Oki25	2019	*Acropora*	cf.	*cytherea*	B

2.2.2 Supplementary Table S2. Statistics summary of variables assessed in this study.

Significance values (*p*-values) from the normality assumption tested (Shapiro-Wilk test, α = 0.05) for all the estimated univariate measures either jointly (S-W) or per species (S-W ~ spp) are displayed: "all", if they met the assumption, or highlighting the species that do not (i.e., A, B, C). All variables that deviated from the assumption were transformed (TR) using the most frequently suggested option after five iterations of the *bestNormalize* function (TR bestNormalize). Homogeneity of variance was also tested on the TR data set (Levene's test, α = 0.05) and corresponding *p*-values are displayed. Variables that did not meet assumptions of normality and homoscedasticity after transformation, were excluded from downstream analyses.

Variable	Type	S-W	S-W ~ spp	TR bestNormalize	S-W TR	S-W ~ spp TR	Levene TR
b_angle_mean	Branch	0.6383	all	N/A	0.6383	all	0.4786
b_angle_var	Branch	0.0020	all	log_x	0.2250	all	0.1917
br_length_mean	Branch	0.1426	all	N/A	0.1426	all	0.4813
br_length_var	Branch	0.0004	B, C	boxcox	0.9758	all	0.8372
br_rate_mean	Branch	0.2714	all	N/A	0.2714	all	0.0981
br_rate_var	Branch	0.0013	C	log_x	0.8669	all	0.9174
br_spacing_v1_mean	Branch	0.0139	all	orderNorm	1.0000	all	0.6831
br_spacing_v1_var	Branch	0.0001	A, C	log_x	0.4921	all	0.7742
br_spacing_v2_mean	Branch	0.0557	all	orderNorm	1.0000	all	0.0941
br_spacing_v2_var	Branch	0.0000	A, C	log_x	0.4481	all	0.8609
d_avg_var	Branch	0.0000	A, B, C	boxcox	0.5823	all	0.4331
da_avg_mean	Branch	0.0000	A, B, C	boxcox	0.2069	all	0.3737
da_mean	Branch	0.0000	A, B, C	boxcox	0.4611	all	0.3714
da_var	Branch	0.0000	A, B, C	boxcox	0.2992	all	0.2950
db_mean	Branch	0.0001	A, C	orderNorm	1.0000	all	0.4155
db_var	Branch	0.0000	A, B, C	orderNorm	1.0000	all	0.5417
dc_mean	Branch	0.0100	A, C	orderNorm	1.0000	all	0.4190
dc_var	Branch	0.0000	A, B, C	log_x	0.0920	all	0.6926
FD	Complexity	0.0622	A	orderNorm	1.0000	all	0.5942
H_kurt	Curvature	0.1187	C	yeojohnson	0.8190	all	0.7095
H_mean	Curvature	0.8538	all	N/A	0.8538	all	0.5621
H_skew	Curvature	0.5515	all	N/A	0.5515	all	0.3555
H_tip_kurt	Curvature	0.5158	all	N/A	0.5158	all	0.3148
H_tip_mean	Curvature	0.3281	B	orderNorm	1.0000	all	0.6047
H_tip_skew	Curvature	0.0081	all	orderNorm	1.0000	all	0.4073
H_tip_var	Curvature	0.0005	B, C	orderNorm	1.0000	all	0.5702
H_var	Curvature	0.0001	B, C	orderNorm	1.0000	all	0.1768
K_kurt	Curvature	0.2770	all	N/A	0.2770	all	0.1579
K_mean	Curvature	0.0001	B, C	orderNorm	1.0000	all	0.7620
K_skew	Curvature	0.0002	A	orderNorm	1.0000	all	0.6720
K_tip_kurt	Curvature	0.8411	all	N/A	0.8411	all	0.0955
K_tip_mean	Curvature	0.1056	C	center_scale	0.1056	all	0.0435
K_tip_skew	Curvature	0.0509	all	log_x	0.7247	all	0.0117

K_tip_var	Curvature	0.0000	A, B, C	orderNorm	1.0000	all	0.3229
K_var	Curvature	0.0000	A, B, C	orderNorm	1.0000	all	0.2573
k1_kurt	Curvature	0.0356	C	N/A	1.0000	all	0.2257
k1_mean	Curvature	0.0031	B, C	orderNorm	1.0000	all	0.1942
k1_skew	Curvature	0.0209	all	boxcox	0.7109	all	0.6118
k1_tip_kurt	Curvature	0.0001	C	orderNorm	1.0000	all	0.2341
k1_tip_mean	Curvature	0.0039	A	orderNorm	1.0000	all	0.8684
k1_tip_skew	Curvature	0.6146	all	N/A	0.6146	all	0.3792
k1_tip_var	Curvature	0.0008	B, C	orderNorm	1.0000	all	0.5904
k1_var	Curvature	0.0001	B, C	orderNorm	1.0000	all	0.2058
k2_kurt	Curvature	0.0874	C	boxcox	0.8008	all	0.8111
k2_mean	Curvature	0.0008	A, B, C	orderNorm	1.0000	all	0.2245
k2_skew	Curvature	0.0072	A, B	orderNorm	1.0000	all	0.2228
k2_tip_kurt	Curvature	0.0472	all	yeojohnson	0.6917	all	0.8340
k2_tip_mean	Curvature	0.2727	C	orderNorm	1.0000	C	0.8376
k2_tip_skew	Curvature	0.5219	all	N/A	0.5219	all	0.0073
k2_tip_var	Curvature	0.0004	B, C	orderNorm	1.0000	all	0.4173
k2_var	Curvature	0.0001	B, C	orderNorm	1.0000	all	0.2253
S	Complexity	0.0127	all	boxcox	0.9444	all	0.9506
SV_ratio	Complexity	0.1301	all	N/A	0.1301	all	0.8204

2.2.3 SUPPLEMENTARY TABLE S3. Results of the analysis of variance (ANOVA).

Variable	*p*-value	Significance
b_angle_mean	4.92E-01	ns
b_angle_var	2.10E-01	ns
br_length_mean	1.34E-05	****
br_length_var	9.07E-05	****
br_rate_mean	3.46E-06	****
br_rate_var	2.40E-05	****
br_spacing_v1_mean	6.96E-07	****
br_spacing_v1_var	0.003352	**
br_spacing_v2_mean	7.29E-05	****
br_spacing_v2_var	4.05E-02	*
d_avg_var	0.17765	ns
da_avg_mean	2.75E-02	*
da_mean	9.33E-03	**
da_var	6.82E-02	ns
db_mean	6.56E-05	****
db_var	1.48E-02	*
dc_mean	9.86E-08	****
dc_var	0.000694	***
FD	5.19E-01	ns
H_kurt	3.89E-01	ns
H_mean	5.61E-03	**
H_skew	8.84E-05	****
H_tip_kurt	0.00126	**
H_tip_mean	2.23E-05	****
H_tip_skew	5.48E-09	****
H_tip_var	5.02E-08	****
H_var	1.29E-08	****
K_kurt	0.003158	**
K_mean	1.75E-06	****
K_skew	0.251868	ns
K_tip_kurt	4.13E-04	***
K_tip_mean	2.18E-08	****
K_tip_skew	7.00E-01	ns
K_tip_var	5.44E-08	****
K_var	3.24E-08	****
k1_kurt	1.37E-01	ns
k1_mean	1.47E-08	****
k1_skew	1.51E-01	ns
k1_tip_kurt	1.33E-02	*
k1_tip_mean	2.92E-07	****
k1_tip_skew	0.000443	***
k1_tip_var	1.19E-07	****
k1_var	5.10E-08	****
k2_kurt	4.97E-02	*
k2_mean	2.27E-08	****
k2_skew	0.109138	ns
k2_tip_kurt	1.37E-05	****
k2_tip_mean	2.11E-08	****
k2_tip_skew	1.34E-03	**
k2_tip_var	3.02E-08	****
k2_var	2.21E-08	****
S	4.16E-02	*
SV_ratio	0.01883	*

Significance (*p*-values):

(<0.0001) ****

(<=0.001)***

(<=0.01)**

(<0.05)*

(>=0.05) ns

2.2.4 SUPPLEMENTARY TABLE S4. Results of the post-hoc Tukey test for interspecific differences.

Significance values (*p*-values) from post-hoc Tukey tests for significant interspecific differences between A (*n*=28), B (*n*=21) and C (*n*=25) are displayed for each variable in the corresponding order: A vs. B, A vs. C and finally B vs. C. The corresponding significance levels according to an α of 0.05 for each pairwise comparison are also shown so that: (<0.0001) ****, (≤0.001)***, (≤0.01)**, (<0.05)* and (≥0.05) ns.

Variable	*p*-value	*p*-value Sig.	Sig. species pair
b_angle_mean	0.6048	ns	ns
b_angle_mean	0.5345	ns	ns
b_angle_mean	0.9980	ns	ns
b_angle_var	0.1819	ns	ns
b_angle_var	0.7149	ns	ns
b_angle_var	0.5709	ns	ns
br_length_mean	0.0032	**	AvsB
br_length_mean	0.0000	****	AvsC
br_length_mean	0.3914	ns	ns
br_length_var	0.0003	***	AvsB
br_length_var	0.0010	**	AvsC
br_length_var	0.8698	ns	ns
br_rate_mean	0.0029	**	AvsB
br_rate_mean	0.0000	****	AvsC
br_rate_mean	0.2305	ns	ns
br_rate_var	0.0002	***	AvsB
br_rate_var	0.0002	***	AvsC
br_rate_var	0.9694	ns	ns
br_spacing_v1_mean	0.9259	ns	ns
br_spacing_v1_mean	0.0000	****	AvsC
br_spacing_v1_mean	0.0000	****	BvsC
br_spacing_v1_var	0.7149	ns	ns
br_spacing_v1_var	0.0030	**	AvsC
br_spacing_v1_var	0.0481	*	BvsC
br_spacing_v2_mean	0.6938	ns	ns
br_spacing_v2_mean	0.0001	****	AvsC
br_spacing_v2_mean	0.0035	**	BvsC
br_spacing_v2_var	0.0605	ns	ns
br_spacing_v2_var	1.0000	ns	ns
br_spacing_v2_var	0.0686	ns	ns
d_avg_var	0.1797	ns	ns
d_avg_var	0.9541	ns	ns
d_avg_var	0.3085	ns	ns
da_avg_mean	0.2608	ns	ns
da_avg_mean	0.0220	*	AvsC
da_avg_mean	0.5862	ns	ns
da_mean	0.3941	ns	ns

da_mean	0.0065	**	AvsC
da_mean	0.2285	ns	ns
da_var	0.0558	ns	ns
da_var	0.4062	ns	ns
da_var	0.5224	ns	ns
db_mean	0.8719	ns	ns
db_mean	0.0001	***	AvsC
db_mean	0.0016	**	BvsC
db_var	0.0113	*	AvsB
db_var	0.2189	ns	ns
db_var	0.3848	ns	ns
dc_mean	0.1209	ns	ns
dc_mean	0.0001	****	AvsC
dc_mean	0.0000	****	BvsC
dc_var	0.0004	***	AvsB
dc_var	0.1007	ns	ns
dc_var	0.1270	ns	ns
FD	0.9150	ns	ns
FD	0.7147	ns	ns
FD	0.5053	ns	ns
H_kurt	0.5145	ns	ns
H_kurt	0.4338	ns	ns
H_kurt	0.9968	ns	ns
H_mean	0.9944	ns	ns
H_mean	0.0123	*	AvsC
H_mean	0.0165	*	BvsC
H_skew	0.0001	****	AvsB
H_skew	0.1999	ns	ns
H_skew	0.0144	*	BvsC
H_tip_kurt	0.0062	**	AvsB
H_tip_kurt	0.0037	**	AvsC
H_tip_kurt	0.9999	ns	ns
H_tip_mean	0.9709	ns	ns
H_tip_mean	0.0001	****	AvsC
H_tip_mean	0.0004	***	BvsC
H_tip_skew	0.0000	****	AvsB
H_tip_skew	0.5614	ns	ns
H_tip_skew	0.0000	****	BvsC
H_tip_var	0.0000	****	AvsB
H_tip_var	0.0163	*	AvsC
H_tip_var	0.0010	***	BvsC
H_var	0.0000	****	AvsB
H_var	0.0021	**	AvsC

H_var	0.0030	**	BvsC
K_kurt	0.0694	ns	ns
K_kurt	0.0027	**	AvsC
K_kurt	0.5745	ns	ns
K_mean	0.0000	****	AvsB
K_mean	0.0004	***	AvsC
K_mean	0.2889	ns	ns
K_skew	0.8401	ns	ns
K_skew	0.4822	ns	ns
K_skew	0.2418	ns	ns
K_tip_kurt	0.0045	**	AvsB
K_tip_kurt	0.0009	***	AvsC
K_tip_kurt	0.9490	ns	ns
K_tip_mean	0.0000	****	AvsB
K_tip_mean	0.0889	ns	ns
K_tip_mean	0.0001	****	BvsC
K_tip_skew	0.6798	ns	ns
K_tip_skew	0.9588	ns	ns
K_tip_skew	0.8407	ns	ns
K_tip_var	0.0000	****	AvsB
K_tip_var	0.0070	**	AvsC
K_tip_var	0.0026	**	BvsC
K_var	0.0000	****	AvsB
K_var	0.0039	**	AvsC
K_var	0.0032	**	BvsC
k1_kurt	0.2606	ns	ns
k1_kurt	0.1647	ns	ns
k1_kurt	0.9847	ns	ns
k1_mean	0.0000	****	AvsB
k1_mean	0.0003	***	AvsC
k1_mean	0.0233	*	BvsC
k1_skew	0.9818	ns	ns
k1_skew	0.2242	ns	ns
k1_skew	0.2014	ns	ns
k1_tip_kurt	0.1902	ns	ns
k1_tip_kurt	0.0105	*	AvsC
k1_tip_kurt	0.5382	ns	ns
k1_tip_mean	0.0000	****	AvsB
k1_tip_mean	0.0002	***	AvsC
k1_tip_mean	0.1681	ns	ns
k1_tip_skew	0.2907	ns	ns
k1_tip_skew	0.0191	*	AvsC
k1_tip_skew	0.0004	***	BvsC
k1_tip_var	0.0000	****	AvsB
k1_tip_var	0.0170	*	AvsC

k1_tip_var	0.0019	**	BvsC
k1_var	0.0000	****	AvsB
k1_var	0.0017	**	AvsC
k1_var	0.0104	*	BvsC
k2_kurt	0.9683	ns	ns
k2_kurt	0.0575	ns	ns
k2_kurt	0.1367	ns	ns
k2_mean	0.0000	****	AvsB
k2_mean	0.0055	**	AvsC
k2_mean	0.0017	**	BvsC
k2_skew	0.1527	ns	ns
k2_skew	0.1913	ns	ns
k2_skew	0.9789	ns	ns
k2_tip_kurt	0.5843	ns	ns
k2_tip_kurt	0.0004	***	AvsC
k2_tip_kurt	0.0000	****	BvsC
k2_tip_mean	0.0000	****	AvsB
k2_tip_mean	0.1749	ns	ns
k2_tip_mean	0.0000	****	BvsC
k2_tip_skew	0.0046	**	AvsB
k2_tip_skew	0.0055	**	AvsC
k2_tip_skew	0.9762	ns	ns
k2_tip_var	0.0000	****	AvsB
k2_tip_var	0.0102	*	AvsC
k2_tip_var	0.0011	**	BvsC
k2_var	0.0000	****	AvsB
k2_var	0.0049	**	AvsC
k2_var	0.0019	**	BvsC
S	0.2920	ns	ns
S	0.0345	*	AvsC
S	0.6461	ns	ns
SV_ratio	0.1068	ns	ns
SV_ratio	0.0204	*	AvsC
SV_ratio	0.8452	ns	ns

2.2.5 SUPPLEMENTARY TABLE S5. Pearson correlation coefficients (*ρ*) for the preliminary selected variables.

Pearson correlation coefficients (*ρ*) and the corresponding *p*-values for pairwise comparisons of preliminary selected variables with | *ρ* | > 0.05 and *p*-value ≥ 0.05 are depicted.

Variable 1	Variable 2	*ρ*	*p*-value
br_length_mean	br_length_var	0.821971	0.00E+00
br_length_mean	br_rate_mean	0.977405	0
br_length_mean	br_rate_var	0.813567	0.00E+00
br_length_mean	br_spacing_v1_mean	0.612135	6.83E-09
br_length_mean	br_spacing_v2_mean	0.564328	1.64E-07
br_length_mean	db_mean	0.573809	9.07E-08
br_length_mean	K_mean	0.50865	3.71E-06
br_length_var	br_rate_mean	0.753196	9.77E-15
br_length_var	br_rate_var	0.940212	0.00E+00
br_length_var	db_mean	0.585441	4.29E-08
br_rate_mean	br_rate_var	0.805754	0.00E+00
br_rate_mean	br_spacing_v1_mean	0.681958	2.24E-11
br_rate_mean	br_spacing_v2_mean	0.595146	2.24E-08
br_rate_mean	db_mean	0.535541	8.81E-07
br_rate_mean	K_mean	0.510711	3.34E-06
br_rate_var	br_spacing_v1_var	0.508633	3.71E-06
br_rate_var	db_mean	0.500248	5.67E-06
br_spacing_v1_mean	br_spacing_v1_var	0.515338	2.62E-06
br_spacing_v1_mean	br_spacing_v2_mean	0.84211	0.00E+00
br_spacing_v1_var	br_spacing_v2_mean	0.684745	1.73E-11
br_spacing_v1_var	db_mean	0.667435	8.35E-11
br_spacing_v1_var	dc_mean	0.610329	7.78E-09
br_spacing_v2_mean	db_mean	0.599635	1.65E-08
br_spacing_v2_mean	dc_mean	0.525311	1.54E-06
db_mean	dc_mean	0.882935	0.00E+00
H_mean	H_tip_mean	0.769637	1.11E-15
H_skew	H_tip_skew	0.728279	1.94E-13
H_skew	K_mean	0.706734	1.98E-12
H_skew	k2_mean	0.756884	6.22E-15
H_tip_kurt	H_tip_var	0.627013	2.27E-09
H_tip_kurt	H_var	0.671125	6.02E-11
H_tip_kurt	K_tip_kurt	0.8954	0
H_tip_kurt	K_tip_var	0.667175	8.54E-11
H_tip_kurt	K_var	0.669626	6.88E-11
H_tip_kurt	k1_mean	0.639956	8.32E-10
H_tip_kurt	k1_tip_mean	0.603323	1.28E-08
H_tip_kurt	k1_tip_skew	0.644084	5.98E-10
H_tip_kurt	k1_tip_var	0.663498	1.18E-10
H_tip_kurt	k1_var	0.699778	4.01E-12
H_tip_kurt	k2_tip_kurt	0.755303	7.55E-15
H_tip_kurt	k2_tip_var	0.63515	1.22E-09
H_tip_kurt	k2_var	0.673067	5.06E-11
H_tip_mean	k1_tip_mean	0.514985	2.67E-06
H_tip_skew	K_mean	0.550019	3.85E-07

H_tip_skew	k2_mean	0.607618	9.43E-09
H_tip_var	H_var	0.942736	0.00E+00
H_tip_var	K_tip_kurt	0.615587	5.32E-09
H_tip_var	K_tip_var	0.991478	0
H_tip_var	K_var	0.954129	0.00E+00
H_tip_var	k1_mean	0.90962	0.00E+00
H_tip_var	k1_tip_mean	0.893528	0.00E+00
H_tip_var	k1_tip_var	0.994328	0.00E+00
H_tip_var	k1_var	0.949508	0.00E+00
H_tip_var	k2_tip_var	0.991872	0.00E+00
H_tip_var	k2_var	0.938409	0.00E+00
H_var	K_tip_kurt	0.653429	2.78E-10
H_var	K_tip_var	0.946437	0.00E+00
H_var	K_var	0.99193	0.00E+00
H_var	k1_mean	0.952507	0.00E+00
H_var	k1_tip_mean	0.845339	0.00E+00
H_var	k1_tip_var	0.936956	0.00E+00
H_var	k1_var	0.992149	0.00E+00
H_var	k2_tip_var	0.954843	0.00E+00
H_var	k2_var	0.995836	0.00E+00
K_mean	k2_mean	0.829018	0.00E+00
K_tip_kurt	K_tip_var	0.645418	5.37E-10
K_tip_kurt	K_var	0.645409	5.37E-10
K_tip_kurt	k1_mean	0.584395	4.59E-08
K_tip_kurt	k1_tip_mean	0.537042	8.10E-07
K_tip_kurt	k1_tip_skew	0.598583	1.77E-08
K_tip_kurt	k1_tip_var	0.655142	2.40E-10
K_tip_kurt	k1_var	0.690724	9.76E-12
K_tip_kurt	k2_tip_kurt	0.636663	1.08E-09
K_tip_kurt	k2_tip_var	0.606152	1.05E-08
K_tip_kurt	k2_var	0.65368	2.72E-10
K_tip_var	K_var	0.961587	0.00E+00
K_tip_var	k1_mean	0.930562	0
K_tip_var	k1_tip_mean	0.91759	0
K_tip_var	k1_tip_var	0.986314	0.00E+00
K_tip_var	k1_var	0.95547	0.00E+00
K_tip_var	k2_tip_var	0.992263	0.00E+00
K_tip_var	k2_var	0.940085	0.00E+00
K_var	k1_mean	0.962754	0
K_var	k1_tip_mean	0.870886	0
K_var	k1_tip_var	0.947507	0.00E+00
K_var	k1_var	0.985157	0.00E+00
K_var	k2_tip_var	0.967258	0.00E+00
K_var	k2_var	0.98694	0.00E+00
k1_mean	k1_tip_mean	0.918782	0
k1_mean	k1_tip_var	0.89504	0.00E+00
k1_mean	k1_var	0.945341	0.00E+00
k1_mean	k2_tip_var	0.930863	0.00E+00
k1_mean	k2_var	0.942417	0
k1_tip_mean	k1_tip_var	0.882499	0.00E+00

k1_tip_mean	k1_var	0.851984	0.00E+00
k1_tip_mean	k2_tip_var	0.899742	0.00E+00
k1_tip_mean	k2_var	0.830025	0
k1_tip_skew	k2_tip_kurt	0.843069	0
k1_tip_var	k1_var	0.947465	0
k1_tip_var	k2_tip_var	0.981884	0.00E+00
k1_tip_var	k2_var	0.932699	0
k1_var	k2_tip_var	0.956563	0.00E+00
k1_var	k2_var	0.986052	0
k2_tip_var	k2_var	0.952764	0
br_length_var	k1_mean	-0.52341	1.71E-06
br_rate_mean	k1_mean	-0.54885	4.13E-07
br_spacing_v1_mean	k1_tip_skew	-0.52138	1.91E-06
br_spacing_v1_mean	k2_tip_kurt	-0.61531	5.43E-09
br_spacing_v1_var	H_tip_mean	-0.65794	1.90E-10
br_spacing_v2_mean	k2_tip_kurt	-0.5085	3.74E-06
db_mean	H_mean	-0.81004	0.00E+00
db_mean	H_tip_mean	-0.89419	0.00E+00
db_mean	k1_tip_mean	-0.50484	4.50E-06
dc_mean	H_mean	-0.71907	5.37E-13
dc_mean	H_tip_mean	-0.82991	0.00E+00
H_skew	H_tip_kurt	-0.58341	4.90E-08
H_skew	H_tip_var	-0.71609	7.40E-13
H_skew	H_var	-0.70968	1.46E-12
H_skew	K_tip_kurt	-0.50939	3.57E-06
H_skew	K_tip_var	-0.69266	8.09E-12
H_skew	K_var	-0.69669	5.45E-12
H_skew	k1_mean	-0.64516	5.48E-10
H_skew	k1_tip_mean	-0.61844	4.32E-09
H_skew	k1_tip_var	-0.72457	2.93E-13
H_skew	k1_var	-0.69521	6.30E-12
H_skew	k2_tip_var	-0.71348	9.78E-13
H_skew	k2_var	-0.73637	7.64E-14
H_tip_kurt	K_mean	-0.68855	1.20E-11
H_tip_kurt	k2_mean	-0.6794	2.84E-11
H_tip_skew	H_tip_var	-0.65541	2.35E-10
H_tip_skew	H_var	-0.62957	1.87E-09
H_tip_skew	K_tip_var	-0.63931	8.76E-10
H_tip_skew	K_var	-0.62048	3.71E-09
H_tip_skew	k1_mean	-0.65588	2.26E-10
H_tip_skew	k1_tip_mean	-0.64812	4.31E-10
H_tip_skew	k1_tip_var	-0.62316	3.04E-09
H_tip_skew	k1_var	-0.59978	1.63E-08
H_tip_skew	k2_tip_var	-0.67606	3.86E-11
H_tip_skew	k2_var	-0.63867	9.22E-10
H_tip_var	K_mean	-0.83974	0.00E+00
H_tip_var	k2_mean	-0.93963	0.00E+00
H_var	K_mean	-0.82041	0.00E+00
H_var	k2_mean	-0.98205	0.00E+00
K_mean	K_tip_kurt	-0.6516	3.23E-10
K_mean	K_tip_var	-0.85764	0.00E+00

K_mean	K_var	-0.84883	0
K_mean	k1_mean	-0.8688	0.00E+00
K_mean	k1_tip_mean	-0.88048	0.00E+00
K_mean	k1_tip_var	-0.84009	0.00E+00
K_mean	k1_var	-0.83269	0.00E+00
K_mean	k2_tip_var	-0.84385	0.00E+00
K_mean	k2_var	-0.81363	0.00E+00
K_tip_kurt	k2_mean	-0.67552	4.05E-11
K_tip_var	k2_mean	-0.93646	0.00E+00
K_var	k2_mean	-0.97427	0.00E+00
k1_mean	k2_mean	-0.91009	0
k1_tip_mean	k2_mean	-0.8053	0.00E+00
k1_tip_var	k2_mean	-0.93839	0.00E+00
k1_var	k2_mean	-0.97668	0.00E+00
k2_mean	k2_tip_var	-0.94423	0.00E+00
k2_mean	k2_var	-0.98329	0.00E+00

2.2.6 SUPPLEMENTARY TABLE S6. Variable selection for dimensionality reduction using Gaussian model-based clustering.

The set of variables that best discriminated groups using normal mixture models (NMMs) defined using the greedy algorithm both in forward and backward directions is summarized. The Mclust model, the supported number of components or groups (G) detected in each case, the corresponding Log-likelihood, degrees of freedom (df), Bayesian information criterion (BIC) and integrated complete likelihood (ICL) are also depicted along with the lists of variables.

	Direction	
	Backward	**Forward**
Mclust model	EEE (ellipsoidal, equal volume, shape and orientation)	EEI (diagonal, equal volume and shape)
Log-likelihood	340.7631	111.8223
df	188	13
BIC	-127.638	167.6918
ICL	-127.638	161.9822
G (# components)	2	2
Initial # variables	29	29
Selected # variables	17	4
Variable subsets	br_length_mean br_rate_mean br_rate_var br_spacing_v1_mean br_spacing_v2_mean dc_mean H_mean H_skew H_tip_var K_tip_kurt K_var k1_tip_mean k1_tip_skew k1_var k2_tip_kurt k2_tip_var H_tip_kurt	br_length_mean H_skew k1_tip_mean H_tip_kurt

2.2.7 **Supplementary Table S7. Results of variance inflation factor (VIF) assessment.**

Variables that did not present collinearity (Variance Inflation Factor (VIF) <10) from the complete data set are listed below. Those that were also present in the preliminary selected subset depicting significant interspecific differentiation are indicated accordingly.

Variables	VIF	Preliminary selected subset
H_mean	6.128335	YES
H_skew	7.747286	YES
K_tip_kurt	9.83418	YES
b_angle_mean	3.59385	
b_angle_var	2.772566	
br_rate_var	4.074808	YES
br_spacing_v1_mean	3.920421	YES
br_spacing_v1_var	5.065652	YES
br_spacing_v2_var	2.643434	
d_avg_var	6.434551	
da_avg_mean	4.359019	
da_var	8.221089	
FD	4.806128	
H_tip_mean	5.538008	YES
H_tip_skew	6.452885	YES
H_kurt	5.397675	
K_skew	3.116537	
k1_tip_kurt	5.612972	
k2_mean	6.399289	YES
k2_tip_kurt	6.550996	YES
S	5.967713	

2.2.8 Supplementary Table S8. Results of the multiple analysis of variance (MANOVA).

The summarized results of a multivariate analysis of variance (MANOVA) using three different tests (i.e., Pillai, Wilks, and Roy) and the variables set are presented. Degrees of freedom (Df) along with *p*-values and MANOVA statistics are reported. Significance levels correspond to: (<0.001)***, (≤0.01)**, (≤0.01)*, (≤0.05). and (≤0.1)‘ ’.

	Df	Pillai	Approx. F	num Df	den Df	*p*-value	Significance
(Intercept)	1	0.99879	2012.83	21	51	< 2.2e-16	***
Species	2	1.47443	6.95	42	104	5.337e-16	***
Residuals	71						
	Df	**Wilks**	**Approx. F**	**num Df**	**den Df**	***p*-value**	**Significance**
(Intercept)	1	0.001205	2012.83	21	51	< 2.2e-16	***
Species	2	0.060179	7.47	42	102	< 2.2e-16	***
Residuals	71						
	Df	**Roy**	**Approx. F**	**num Df**	**den Df**	***p*-value**	**Significance**
(Intercept)	1	828.81	2012.83	21	51	< 2.2e-16	***
Species	2	4.93	12.21	21	52	1.982e-13	***
Residuals	71						

2.2.9 Supplementary Table S9. Linear discriminant analysis (LDA) prediction accuracy.

The summarized results of the linear discriminant analysis (LDA) are reported for the complete data set and the randomly selected training (68% of data) and testing (32% of data) subsets using prediction accuracy or confusion matrices. The central diagonal represents the number of specimens that were correctly identified by the linear model in each one of the cases. The accuracy percentage was calculated for each subset based on the total number of specimens in each set.

Complete (*n*= 74)	Is species A	Is species B	Is species C
Allocated to species A	26	0	0
Allocated to species B	1	21	0
Allocated to species C	1	0	25
		Accuracy %	97.30%

Training (*n*= 50)	Is species A	Is species B	Is species C
Allocated to species A	21	0	0
Allocated to species B	0	12	0
Allocated to species C	0	0	17
		Accuracy %	100%

Testing (*n*= 24)	Is species A	Is species B	Is species C
Allocated to species A	5	1	3
Allocated to species B	1	8	0
Allocated to species C	1	0	5
		Accuracy %	75%

2.2.10 Supplementary Table S10. Prediction accuracy of discriminant analysis based on Gaussian mixture modelling.

The summarized results of the discriminant analysis based on Gaussian mixture modelling (Supplementary Materials and Methods) are reported for the randomly selected training (68% of data) and testing (32% of data) subsets using two different approaches: the eigenvalue decomposition discriminant analysis (EDDA), which assumes that the density for each class can be described by a single Gaussian component; and the MclustDA approach that uses a finite mixture of Gaussian distributions within each class (mclust R package v5.4.7). Log-likelihood (Log-L), number of specimens used (n), degrees of freedom (df), Bayesian information criterion (BIC), number of components in each class (G, groups), accuracy (AC%), and the cross-validation classification error (CE) and standard error (SE) of the cross-validated statistic are also reported for each approach.

EDDA				**MclustDA**			
Log-L	**n**	**df**	**BIC**	**Log-L**	**n**	**df**	**BIC**
-303.8129	50	99	-994.9162	-284.1052	50	169	-1229.342

Classes	**n**	**%**	**Model**	**G**	**Classes**	**n**	**%**	**Model**	**G**
A	17	34	EEE	1	A	17	34	XXI	1
B	15	30	EEE	1	B	15	30	EEE	4
C	18	36	EEE	1	C	18	36	EEI	2

Training (*n*= 50)	Allocated to species A	Allocated to species B	Allocated to species C	**Training (*n*= 50)**	Allocated to species A	Allocated to species B	Allocated to species C
Is species A	17	0	0	Is species A	16	0	1
Is species B	2	13	0	Is species B	0	15	0
Is species C	1	1	16	Is species C	2	0	16
		AC%	92%			**AC %**	94%
Testing (*n*= 24)	Allocated to species A	Allocated to species B	Allocated to species C	**Testing (*n*= 24)**	Allocated to species A	Allocated to species B	Allocated to species C
Is species A	4	4	3	Is species A	5	0	6
Is species B	2	4	0	Is species B	3	0	3
Is species C	0	2	5	Is species C	0	0	7
		AC %	54.17%			**AC %**	50%
CE	0.20	**SE**	0. 0.0495	**CE**	0.380	**SE**	0.0526

3 Supplementary References

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DATA DESCRIPTOR

An Indo-Pacific coral spawning database

Andrew H. Baird *et al.*[#]

The discovery of multi-species synchronous spawning of scleractinian corals on the Great Barrier Reef in the 1980s stimulated an extraordinary effort to document spawning times in other parts of the globe. Unfortunately, most of these data remain unpublished which limits our understanding of regional and global reproductive patterns. The Coral Spawning Database (CSD) collates much of these disparate data into a single place. The CSD includes 6178 observations (3085 of which were unpublished) of the time or day of spawning for over 300 scleractinian species in 61 genera from 101 sites in the Indo-Pacific. The goal of the CSD is to provide open access to coral spawning data to accelerate our understanding of coral reproductive biology and to provide a baseline against which to evaluate any future changes in reproductive phenology.

Background & Summary

Scleractinian corals are the ecosystem engineers of coral reefs, the most species-rich marine ecosystems. Scleractinian corals have a bipartite life history, with a sessile adult stage and a planktonic larval stage that allows dispersal among reefs. Corals produce larvae in one of two ways: gametes are broadcast-spawned for external fertilization or the eggs are retained for internal fertilization, followed by the release of planula larvae from the polyp. The discovery of multi-species synchronous spawning on the Great Barrier Reef[1] stimulated a large effort to document coral spawning times in other regions of the world. Similar multi-species spawning events *sensu*[2] have now been documented in over 25 locations throughout the Indo-Pacific[3–5]. However, much additional data on coral sexual reproductive patterns remain unpublished. Even when spawning data are published, there is often insufficient detail, such as the precise time and duration of spawning, to address many important questions. Consequently, predicting the month of spawning has been the focus of many studies to date[6].

Coral spawning times can be used to address many significant and fundamental questions in coral reef ecology. Most coral species are notoriously difficult to identify and spawning times have been used to infer pre-zygotic barriers to fertilization and thus assist decisions about species boundaries[7,8]. While proximate cues associated with the month of spawning are reasonably well understood in some taxa[6,9], the relationship between cues for the date and time of spawning are poorly understood. Similarly, potential phylogenetic patterns and geographical variation in spawning times are only beginning to be explored[10]. Knowing when corals spawn is also important for managing coastal development. For example, in Western Australia, legislation requires dredging operations to cease during mass spawning events[11,12]. Coral spawning is also an economic boon for tourist operators in many parts of the world, such as the Great Barrier Reef. Furthermore, population level records of spawning times provide a baseline against which to evaluate potential changes in spawning synchrony or seasonality associated with anthropogenic disruptions to environmental cues, in particular, sea surface temperature[13]. Knowledge of the timing of spawning is also essential for accurately estimating levels of connectivity among populations, given season differences in current flow[14]. The value of long-term species level data on coral spawning has recently been demonstrated in a test of the influence of temperature and wind on the night of coral spawning[15].

In this data descriptor, we present the Coral Spawning Database (CSD). The CSD includes spawning observations for reef building coral species from the Indo-Pacific. The CSD includes 6178 observations (3085 of which were unpublished) of the time or day of spawning for 300+ scleractinian species in 61 genera (Online-only Table 1) from 101 sites (Fig. 1) in the Indo-Pacific. The goals of the CSD are: (i) to assemble the scattered and mostly unpublished observations of scleractinian coral spawning times and (ii) to make these data readily available to the research community. Our vision is to help advance many aspects of coral reef science and conservation at a time of unprecedented environmental and societal change.

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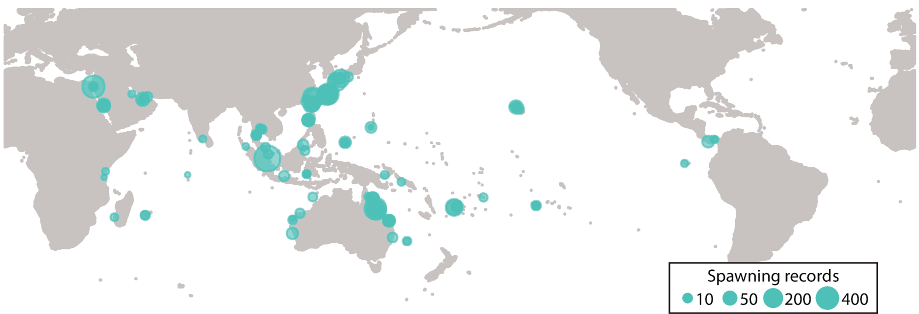

Fig. 1 The number of spawning records by site.

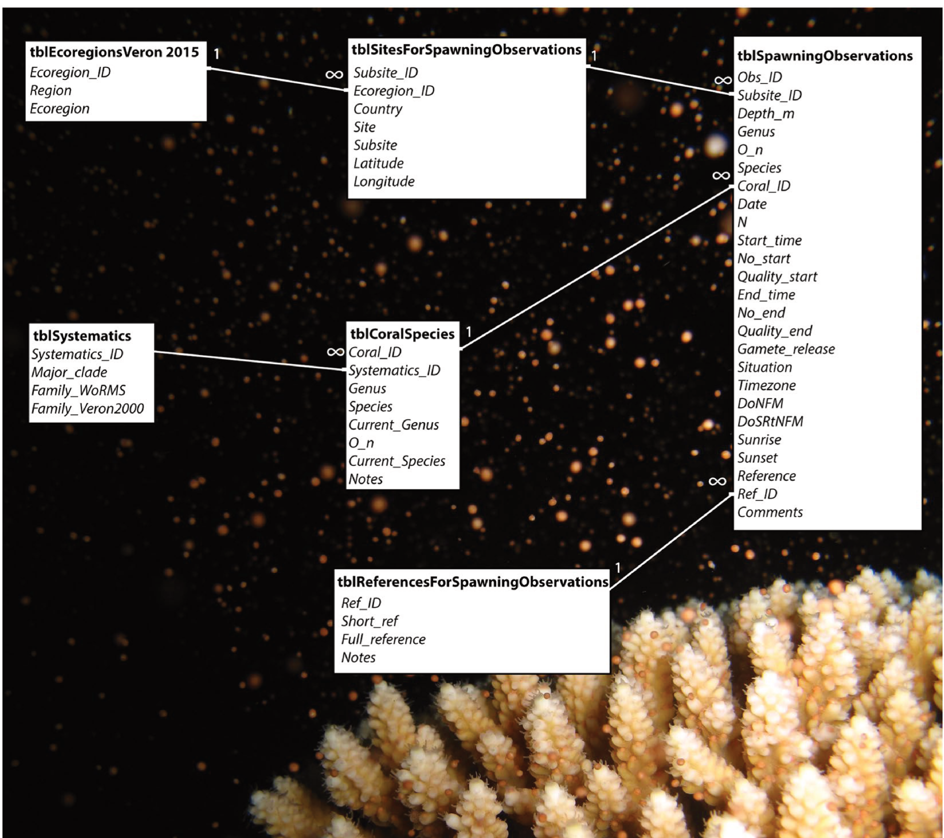

Fig. 2 Arrangement of data tables in the Access relational database.

Methods

The CSD includes spawning times for broadcast spawning scleractinian coral species in the Indo-Pacific. There are two sources for these data: the literature and unpublished observations. Published literature was selected based on the authors' knowledge of the subject area and a literature search using the terms "coral AND spawn*". Over 50 researchers known by the authors to have extensive data on coral spawning times were approached to contribute unpublished data. This initial invitation led to a subsequent round of invitations to additional contributors. Of course, we encourage any researchers with data we have missed to contribute their observations in the annual update of the database. The database focusses on spawning times. Many other biological variables related to coral reproduction, such as fecundity, are available in the Coral Traits Database[16].

The database is available as a Microsoft Access relational database or an Excel spreadsheet. To minimise repetition in data entry, spawning observation information is entered in three primary tables (Fig. 2). The first ("tblSitesForSpawningObservations") is used to enter geographic information on each study site; the second

("tblSpawningObervations") contains details of the spawning activity recorded at each site; the third ("tblReferencesForSpawningObervations") contains either full bibliographic details for published studies or details of the source of unpublished data. To assist with data analysis, three accessory tables are also linked. The first ("tblEcoregionsVeron2015") allows sites to be grouped into the biogeographical Ecoregions proposed by[17] or by broader region (e.g. Indian Ocean, Western and Central Pacific, Eastern Pacific). The remaining two tables allow the coral species to be grouped systematically for analysis. The first ("tblCoralSpecies") has a list of over 1600 coral species with genus and species names (primarily from[18] or subsequent descriptions of new species) mapped to currently accepted names (primarily from[19]) where the taxonomy has changed. The second ("tblSystematics") allows species to be grouped into major clades or currently accepted families[19] as revealed by molecular studies[20–22].

Data entry. *Coral Spawning Database fields.*

1) **Site information (in tblSitesForSpawningObservations):**
Ecoregion_ID link to Ecoregions (150) as defined by[17]
Country the country, territory (e.g. Guam) or island group (e.g. Hawaiian Islands) where spawning observation was made
Site accepted name for broad geographical location (e.g. archipelago, island, offshore reef, bay, etc.) of the observation
Subsite more precise site name within location (where applicable; na entered where no subsite)
Latitude in decimal degrees (-ve values for sites South of the Equator).
Longitude in decimal degrees (-ve values for sites West of the Greenwich Meridian).

2) **Spawning observations (in tblSpawningObservations):**
Depth_m the approximate depth at which the colony was collected (for *ex situ* observations) or observed (for *in situ* observations). If not recorded then −99 entered.
Genus currently accepted genus name[19]
O_n open nomenclature qualifier: see explanation below under "Species identifications".
Species the species name used by the observer
Date date of spawning observation in the format day/month/year (e.g. 24/11/1983)
N number of colonies or individuals observed spawning. Used −99 if not known. If exact number of colonies not counted but more than a specific number were observed to spawn (e.g. > 25), then minimum number counted was entered (e.g. 25).
Start_time time of first observation of spawning for colony(ies) of species: time (hh:mm) on a 24 hour clock e.g. 18:30. See "recording the time of spawning" below for ways to use the time fields to capture the various ways spawning is usually observed. No threshold applied to the intensity of spawning.
No_start no information on time that spawning started: True or False.
Quality_start if No_start is False, Exact or Approx.
End_time time of last observation of spawning for colony(ies) of species (if later than start time, normally): time (hh:mm) on a 24 h clock e.g. 18:30
No_end no information on time that spawning ended: True or False
Quality_end if No_end is False, Exact or Approx
Gamete_release (five character states as follows)

- Bundles – eggs and sperm released together packaged in bundles
- Eggs – only eggs released
- Sperm – only sperm released
- Both separately – eggs and sperm released separately from the same colony. Examples include *Lobophyllia hemprichii* and *Goniastrea favulus*
- Not recorded – release of gametes not observed or not reported

Situation *In situ* = spawning observed underwater or *Ex situ* = spawning observed in tanks of colony(ies) recently removed from the reef.
Timezone local time zone on the date of the spawning observation. This allows local time of spawning to be related to local time of sunset (or occasionally sunrise, for daytime spawners). This field is not an integer to accommodate 30 minute time differences (e.g. India and Sri Lanka are on UTC + 5.5). Enter -ve values for sites west of the Greenwich Meridian: e.g. −11 for Hawaii. (Note: Daylight Saving Times mean that time zones at some sites vary with date, e.g. Fiji goes from UTC + 12 to UTC + 13 from early November to early January).

The next four fields contain benchmarks for comparing spawning among sites for different species or groups of species[23]. The first is the date of the nearest full moon (DoNFM) to the date of spawning (with 75% of spawning recorded in the week after the full moon). This allows all spawning dates to be calculated in terms of days before or after the full moon (DoSRtNFM). Sunset provides a benchmark for comparing the times of spawning for most spawners (over 90% of spawning started within 4 hours of sunset) and sunrise for a few daytime spawners such as *Pocillopora verrucosa*. Dates of full moon and times of sunrise and sunset are available for given locations from the web (e.g. www.timeanddate.com) and can be entered manually. However, they can also be calculated automatically in the database based on the date, time zone and, for sunrise and sunset, the latitude and longitude. Excel spreadsheets are also available on request from the corresponding authors to calculate dates of full moon and times of sunrise and sunset in addition to a data entry template.

DoNFM	Date of Nearest Full Moon. Calculated automatically and corrected for longitude based on the local time zone.
DoSRtNFM	Date of Spawning Relative to Nearest Full Moon. Calculated automatically using time zone and date of observation in days before (-ve) or after (+ve) the nearest full moon (ranges from −15 days to +14 days).
Sunset	local time of sunset using a 24 h clock e.g. 18:30. Sunset and sunrise times were calculated for each observation based on latitude, longitude and time zone of the site and the date, using the method in the NOAA solar calculations day spreadsheet at https://www.esrl.noaa.gov/gmd/grad/solcalc/calcdetails.html. An Excel spreadsheet (Sunrise_Sunset_DoNFM_Calculations.xlsx) is provided for anyone wishing to use the Excel version of the dataset.
Sunrise	local time of sunrise using a 24 h clock e.g. 05:30. See above.
Ref_ID	a link to reference information for the data if available. If not the names of the observers are listed (e.g. Baird, Connolly, Dornelas and Madin unpublished)
Comments	any additional details provided

3) **Reference information (in tblReferencesForSpawningObservations):**

Each set of observations is referenced to its published or unpublished source in this table via a Ref_ID. The table contains two main fields: "Short_ref" (e.g. Baird *et al.* 2015) and "Full_reference" (e.g. Baird AH, Cumbo VR, Gudge S, Keith SA, Maynard JA, Tan C-H, Woolsey ES (2015) Coral reproduction on the world's southernmost reef at Lord Howe Island, Australia. Aquatic Biology 23:275–284). These can be filled in before or after entering spawning observations. An email address is provided for all unpublished contributions.

Notes to recording the time of spawning. For the quality of a start or end time to be 'Exact', a colony must be under continuous observation and the time of onset or end of spawning be observed and recorded. Most *in situ* observations would be expected to be approximate ('Approx').

The Quality_start, Quality_end, No_start and No_end fields are designed to accommodate the most common ways spawning is observed. A series of examples are given below.

1. A colony is observed spawning but it is not known exactly when it started. No end time is recorded.
   Here enter the time the colony was first observed spawning as the Start_time and the Quality_start as 'Approx'. Leave the End_time blank and set No_end to True.
2. A colony is followed closely until spawning is observed to begin but the precise time when spawning ends is not recorded. However, the colony is observed to be still dribbling spawn 30 minutes after spawning started.
   Here enter the Quality_start_ as 'Exact' with the End_time set to 30 minutes after the Start_time and the Quality_end set to 'Approx'.
3. A colony is followed closely from the beginning until the end of spawning.
   Here enter the times and note Quality_start and Quality_end as 'Exact'.
4. A colony is placed in a bucket and checked every 30 minutes. At the first observation there is no evidence of spawning, 30 min later the surface of the water is covered in bundles and the colony is no longer spawning.
   Here enter the time of the first observation as the start time and the time of the second observation as the end time and set Quality_start and Quality_end to 'Approx'.
5. Only the night of spawning is known, for example, gametes are no longer apparent in a tagged and sequentially sampled colony.
   Here don't enter either a start time or an end time and leave Quality_start and Quality_end blank. Set No_start and No_end to True.

Species identifications. Species were generally identified following[18,24] or by comparing skeletons to the type material or the original descriptions of nominal species. Specimens identified following[18,24] were updated to the currently accepted names at the World Register of Marine Species[19]. The database also allows for uncertainties in species identifications to be indicated with the use of a series of open nomenclature qualifiers[25,26] that allow the assignment of specimens to a nominal species with varying degrees of certainty. Specimens that closely resemble the type of a nominal species are given the qualifier cf. (e.g. *Acropora* cf. *nasuta*). Specimens that have morphological affinities to a nominal species but appear distinct are given the qualifier aff. (e.g. *Acropora* aff. *pulchra*): these specimens are either geographical variants of species with high morphological plasticity or potentially undescribed species. Species that could not be matched with the type material of any nominal species were labelled as sp. in addition to the location where they were collected (e.g. *Acropora* sp_1_Fiji). These specimens are most probably undescribed species. For 1% of records spawning colonies were only identified to genus (e.g. *Montipora* sp.). Contact the sources of these data for further information on the species identity.

Data Records

A snapshot of the data contained in this descriptor can be downloaded from figshare[27]. The data includes 6178 observations, 3085 of which were unpublished with the remainder gleaned from the literature[28–128]. These data have been through a rigorous quality control and editorial process. Annual updates of the dataset will be uploaded to figshare as new version and also made available at any time on request from the Editor (JRG). Contributions to the CSD are welcome at any time and should be sent to the Editor (JRG).

Technical Validation

The database is governed on a voluntary basis, by an Editor (JRG), Assistant Editors (JB & AGB), a Taxonomy Advisor (AHB) and a Database Administrator (AJE). Quality control of data and editorial procedures include:

1. **Contributor approval**. Database users must request permission to become a database contributor.
2. **Editorial approval**. Once a contributor sends data to the Editor, the data will be checked and if correctly formatted will be forward to the Database Administrator
3. **User feedback**. Data issues can be reported for any observation by email to the Editor

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A coral spawning calendar for Sesoko Station, Okinawa, Japan

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Abstract Sesoko Station, Okinawa, has been the site of many significant advances in coral reproductive research and it continues to be a preferred destination for both Japanese and international researchers. Consequently, there are decades of spawning observations, which we present and explore here with the aim of making it easier to predict when species spawn at Sesoko Station. The data include over 700 spawning observations from 87 species of reef-building hermatypic corals. Almost all spawning occurred between dusk and dawn, with most spawning activity concentrated in the 2 to 4 hours after sunset. Some phylogenetic patterns were evident: most *Acropora* species spawn on or around the 6th full moon after December 21st (the northern hemisphere winter solstice); spawning in common species of merulinids and *Porites* appears to be concentrated around the 7th full moon and spawning in the fungiids around the 8th and subsequent full moons. The night of peak spawning with respect to the night of the full moon varied considerably among years in common *Acropora* species, but was dependent on the calendar date of the full moon in May or June. Therefore, despite

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an extended spawning season of over three months and considerable variation in the calendar date of spawning in many species among years, the month and night of spawning are reasonably predictable for many species enhancing the value of Sesoko Station as a site for coral reproductive research.

Keywords Coral reefs, Phenology, Reproduction, Multi-species synchronous spawning

Introduction

Most scleractinian corals broadcast spawn their gametes for external fertilization (Harrison and Wallace 1990; Baird et al. 2009). In general, each colony spawns once per year, often in high synchrony with nearby conspecifics. These multi-specific spawning events are an excellent opportunity for scientists to access coral propagules for experiments. The time of spawning is often predictable in terms of the month of the year, the day relative to the full moon and the time of day, however, there is considerable annual variation at most temporal scales that has yet to be fully explored. Being able to accurately predict spawning dates and times is essential for planning field trips to conduct coral spawning work and for managing human activities that affect coral reproduction, such as dredging (Baird et al. 2011; Styan and Rosser 2012).

The Tropical Biosphere Research Center (TBRC) of the University of the Ryukyus, based on Sesoko Island in the prefecture of Okinawa (hereafter referred to as Sesoko Station), was opened in 1971. Sesoko Station has been the site of many significant advances in coral reproductive research and it continues to be a preferred destination for both Japanese and international researchers. The first records for coral spawning times at Sesoko Station are those of Heyward et al. (1987). Other landmark studies on coral reproductive biology and larval ecology from Sesoko include the first records of daytime spawning (Kinzie 1993), the first records of a single colony being able to both brood and broadcast spawn propagules (Sakai 1997), the first evidence to suggest that individuals of some fungiids can change sex (Loya and Sakai 2008), some of the first work to explore the timing of the acquisition of zooxanthellae in the early life-history of corals (Harii et al. 2009), the effect of increased temperature on pre-competent periods in coral planulae (Figueiredo et al. 2014) and evidence of the first naturally occurring coral hybrids in the Indo-Pacific (Morita et al. 2019). Sesoko Station is also the site of some of the most exhaustive and detailed observation of coral spawning *in situ*. In particular, Dr Satoshi Nojima spent up to 4 hours a night on the reef in front of Sesoko Station every night for over 30 days in 1993 to record coral spawning, a feat which was recently repeated by Dr Takuma Mezaki.

Many interesting and important questions can be addressed with data on the timing of coral spawning. Spawning times are a useful line of evidence in taxonomic studies. For example, if two putative species spawn at different times they are likely to be different species (Wolstenholme 2004; Furukawa et al. 2020). Effective conservation and management of coral reefs is also dependent on knowing when corals spawn. For example, potentially damaging activities, such as dredging, can be prohibited at times that corals are known or predicted to spawn (Baird et al. 2011; Jones et al. 2015). Coral spawning is also a significant attraction for tourists in many parts of the world. Knowing when corals spawn is also fundamental to understanding and predicting patterns of connectivity, given that currents vary seasonally in many parts of the world (Hock et al. 2019).

The aim of this paper is to provide a spawning calendar and some predictive tools to allow researchers to better manage human activities and plan field trips to Sesoko Station.

Materials and methods

Site description: The fringing reefs of Sesoko Island, Okinawa, Japan (26°38′42″N 127°51′52″E). For a description of the reef see Sakai and Yamazato (1987).

Source of data: The data are a subset of the dataset published by Baird et al. (2021). We included all data from sites around Sesoko. The only changes were that the open nomenclature status of two species *(Acropora* cf. *hyacinthus* and *Acropora* aff. *hyacinthus*) were dropped because at this location they appear to be one species.

Data exploration: The date of coral spawning is usually expressed in days relative to the date of the nearest full

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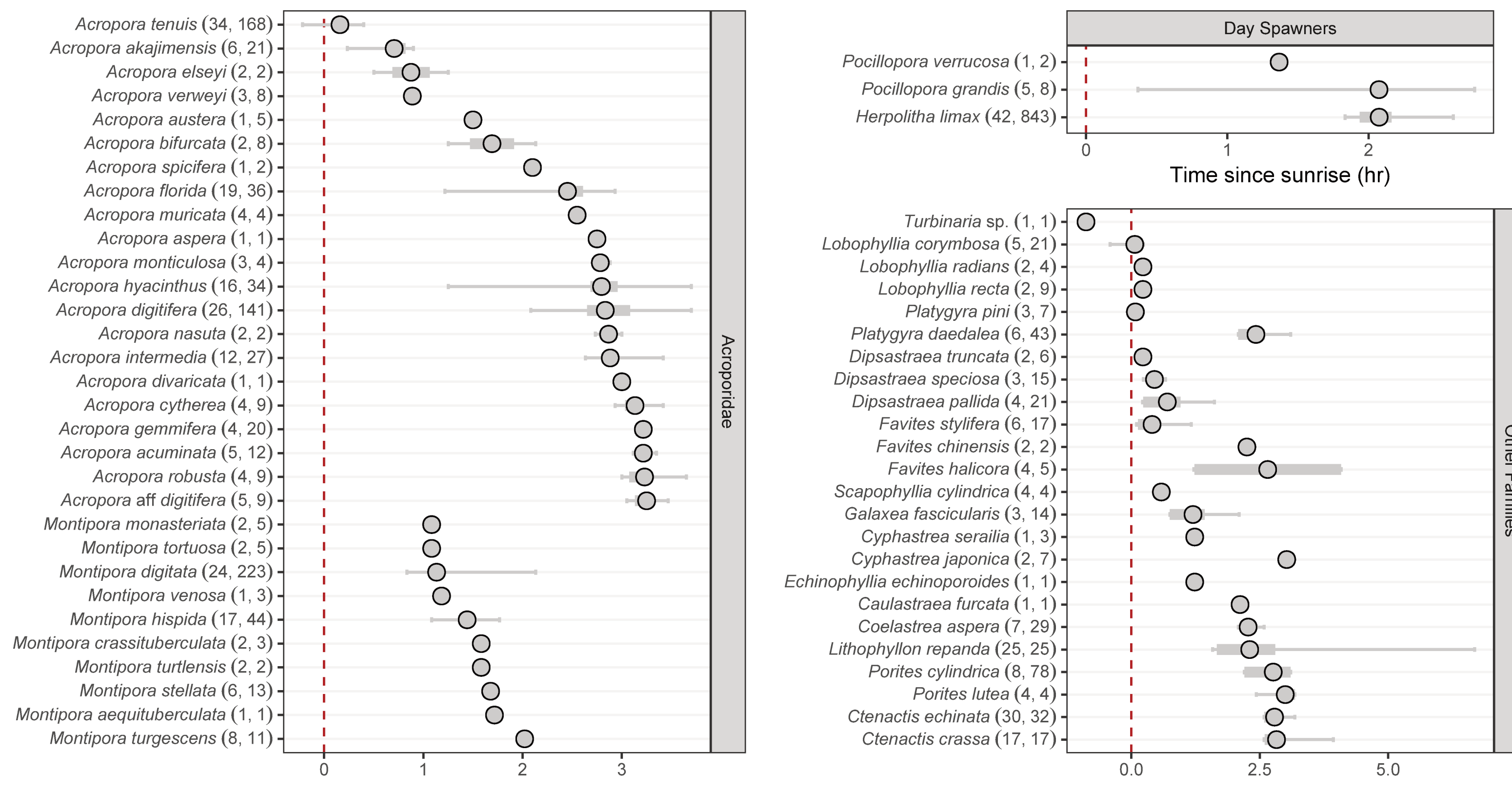

Fig. 1 The time of coral spawning relative to sunset or sunrise. The grey filled circle is the mean observed start time and the bars are the range of start times, showing the minimum and maximum (thin bars) and the 25th to 75th percentiles (thick bars). Within the brackets after each species, the first value represents the total number of records (e.g., nights when timing was noted) and the second value represents the total number of colonies observed to spawn (note: if the number of colonies was not recorded then at least one colony was assumed to have spawned).

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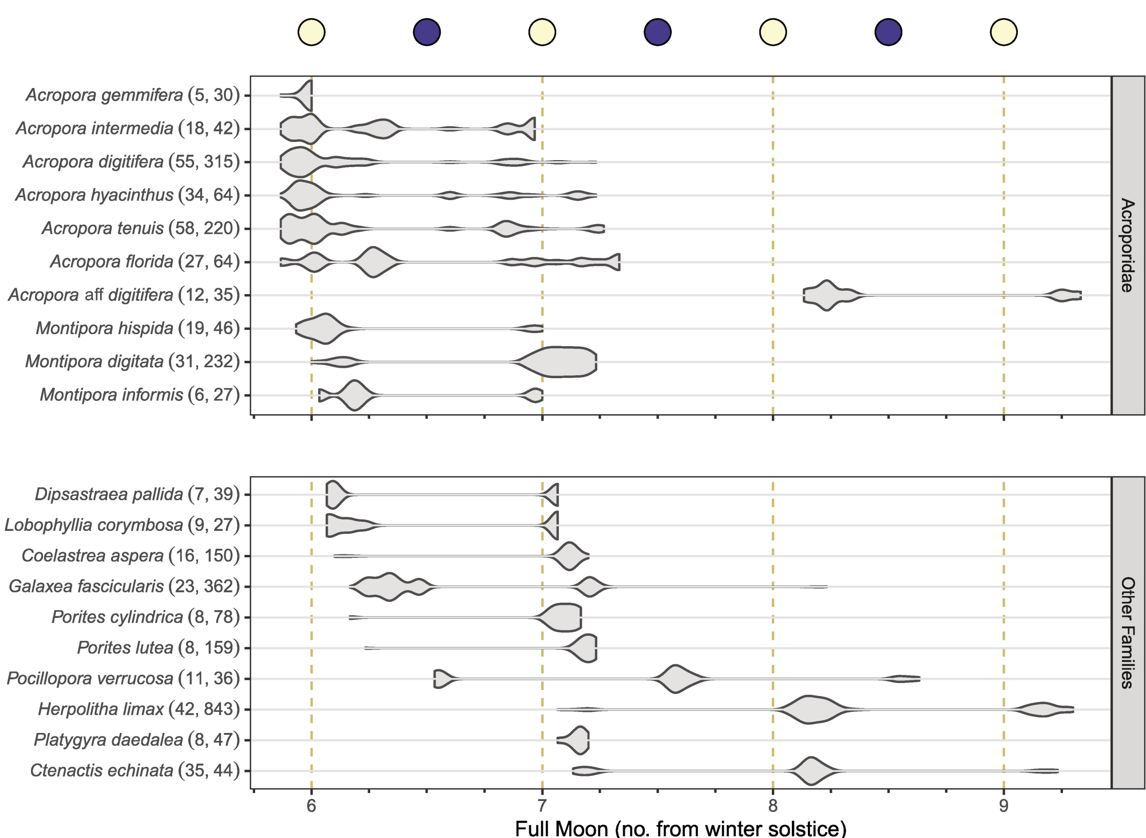

Fig. 2 A spawning calendar for some common coral species at Sesoko Island. The x-axis divides the year into lunar months with full moon 1 defined as the first full moon on or after December 21st of the previous calendar year. Full and new moons are represented as pale and dark circles, respectively. Within the brackets after each species name, the first value represents the total number of records (e.g., nights when spawning was observed) and the second value represents the total number of colonies observed to spawn (note: if the number of colonies was not recorded then at least one colony was assumed to have spawned). Violin contours show the probability density of spawning being observed at a specific date in the lunar calendar. A daily breakdown of the total number of colonies observed to spawn for each species, including rare species not shown here, can be found in the spawning calendar table in the electronic supplementary materials. All years of data are pooled.

moon. For the purpose of our spawning calendar, full moons were numbered consecutively from December 21 (the typical date of the northern hemisphere winter solstice). This resulted in full moon 1 in the lunar year being the first full moon on or after December 21 in the previous calendar year to spawning. This is in contrast to the Gregorian calendar which has spawning commencing in May in some years and June in others. Coral spawning relative to the lunar calendar was visualized for the 20 species with the greatest number of spawning observations. Probability density curves, presented as violin plots, were computed for each taxon to show the distribution of spawning observations through time. This required a decision on the smoothing bandwidth parameter value (standard deviation of the smoothing kernel). A value of 0.03 avoided over-smoothing, whilst still clearly showing dates without spawning observations. Observations with no spawning times recorded were removed from the analysis of spawning times relative to sunset resulting in a different number of observations for the same species between Fig. 1 & 2.

Results

Spawning times relative to sunset

Of the 58 species for which there are observations on the diel timing of spawning at Sesoko, most observations

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Table 1 Summary of diel spawning times of 58 species on the fringing reefs of Sesoko Island, Okinawa, Japan. The # colonies column indicates the total number of colonies observed (note: if the number of colonies was not recorded then at least one colony was assumed to have been observed).

Night spawners			**All times decimal hours relative to sunset**					
Taxon	Observations	# colonies	Min_start	Mean_start	Max_start	Min_end	Mean_end	Max_end
Acropora acuminata	5	12	3.12	3.22	3.35	3.93	3.93	3.93
Acropora aff. *digitifera*	5	9	3.05	3.25	3.47			
Acropora akajimensis	6	21	0.23	0.71	0.90			
Acropora aspera	1	1	2.75	2.75	2.75			
Acropora austera	1	5	1.50	1.50	1.50			
Acropora bifurcata	2	8	1.25	1.69	2.13	2.13	2.13	2.13
Acropora cytherea	4	9	2.93	3.13	3.42	3.57	3.57	3.57
Acropora digitifera	24	128	2.08	2.84	3.70	2.08	3.53	4.25
Acropora divaricata	1	1	3.00	3.00	3.00	4.25	4.25	4.25
Acropora elseyi	2	2	0.50	0.88	1.25			
Acropora florida	19	36	1.22	2.45	2.93	2.62	2.86	3.32
Acropora gemmifera	4	20	3.18	3.22	3.25			
Acropora hyacinthus	15	32	1.25	2.80	3.70	3.70	3.70	3.70
Acropora intermedia	12	27	2.63	2.88	3.42	2.63	3.14	4.08
Acropora monticulosa	3	4	2.72	2.78	2.88	4.25	4.25	4.25
Acropora muricata	4	4	2.52	2.55	2.58			
Acropora nasuta	2	2	2.73	2.87	3.00	3.48	3.87	4.25
Acropora robusta	4	9	3.00	3.23	3.65	4.25	4.25	4.25
Acropora spicifera	1	2	2.10	2.10	2.10			
Acropora tenuis	34	168	−0.22	0.16	0.40	0.07	0.48	0.90
Acropora verweyi	3	8	0.85	0.89	0.92			
Caulastraea furcata	1	1	2.12	2.12	2.12	2.12	2.12	2.12
Coelastrea aspera	7	29	2.08	2.27	2.58	2.33	3.31	3.68
Ctenactis crassa	17	17	2.58	2.83	3.93	3.93	5.18	5.55
Ctenactis echinata	30	32	2.58	2.79	3.18	3.18	4.99	5.55
Cyphastrea japonica	2	7	2.88	3.03	3.17	3.17	3.17	3.17
Cyphastrea serailia	1	3	1.23	1.23	1.23			
Dipsastraea pallida	4	21	0.22	0.70	1.62	0.98	1.30	1.62
Dipsastraea speciosa	2	11	0.23	0.45	0.67	0.67	0.67	0.67
Dipsastraea truncata	2	6	0.22	0.23	0.23			
Echinophyllia echinoporoides	1	1	1.23	1.23	1.23			
Favites chinensis	2	2	2.25	2.25	2.25	3.58	3.58	3.58
Favites halicora	4	5	1.22	2.65	4.08			
Favites stylifera	6	17	0.10	0.40	1.17	0.10	0.49	1.17
Galaxea fascicularis	3	14	0.75	1.20	2.10	2.08	2.09	2.10
Lithophyllon repanda	25	25	1.58	2.30	6.68	7.68	7.68	7.68
Lobophyllia corymbosa	5	21	−0.42	0.07	0.23	−0.42	−0.04	0.15
Lobophyllia radians	2	4	0.22	0.23	0.23			
Lobophyllia recta	2	9	0.22	0.23	0.23			
Montipora aequituberculata	1	1	1.72	1.72	1.72			
Montipora crassituberculata	2	3	1.58	1.58	1.58			
Montipora digitata	24	223	0.83	1.13	2.13	1.58	2.11	3.17
Montipora hispida	17	44	1.08	1.44	1.77	2.10	2.10	2.10
Montipora monasteriata	2	5	1.08	1.08	1.08			
Montipora stellata	6	13	1.65	1.68	1.72			
Montipora tortuosa	2	5	1.08	1.08	1.08			
Montipora turgescens	8	11	1.98	2.02	2.07			
Montipora turtlensis	2	2	1.58	1.58	1.58			
Montipora venosa	1	3	1.18	1.18	1.18	3.18	3.18	3.18
Platygyra daedalea	6	43	2.08	2.43	3.10	2.58	3.41	3.85
Platygyra pini	3	7	0.00	0.08	0.23	1.08	1.08	1.08
Porites cylindrica	8	78	2.20	2.76	3.10	4.60	4.60	4.60
Porites lutea	4	4	2.43	3.00	3.18			
Scapophyllia cylindrica	4	4	0.58	0.58	0.58			
Turbinaria sp.	1	1	−0.88	−0.88	−0.88			
	359	**1180**						

Day spawners			**All times decimal hours relative to sunrise**					
Taxon	Observations	# colonies	Min_start	Mean_start	Max_start	Min_end	Mean_end	Max_end
Herpolitha limax	42	843	1.83	2.07	2.60	3.30	4.50	4.78
Pocillopora grandis	5	8	0.37	2.07	2.75	3.25	4.75	5.25
Pocillopora verrucosa	1	2	1.37	1.37	1.37	1.87	1.87	1.87
	48	**853**						

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are concentrated in the first 4 hours after sunset (Table 1; Fig. 1; ESM). In contrast, *Herpolitha limax*, *Pocillopora grandis* and *P. verrucosa* start to spawn between 1 and 2 h after sunrise (Table 1; Fig. 1). The majority of *Acropora* spp. spawn between 2.5 and 3.5 hours after sunset (ESM). The exceptions are a few *Acropora* spp. that spawn within one hour of sunset, including *A. tenuis* and *A. akajimensis*. Some *Acropora* spp. have a large range of spawning times, e.g. *A. digitifera*, *A. hyacinthus* and *A. florida* (Fig. 1; ESM). *Montipora* spp. spawn between 1 and 3.5 hours after sunset, with most species spawning 1−2 hours after sunset (Table 1; Fig. 1; ESM). The majority of non-acroporid taxa spawn within 2 hours of sunset (ESM).

Lunar moon and night of spawning

The vast majority of spawning observations in the 87 taxa occur on or around full moons (ESM). The only species that does not follow this trend is *Pocillopora verrucosa* which spawns on the new moon (Fig. 2). For the *Acropora* spp., the majority of spawning observations are concentrated around the 6th moon following the winter solstice (ESM). Nonetheless, for species with greater than approximately 20 observations, spawning also occurred around the 7th moon following the winter solstice (ESM). In all species, spawning occurred over a considerable range of nights (ESM) and the night of peak spawning in some *Acropora* species is affected in part by the date of the full moon (Fig. 3). For example, if the 6th full moon falls before May 30 the *Acropora* tend to spawn on the nights after the full moon, whereas, if it falls after May 30 the *Acropora* tend to spawn on nights prior to the full moon (Fig. 3). One species, *A.* aff. *digitifera* (previously referred to as *Acropora* sp_1; e.g. Hayashibara and Shimoike (2002)) spawns two months later than the other *Acropora* species following the 8th moon after the winter

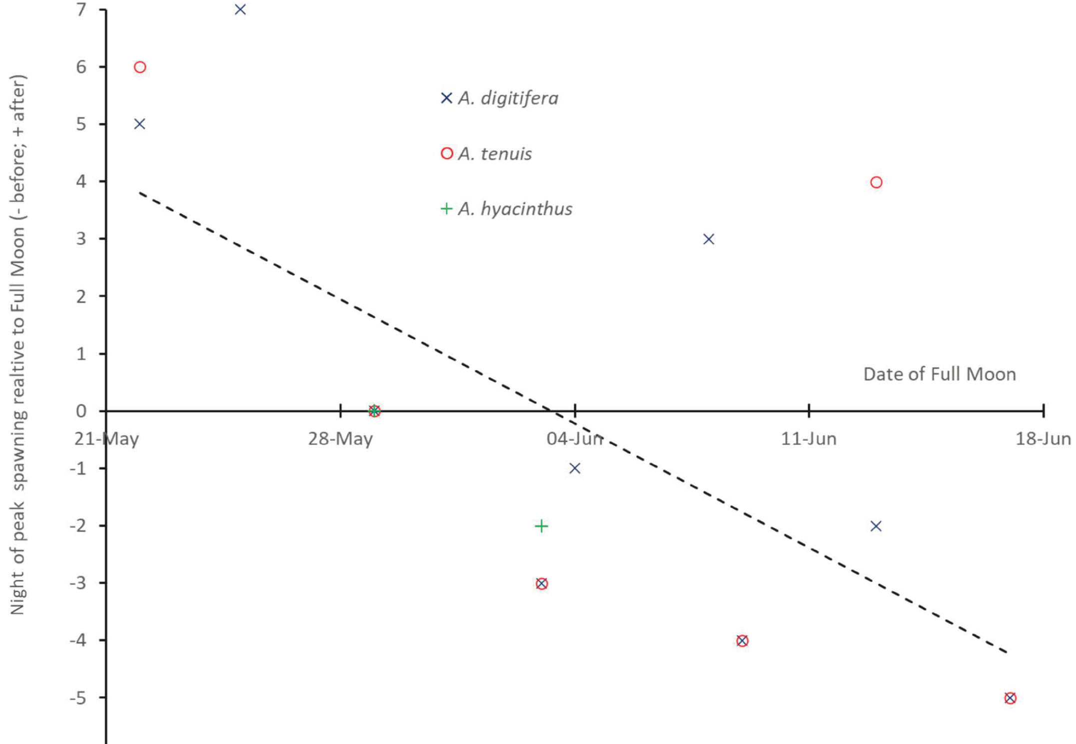

Fig. 3 The night of peak spawning in three species of the genus *Acropora* as a function of the calendar date of the full moon at Sesoko Station. The x-axis is the calendar date of the full moon, and the y-axis is the night of peak spawning in *Acropora tenuis*, *A. digitifera* and *A. hyacinthus* relative to the full moon. Peak spawning was defined as the night on which the highest proportion of *Acropora* colonies were observed to spawn. The dashed line is the trend line of all the points.

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solstice.

Some *Acropora* spp. have two peaks in spawning observations within the 6th month after the winter solstice. For example, *A. intermedia* and *A. florida* have a peak in spawning observations just before the 6th moon and another approximately a week later.

The *Montipora* spp. have very similar patterns with respect to the lunar month to the *Acropora*, with most spawning observations concentrated around the 6th full moon and fewer on the 7th full moon (ESM). In contrast, spawning observations for the two *Porites* spp. are concentrated around the 7th full moon (ESM). In addition to *A.* aff. *digitifera* mentioned above, the only other species with spawning observations later in the lunar year are four fungiid spp. (Fig. 2, ESM 1) that spawn following moons 7, 8 and 9 plus some *Galaxea* colonies following the 8th full moon (ESM).

Discussion

The vast majority of spawning observations in Sesoko occur at night. These results are similar to observations from other regions in the Indo-Pacific, including the Great Barrier Reef (Harrison et al. 1984; Babcock et al. 1986) and the Red Sea (Shlesinger and Loya 1985; Bouwmeester et al. 2015), however, this in part reflects the fact that people are generally only looking for spawning at night. Species known to release gametes during the day include *Pavona* sp. (Plathong et al. 2006) and *Porites rus* (Bronstein and Loya 2011). Even with all the coral reproductive research at Sesoko over a 30 year period, there are still data on the night of spawning for only 87 species and data on the time of spawning for 58 species of the approximately 143 species recorded at Sesoko (Sakai and Yamazato 1987). Furthermore, the number of observations for many species is low. More work is needed at other times of the day to determine when these other species are spawning, in particular, species that are not from families well represented in the spawning observations to date, such as the Agariciidae and Coscinaraeidae.

Interestingly, there are no spawning observations before the 6th moon following the 21 December. While there are 114 records of spawning between 20th and 31st May (out of 711 records in total) all of these are from -3 to +6 days from the 6th full moon after the 21 December. Clearly, a lunar calendar commencing on 21 December is a better predictor of the month of coral spawning than the Gregorian calendar at Sesoko. Whether or not this predictive tool works in other locations in which there is annual variation in the first month of spawning, such as the GBR, needs to be tested.

Phylogeny appears to have an effect on the lunar month of spawning. Most acroporid and lobophylliid corals spawn on the 6th moon after 21 December; pocilloporids and poritids around the 7th and fungiids on the 8th moon after the winter solstice. Phylogeny also appears to influence the night of spawning within the mass spawning period on the Great Barrier Reef (Willis et al. 1985). Further research is required to identify whether there are similar patterns in other regions and to identify the causes of such patterns.

The night of peak spawning (defined as the night on which the most colonies were observed to spawn within a species) varied considerably among years in some species. For example, peak spawning of *A. tenuis* occurred anywhere from 5 days before to 6 days after the closest full moon (Fig. 3). A similar range in spawning nights was also evident in the *Acropora* spp. in Taiwan (Lin and Nozawa 2017). A similar range in spawning nights is not seen at sites on the Great Barrier Reef, such as Lizard Island or Orpheus Island (Baird et al. 2021). However, the night of spawning is associated with the calendar date of the full moon (Fig. 3) with spawning occurring earlier relative to the full moon the later the calendar date of the full moon. This pattern has recently been shown to be influenced, in part, by environmental conditions in the weeks and months prior to spawning, in particular, cumulative sea temperatures (Sakai et al. 2020).

Some caveats apply to these data, in particular, the value of the observed data to make predictions will be strongly dependent on the number of observations. However, the fact that the variability in these data increases with the number of observations suggests that making accurate predictions might always be difficult, particularly for variables such as the night of spawning. Furthermore, it remains to be tested whether the patterns observed at Sesoko apply in other parts of the world. For example, it would not be wise to predict the night of

spawning for a given species on the Great Barrier Reef based on these data from Sesoko. Further research is required to test the generality of the patterns identified at Sesoko.

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Electronic supplementary material

ESM can be downloaded from the J-STAGE website: https://doi.org/10.3755/galaxea.G2021_S10O

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(Veron, 2013)

Ta-taki falls
Kunigami, Okinawa, Japan
Photo by J.-F. Flot

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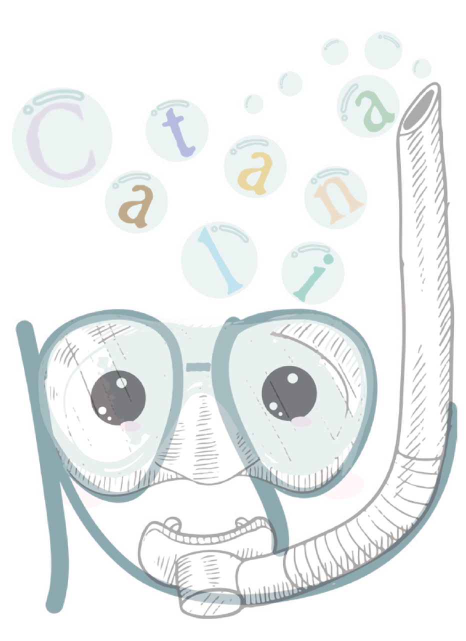
(Veron, 2013)

Stairs down Cape Hedo
Kunigami, Okinawa, Japan
Photo by C. Ramírez-Portilla



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