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„Systematic investigation of *Pimpinella saxifraga* in Germany “

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Dedication

To my father pure soul

A sheet of the *Pimpinella saxifraga* was included in the morphological traits study as a case study of how much variation can be observed within taxa. Photo credit: Mohammad jawarneh.

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List of Abbreviation

DNA	Deoxyribonucleic acid
dd H_2O	Double distilled water
Ta	Annealing temperature
Sec	Second
AMOVA	Analysis of Molecular Variance
PCoA	Principal Coordinates Analysis
PCR	Polymerase chain reaction
SSR	Simple Sequence Repeat
ng	Nanogram
µl	Microliter
bp	Base pair
µg	Microgram
Min	Minute
dNTPs	Deoxyribonucletide triphosphate
UV	Ultra Violet
U	Unit
Pmol	Picomole
Tm	Melting temperature
Taq	Thermus aquaticus
PVP	Polyvinylpyrrlidon
GBIF	Global Biodiversity Information Facility
TE	Tris-EDTA buffer
BSA	Bovine Serum Albumin
DMSO	Dimethyl sulfoxide
$MgCl_2$	Mangnesium chloride
ROX	Carboxyrhodamie
FAM	6-Carboxyfluorescein
HEX	Hexa chloro- Fluorescien
GSSRs	Genomic microsatellates
BSSRs	BAC end sequences
R	Reverse Primer
F	Forword Primer
H	Shannon Diversity Index
ANOVA	Analysis of variance
PIC	Polymorphic information content
SD	Standard deviation

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Abstract

Introduction: This study focuses on *Pimpinella saxifraga* L. (Apiaceae), native in Germany. *Pimpinella* contains about 180 species all over the world being one of the largest genera in the Apiaceae. Due to large morphological variation and the wide range of geographical distribution, it is taxonomically complex. We hypothesize, that 1) molecular data can separate individuals from biogeographic regions in Germany; 2) that morphological features discriminate taxa from Germany; 3) molecular and morphological data will be congruent in their information content about diversity of *P. saxifraga* in Germany.

Material and methods: We investigated 78 individuals from different German herbaria by morphology and genetics. 44 SSR Nuclear microsatellite markers evolved for different Apiaceae taxa were estimated for inter-generic transferability to *P. saxifraga*. We investigated five quantitative and six qualitative morphological traits to compare individuals from biogeographical regions and different altitudes.

Result: The transferability of nine nuclear microsatellite markers from *D. carota* to *P. saxifraga* were successful. A total of 122 alleles were gained with a mean of 12.4 alleles per marker. The level of expected and observed heterozygosity per markers extended from 0.31 to 0.95 and 0.27 to 0.82 respectively in 9 primers The average polymorphic information content values ranged from 0.25 to 0.85 with an average of 0.57. High genetic diversity (He = 0.61) within and among *P. saxifraga* individuals were detected. Mantel tests detected significant correlation between genetic and geographical distance. Analysis of molecular variance (AMOVA) specified that the molecular variance existed within regions (98%) rather than among regions (2%). STRUCTURE analysis presented two genetic clusters (K=2) described the SSR data supported by factorial analysis and principal coordinate analysis (PCoA). Hierarchical neighbor-joining tree (NJ) showed also low level of genetic differentiation between 78 individuals according to biogeographical regions. Factorial analysis and STRUCTURE analysis using leaflet morphological character states detected two major clusters. Cluster I with pinnatifid lobed linear teeth; cluster II with ovate serrate teeth, confirmed by unweighted Neighbor-Joining tree (NJ) and Median-joining network. No match of leaf morphological differences with geographical distances was found.

Conclusion: This is study is the first of the genetic population structure and genetic diversity of German *P. saxifraga* using nine SSR markers. The correlation of the individuals with their geographical regions was very low. Neither genetic markers nor morphological traits revealed any connection to the geographic region but separate taxa as groups. Morphological characters of *P. saxifraga* provide a key for taxa identification but do not explain their taxonomic status as species. The applicability of our results to the formerly published morphological records is limited, mainly due to ambiguous terminology. More work is needed to define the effect of cultivation on qualitative vegetative and reproductive traits of *P. saxifraga* taxa, in addition to the application of further specific molecular markers.

Zusammenfassung

Einführung: Im Mittelpunkt dieser Studie steht die in Deutschland beheimatete *Pimpinella saxifraga* L. (Apiaceae). *Pimpinella* ist eine der größten Gattungen der Apiaceae. Aufgrund der großen morphologischen Variation und der weltweiten geographischen Verbreitung ist sie taxonomisch komplex. Hypothesen dieser Studie sind, dass 1) Molekulare Daten Individuen aus biogeografischen Regionen Deutschlands trennen können; dass 2) morphologische Merkmale Taxa in Deutschland unterscheiden; 3) molekulare und morphologische Daten in ihrem Informationsgehalt über die Diversität von *P. saxifraga* in Deutschland kongruent sein werden.

Material und Methoden: Wir untersuchten 78 Individuen aus verschiedenen deutschen Herbarien nach Morphologie und Genetik. 44 SSR Nukleare Mikrosatelliten-Marker, die für andere Apiaceae-Taxa entwickelt wurden, wurden auf intergenerische Übertragbarkeit auf *P. saxifraga* getestet. Wir untersuchten fünf quantitative und sechs qualitative morphologische Merkmale, um Individuen aus biogeografischen Regionen und unterschiedlichen Höhenlagen zu vergleichen.

Ergebnis: Neun nukleare Mikrosatelliten-Marker von *Daucus carota* konnten erfolgreich auf *P. saxifraga* übertragen werden. Insgesamt wurden 122 Allele mit einem Mittelwert von 12,4 Allelen pro Marker gewonnen. Das Niveau der beobachteten und erwarteten Heterozygosität pro Marker lag zwischen 0,31 und 0,95 bzw. 0,27 bis 0,82. Die durchschnittlichen Werte des polymorphen Informationsgehalts lagen zwischen 0,25 und 0,85 mit einem Mittelwert von 0,57. Es wurde eine hohe genetische Diversität (He = 0,61) innerhalb und zwischen *P. saxifraga* Individuen festgestellt. Mantel-Tests ergaben eine signifikante Korrelation zwischen genetischer und geographischer Entfernung. Die molekulare Varianzanalyse (AMOVA) zeigte, dass die molekulare Varianz innerhalb von Regionen (98%) und nicht zwischen Regionen (2%) existierte. STRUKTUR-Analyse präsentierte zwei genetische Cluster (K=2) die die SSR-Daten beschrieben, welche durch die Faktoranalyse und die Hauptkoordinatenanalyse (PCoA) unterstützt wurden. Der hierarchische Neighbor-Joining Baum (NJ) zeigte ebenfalls eine geringe genetische Differenzierung zwischen den 78 Individuen nach biogeographischen Regionen. Die faktorielle Analyse und die Strukturanalyse unter Verwendung von morphologischen Merkmalszuständen der Blätter ergaben zwei Hauptcluster. Cluster I mit gefiederten, linearen Zähnen; Cluster II mit eiförmigen gezackten Zähnen, bestätigt durch einen ungewichteten Neighbor-Joining Baum (NJ) und einem Median-Joining-Netzwerk. Es

wurde keine Übereinstimmung zwischen den morphologischen Unterschieden der Blätter mit den geographischen Abständen gefunden.

Schlussfolgerung: Dies ist die erste Studie zur genetischen Diversität und Populationsstruktur von *P. saxifraga* mit neun SSR-Markern. Die Korrelation der Individuen mit ihren geographischen Regionen war sehr gering. Weder genetische Marker noch morphologische Merkmale zeigten eine Verbindung zur geographischen Region, sondern trennten Taxa als Gruppen. Morphologische Merkmale von *P. saxifraga* liefern einen Schlüssel zur Taxa-Identifikation, erklären jedoch nicht ihren taxonomischen Status als Art. Die Anwendbarkeit unserer Ergebnisse auf die bisher publizierten morphologischen Aufzeichnungen ist vor allem aufgrund der mehrdeutigen Terminologie eingeschränkt. Neben der Anwendung weiterer spezifischer molekularer Marker sind weitere Arbeiten erforderlich, um die Wirkung der Kultivierung auf qualitative vegetative und reproduktive Merkmale von *P. saxifraga*-Taxa zu definieren.

CHAPTER I
INTRODUCTION

***Pimpinella saxifraga* L., © Konrad Lauber – Flora Helvetica – 2012 Haupt Bern**

1. Introduction

1.1. The Apiaceae family

Apiaceae (Umbelliferae) is one of the most important flowering plant families. "*Despite its long taxonomic history dating as far back as Morrison's (1672) Plantarum Umbelliferarum, the earliest systematic study of any group of plants (Constance, 1971), the family still awaits a modern classification. The most recent treatment of umbellifers (Pimenov and Leonov, 1993) was an adaptation of the century-old system of Drude (1898), highly criticized for using subtle or poorly defined diagnostic characters (Heywood 1982)"* (Downie *et al*., 2000). The Apiaceae nowadays comprise 300-450 genera and 3000-3750 species (Constance, 1971; Pimenov and Leonov, 1993 ; Stevens 2001). The current system of Apiaceae classification is based on molecular and morphological data four subfamilies are separated, such as Saniculoideae, Mackinlayoideae, Azorelloideae, and Apioideae (Plunnkett and Lowry II, 2011).The Apioideae is the biggest clade of this family and divides into 41 major clades (Downie *et al*., 2001 ; Downie *et al*., 2010).Its distribution is nearly worldwide with two centers of diversity, namely, in the northern temperate regions and in the high altitudes of the tropical region. (Tutin, 1968; Constance, 1971; Willis, 1973; Pimenov & Leonov, 1993; Mabberley, 1997; Tabanca *et al*., 2007; Plunkett *et al*., 2018).

The Apiaceae are present in almost all types of habitat, from sea-level to alpine zones, and include forests, grasslands, grazed pastures, aquatic biotopes, as well as their clearings and margins, rocky hills, gravelly soils, screes, cliffs, open sandy and cultivated fields, fallows, steppes, roadsides and waste grounds (Plunkett *et al*., 2018).

The main morphological characteristics of the Apiaceae species are as follows: most species are of an aromatic herbaceous nature, with an aromatic oil mainly located in the fruit but also in the tissue of the mostly hollow stems, the single flowers are small with floral parts in 5:5 petals, 5 stamens are arranged in whorls and grouped in shaped inflorescences either in simple or compound umbel, pinnate or palmate alternate compound leaves with reticulate venation, the dry fruit can be divided into two parts, namely, schizocarps and mericarps, separate from the sterile carpophores seeds that are in pairs, commonly conspicuously ribbed, and sometimes winged (Heywood, 1971; Tutin, 1980 and Drew *et al*., 2009).

Apiaceae are insect-pollinated. Wind pollination has not been shown to be of importance in the Apiacaae family (Drabble and Drabble, 1927; Koul *et al*., 1993; Proctor *et al*., 1998). Numerous small flowers are densely aggregated into a flat umbrella such as umbel and visited by many insect taxa such as Lepidoptera, Hymenoptera, Diptera, Coleoptera and Hemiptera. The pollinators are attracted by nectar (produced by the stylopodium) and pollen throughout the whole flowering period (Lindsey, 1984 ; Lindsey and Bell, 1985; Ellis and Ellis-Adam, 1993 and 1994; Zych *et al*., 2004).

Apiaceae is a family of great economic importance; this is so because the plants contain essential oil, from which spices or medicines can be prepared. Aromatic oil is produced in nearly all parts of the plant. Even the roots contain secretory cavities (Acimovic and Milic, 2017). This essential oil is used on many common vegetables, such as carrots, celery, coriander, parsley, dill and parsnips, but other Apiaceae species provide spices, such as cumin, fennel and anise. This oil has distinct flavours, largely due to the various volatile compounds in the fruit and leaves (Plunkett and Downie, 1999). Several species, such as carrots, heracleum and angelica, provide fodder for cattle and horses (Dowine *et al*., 2000; Rathore *et al*., 2013; Aćimović *et al*., 2015).

Apiaceae are widely used and applied in folk medicine to prohibit and treat various diseases of the endocrine and digestive systems (Sayed-Ahmad *et al.*, 2017) In addition, numerous authors have shown their antimicrobial, hypoglycemic, hepatoprotective, hypolipidemic, and anticancer activity (Aćimović *et al*., 2015), and in crop species, such as centella, cicuta, and aethusa, which confirms the potential application of seed spices and their extracts in functional food and cosmetic and pharmaceutical industries.(Bakkali *et al.*, 2008; Kannapan *et al*., 2011 Aćimović *et al*., 2015). However, the Apiaceae family also includes some widespread weeds and toxic plants, such as the poisonous spotted hemlock (Dowine *et al*., 2000) among others.

1.2. The genus *Pimpinella*

This study focuses on the genus *Pimpinella* from the Apiaceae. *Pimpinella* include about 170-180 species all over the world. It is also considered to be one of the largest genera in the Apiaceae subfamily, Apioideae (Abebe, 1992; Pimenov and Leonov, 1993). It is actually the major constituent of the Pimpinelleae tribe (Downie *et al*., 2010) and it is deemed to be

taxonomically complex due to its high extent of morphological variation and its wide range of geographical distribution (Abebe, 1992).

The *Pimpinella* species are annual, biannual or perennial herbs, the leaves are compound, entire or sometimes trisect 1-3 pinnate, and the flowers develop in a compound umbel, but bract, bracteoles and sepals are absent. The 5 petals are white or yellow but rarely purple or pinkish. They are pollinated by insects. Fruits are ovate, laterally compressed in shape, mericarps are homomorphic with prominent ridges, and carpophores are present, vittae are reddish-brown and are important diagnostic characteristics (Druce, 1918; Hegi, 1926; Wolff, 1927; Tutin *et al*., 1968 and Grime *et al*., 2007). The plants grow on rocky crevices, meadows, grassland, dry rocky places and mountain pastures (Engstrand, 1987 and Velayos, 2003).

1.3. The species *Pimpinella saxifraga*

1.3.1. Plant description.

P. saxifraga is a hemicryptophyte, a perennial, erect herb, derived from a stock which often has fibrous remains of petioles winter green petioles that form a vegetative rosette in the first years. Its total height varies from 60 to 100 cm. according to the environmental conditions and competition with other plants from the second year. The vegetative parts have a main, long-shaped fusiform and a deeply penetrating taproot with limited branching of lateral roots (Hegi, 1965). This root is spindle-shaped and cylindrical, and is either simple or not knotty at the top the root is often multi-headed, fissured, dirty yellowish-white or ringed on the outside and whitish inside with white milky juice.

The stems are solid, glabrous or a little puberulent with branched hairy, upper internodes ranging from being angular to being slightly sulcate (Thellung, 1926; Weide, 1962; Reduron, 2008). The stem is up to 100 cm. high, pale green, rounded, subterete, thin-ribbed, and branched above (Sell and Murrell, 2009). Figure 1.1 shows the stem basal leaves are pinnate and separated into linear segments (Knuth, 1908), with oval toothed leaflets. Leaves are medium dull green on the upper surface, but paler beneath, alternate (Sell and Murrell 2009), oblong-lanceolate to broadly pinnate with ovate leaflets, either obtuse or acute, veiny, serrate to pinnatified, sessile or subsessile short petiolate 3-7 of segments which modify from simple, ovate, to bipinnate**.** The

upper leaves are pinnate small blade with longer sheath imperfect leaf plates, have a lanceolate or a sub-linear lobe with entire segments, while the uppermost leaves have a reduced blade and a petiole sheath (Tutin *et al*., 1980 and Sell and Murrell, 2009).

Inflorescences are compound umbels, with 10-22 rays, which are 1-4.5 cm. long and smooth; these rays are straight or slightly bent inwards, and the peduncles are longer than the rays and glabrous, but the bracts and bracteoles absent. The umbels are in general mostly hermaphroditic flowers that are white, polygamous, not or only slightly emarginated, and their apex is inflexed. The flowering time is from June to September. There are 5 stamens with whitish filaments and yellowish anthers. The ovary is inferior and comprises two fused carpels. Two styles shorter are than the petals, but are later elongated with an enlarged base forming the stylopodium; the stigma is capitate (Thellung 1926; Roger, 1961; Weide, 1962; Tutin and Moore, 1989; Reduron, 2008; Sell and Murrell, 2009). The fruits are long schizocarps, 2-2.5 mm long, round egg-shaped, but laterally flattened as the fruits ripen; they elongate but later often break off. The mericarps have slender ridges while the vittae are reddish-brown, darker than the slender ridges that form from midsummer to late summer.

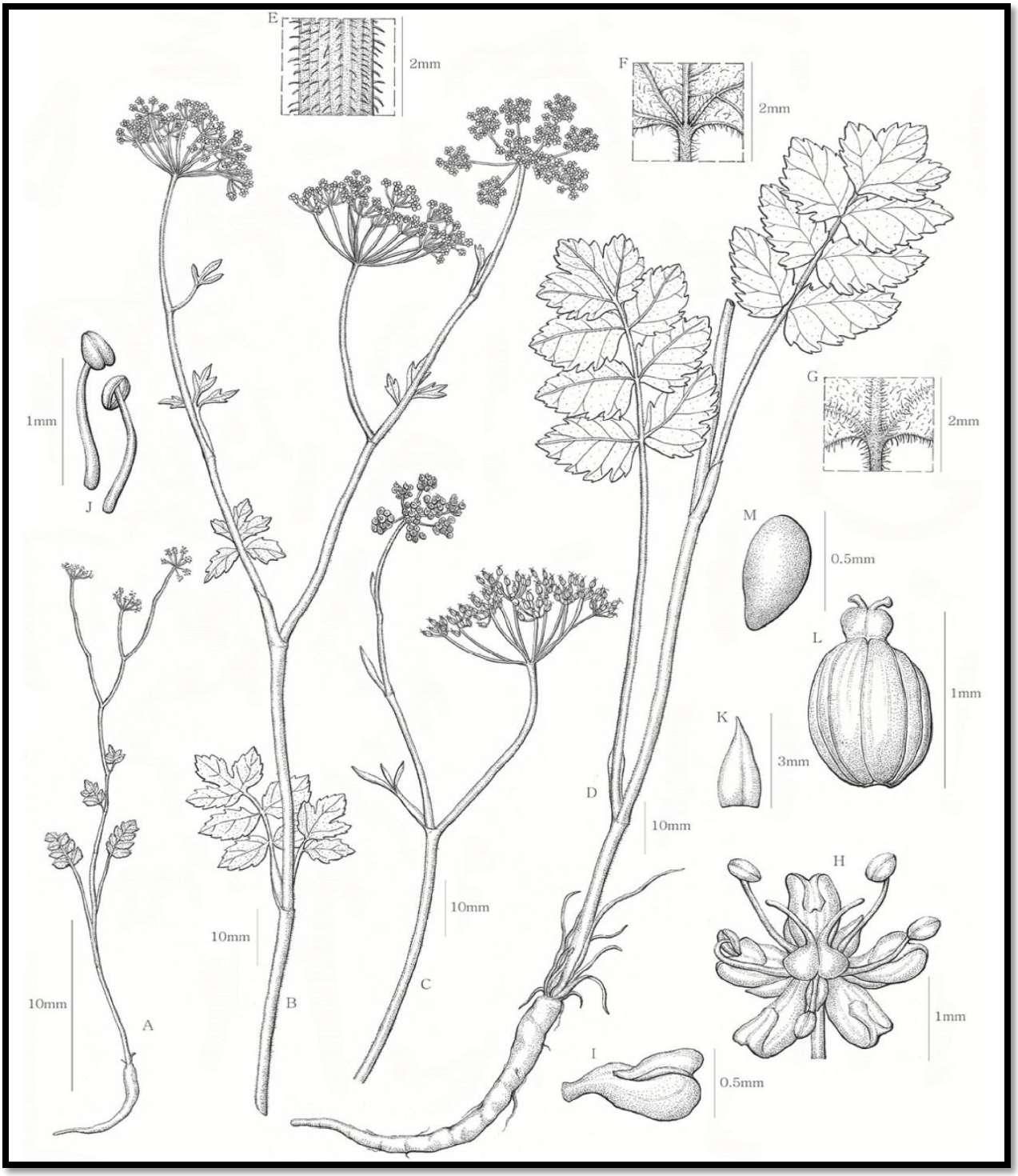


Figure 1.1: Typical morphology of *Pimpinella saxifraga*: A. Habit. B. Flowering branch. C. Fruiting branch. D. Root with branch. E. Stem. F. Abaxial view of leaf. G. Adaxial view of leaf. H. Flower. I. Petal. J. Stamen. K. Calyx. L. Fruit. M. Seed. (Lee, 2018).

1.3.2. Geographical and altitudinal distribution.

P. saxífraga is widely distributed throughout Europe an, Western Asia and it has also been introduced to the northern parts of North America (Figure 1.2; Hegi, 1926; Tutin, 1968; Matthews, 1972; Hamet-Ahti, 1980 and Kisiel *et al*., 1998). *P. saxífraga* is recorded throughout Western Europe (Belgium, France, Netherlands, Luxembourg, the United Kingdom and Ireland) but it is absent from the Faroe Islands and Iceland (Thellung, 1926; Weide, 1962 and Reduron, 2008). It is also less common to rare in Northern Scotland and Ireland (Kelly 1964 & Godwin, 1975, Carey *et al*., 1995). It has been recorded on the Isle of Man, the Isles of Scilly, and the Isle of Wight but not on the Channel Isles. In Southern Europe it has been found in the south-west areas of central Spain but is absent in Portugal. It has also been found throughout most of Italy. In Central Europe *P. saxífraga* is common in Germany, the Czech Republic, Austria, Poland, Slovakia, Hungary, Switzerland, Croatia, Poland, Lithuania, Latvia, and Estonia) (Hegi, 1926; Berger *et al*., 1975; Hegi ,1987). In Northern Europe the species extends south from central Norway, and from the northeast mountains to the southern part of Sweden (Sterner 1922), but it is scattered to rare in the Scandinavian northern lowlands. In southeastern Europe, it can be found in Romania and the Balkan countries and even as far as central Greece, southern Russia and Crimea, but it remains only scattered in some regions of the Caucasus (Hegi, 1926; Berger *et al.*, 1975; Hegi, 1987; Kubeczka, 1989).

Outside Europe, *P. saxifraga* has been recorded in the north part of Turkey (Davis 1972), western Iran, the Himalayas and both, West and East Siberia. It has even been observed in New Zealand too, but it was introduced there (Hegi, 1926; Matthews, 1972; Seidemann, 2005). Similarly, it was also found as a newly introduced species in the southern part of South Korea, namely, in Seogwipo and on Jejudo Island (Lee *et al*. 2018). Altitude distribution of *P. saxifraga* is generally found in the various geographical regions in Germany with diverse altitude, ranging from North Germany and the Baltic Coast to the Bavarian Alps, where it has been found at an elevation of 2240 m (Schlechtendal, 1886; Hegi, 1965).

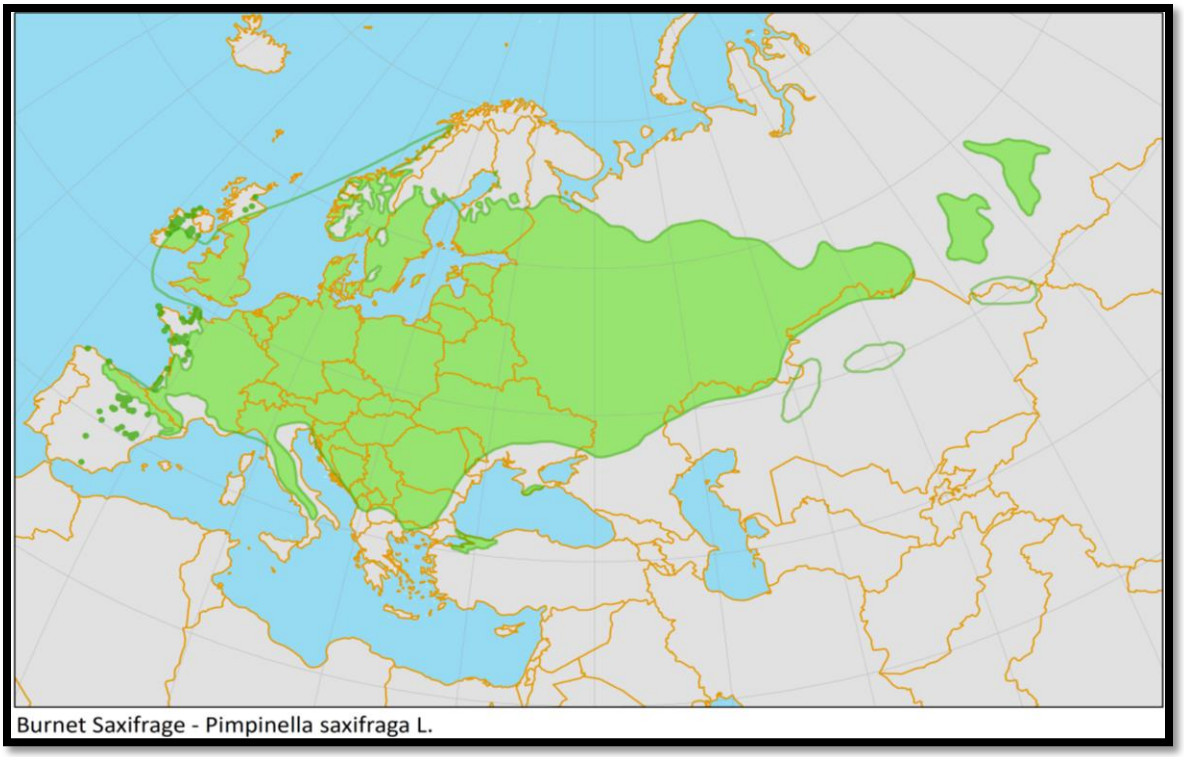


Figure 1.2: Native distribution range of *Pimpinella saxifraga* in Europe and its adjacent regions (from Meusel *et al.*(1965) but redrawn by Dr. Erik Welk).

1.3.3. Climate and topographical region in Germany.

Germany is in the centre of Europe the German territory has a considerable share of three biogeographical regions, namely, the Continental and Atlantic regions as well as an extension of the Alpine area (Figure 1.3) (Natura 2000). The climate in most of Germany is temperate continental, characterized by cold winters with average daily temperatures around 0°C or sometimes slightly higher and warm summers with temperatures nearly of 22/24 °C in July and August (Natura 2000). The northern region is a bit more temperate, but it is also rainy and windy due to the influence of the Atlantic Ocean. The massifs of south central Germany and a small part of the Alps in the far south have a mountainous climate, which of course becomes cooler with increasing altitude. Since Germany is exposed to temperate air masses from the Atlantic Ocean and cold air masses from Russia or the Arctic, the weather is often unstable, with noticeable changes and different weather conditions, such as heat, cold, fog, wind, snow, and thunderstorms. However, precipitation varies according to the region. Average precipitation in the North German Plain is 508-737 mm annually whereas average rainfall in the highlands of Central Germany is 712-1500 mm. Alpine regions often experience and exceed 1985 mm. of

rainfall. Mountain winds can appear during any season causing an instant change in temperature along with extremely warm conditions and clear skies. Summer is usually hot and dry while snow can begin between November and January, and temperatures can then often drop below zero (Natura 2000).

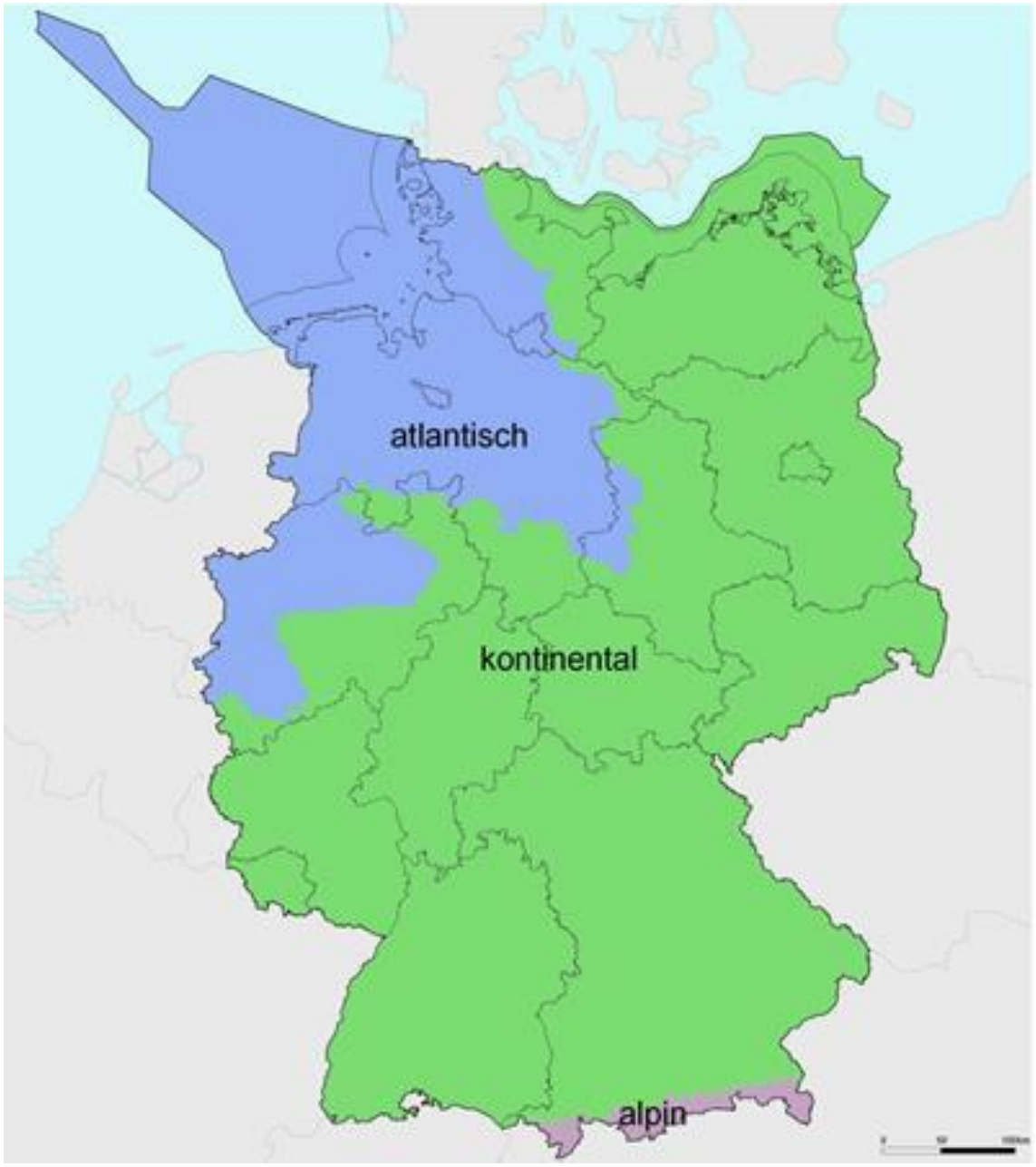


Figure 1.3: A map of Germany's biogeographical regions. From this it can be seen that Germany is divided into the following three biogeographical regions : Atlantic, Continental and Alpine) (Source: Bmub-Bundesministerium for the Environment, Nature Protection, Building and Nuclear Safety (2007)).

1.3.4. Habitats and ecological factors

P. saxifraga L. is a widespread species in temperate meadow communities that develop on sunny, moderately dry slopes and on calcareous pastures (Hegi *et al*.,1965 and Hänsel *et al*., 1994). It is also known to flourish in broad-leaved forests and rocky slopes and frequently in both grazed and un-grazed grassland, with occasional local occurrences in limestone pile quarries, cliff spoil and flushed rock ledges as well as road verges and scree (Balme, 1953; Hegi *et al*.,1965; Wells *et al*., 1976; Hamet-Ahti, 1980; Grime *et al*., 1988; Auested *et al*., 2010 and Akalin *et al*., 2016) (Figure 1.4). It might also develop on sites with varying soil water acidity and availability, As well as with the availability of varying lighting above the ground It also occurs in anthropogenic disturbed areas, such as agricultural margins and along road sides (Wells et al., 1976; Hamet-Ahti, 1980). Notwithstanding this, it is still less carefully related to a specific habitat (Delarze and Vust, 2015), especially on sites with well-developed turf. Its huge ecological potential and widespread prevalence contribute significantly to the mosaic structure of numerous pasture communities (Niinemets, 2005). In Germany, according to the flora website (https://www.floraweb.de/pflan zenarten/oekologie .xsql?suchnr=4282&), habitats are divided into main occurrence locations, such as fresh meadows, dwarf shrub heaths, and pastures (Hegi, 1965 and Grime *et al*., 2007). There are also, secondary occurrences such as rock, wall and stone corridors outside the high mountains and even occurrence on mountain slopes and in arid forest areas. Furthermore, in all types of soil, but especially in dry acid soils, (Ellenberg, 2009) and chalky limestone soil, *P. saxifraga* is able to exist and flourish.

Figure 1.4: The growth habit of *Pimpinella. saxifraga* – A. Rock ledges *(*https://www.brc.ac.uk/plantatlas/plant/*P. saxifraga) B.* grassland *(*https://www.aphotoflora.com/d_*P._saxifraga*_burnet_saxifrage.html*)*.

The Ellenberg ecological indicator factors for *P. saxifraga* are based on its ecological demands when in competition with other species and are available in Central Europe (Ellenberg, *et al.*, 1992) and Great Britain (Hill *et al*., 2004). The number ratio of light is equal to 7, which indicates that *P. saxifraga* is able to adapt to different light availability above-ground; the different temperature readings also show its adaptation from cold to heat. The moisture index is equal to 3: this means that *P. saxifraga* is often found in dry surroundings and is frequently perceived as a plant that prefers dry soil (Franz, 1981; Schulz *et al*., 1983; Schmid and Imhof, 1988 ; Jodral, 2004). *P. saxifraga* can tolerate dry surface conditions due to its long and slender taproot, which can reach depths of more than 2.5 meters (Kutschera *et al.,* 1997 and Niinemts, 2005). The amount r of nitrogen equals 2, which indicates poor soil fertility (Grime *et al*., 2007). The amount of salt is equal to 0, which means *P. saxifraga* also does not tolerate salt, resulting in it being unable to resist heavy metals.

The main community of *P. saxifraga* in Germany is reported to be in Festuco-Brometea (base-rich dry grassland) (Oberdorfer & Korneck, 1978 ; Toman, 2008), within the *Festucetalia valesiacae, Festucion valesiacae* alliance (continental steppe grasslands dominated by Stipa capillata and Stipa tira). *P. saxífraga* is also found, in the Xerobromion alliance, which corresponds with *Festucion valesiacae*, a relatively extreme dry habitat (Oberdorfer & Korneck, 1978; Ellenberg, 1988). *P. saxifrga* can reach higher levels of abundance within the Mesobromion alliance (chalk and limestone grasslands) and these grasslands occur mainly in the western part of Central Europe and in certain areas of sub-continental Western Europe (Chytry, 2010).

1.3.5. Chromosomal number and ploidy level

Chromosome counts for *P. saxifraga* vary between 2n = 18, 20, 36, or 40 (http://ccdb.tau.ac.il/ 19.06.2018). In Austria, Croatia and Slovenia the count is also 2n = 20 (Hunkeler and Favarger, 1967; Vidic *et al*. 2009); however, in Germany it is recorded as 2n = 40 (Gawlowska, 1969 ; Alber, 1998).

1.3.6. Floral and pollination biology

Small hermaphrodite flowers have five white petals and five stamens that are protandrous and can be seen on a single flower (Knuth, 1908). They are mostly andromonoecious, with the umbelluls of primary umbels bearing hermaphrodite flowers, namely, those of the secondary umbel marginal hermaphrodite and central males discussed by Ajani *et al* (2016). The functional male flowers showing the same pattern of development as hermaphrodite flowers except for female flowers, which is inhibited at a late stage of development (Ajani *et al.,* 2016). The accumulation and density of flowers in inflorescences is an important biological feature that depends on the type of species. The accumulation of flowers in large umbel inflorescences attracts insects from long distances; This is useful for pollination (Linder, 1998).

Pollen grains are radiosymmetric cold prolate, and subprolate but all form prolate (Erdtman, 1986). In plants, the relocation of mature pollen grains to the stigma is defined as pollination (King and Brooks, 1947). Pollination can be either non-biotic or biotic. Within angiosperms more than 80% of the species depend on biotic pollination (Ackerman 2000 ; Ollerton *et al.*, 2011). Biotic pollination is an interaction between animals and plants, and, in general, both plants and animals benefit from pollination. The transfer of pollen by animals will also increase the likelihood of outcrossing, which is the goal of the plant. The benefit of the animal is the reward, namely, the production of nectar, pollen, resin and oil). It is the main pollinator for *P. saxifraga,* such as Coleoptera, Hymenoptera, Neuroptera and Diptera (Knuth, 1908; Willemstein, 1987). It is predominantly outcrossing but, according to Knuth (1908), East (1940), and Berger *et al*. (1975) self-pollination is also possible.

1.3.7. Germination and Phenology

The seed requires a time of chilling to break its dormancy and to stimulate germination (Thompson and Grime, 1979). Therefore, sowing *P. saxifraga* seeds in the autumn requires there to be exposure to lower temperatures, which then start to germinate synchronously in late winter or early spring. (Grime, 1997). *P. saxifraga* overwinter as green rosette leaves but, as spring proceeds, the plants start with slow vegetative growth. *P. saxifraga* is then showing a certain leafiness, one that reaches a maximum of per rosette by July (Mitchley, 1988). The flowers then

start opening in July until September, with the beginning of the seed set being at the end of September. The shoots then die in the autumn (Grime, 1988).

1.3.8. Economic importance and use

P. saxifraga has been frequently used in folk medicine. Extracts of the herb can be used as an expectorant for prolonged coughs, pharyngitis, laryngitis, tracheitis and chronic bronchitis, and also in the treatment of gastric diseases (Karsten, 1962; Wagner, 1982; Grieve, 2016 and Parashchuk *et al*., 2019). It is also used as an anti-inflammatory and anti-hypertensive agent (Grieve, 2016) and has even been used in traditional sources to motivate milk production in lactating women.

P.saxifraga roots are registered in the German Pharmacopoeia as 'Pimpinellae Radix' and are used as expectorants and for increasing bronchial secretions (Bohn *et al.,* 1989). In Turkey, the roots are used to combat various infections as a soothing expectorant and tonic (Tabanca, 2006). The essential oil extracted from the *P.saxifraga* roots is used as medical weapon against heart disease and coughs in various countries (Grieve, 2016). This essential oil is also able to dissolve urinary stones and airway mucus and furthermore can be used to treat a cough, a sore throat, bronchitis and other infections of the upper respiratory tract (Redzic, 2010 and Marchyshyn *et al.*, 2018).

The addition essential oil of *P. saxifraga* to sodium alginate coating on the bacterial and oxidative stability of cheese was deliberate during cold storage which improved cheese preservation by lowering weight loss, maintaining pH and color and enhancing oxidative and bacterial stability without unpleasant flavor to consumers (Ksouda *et al.,* 2019).

P. saxifraga can also be used to remove a maximum of over 90% of active oxygen and it is even supposed to help combat wrinkles, spots and other visible signs of skin aging (JSP 1947). The young leaves can also be used as a flavoring in drinks and salads The dried leaves are used for flavor in vinegar and even as herbal tea (Mehdiyeva *et al*., 2016).

1.4. Taxonomic delimitation

1.4.1. Species delimitation

Species is the basic unit of biological classification and is thus important for any kind of communication among evolutionists (Agapow *et al*., 2004). The first classifications were dependent on simple morphological observations, but then new and more complex concepts of the species emerged, adding more and more distinctive characteristics from ecology to reproduction and genetics (Mayden, 1997; Harrison, 1998 and de Queiroz, 1999).

The theoretical concept of species is closely linked to the delimitation of species, as a means to determine species boundaries (de Queiroz, 2007) Many different methods have been proposed here (Carstens *et al*., 2013). A number of concepts for species have been developed, based on the idea that species are evolutionary groups; one such concept is that of evolutionary species (Simpson, 1961 and Mayden, 1997) and another involves variants of forms of phylogenetic species concepts (Donoghue, 1985 ; Coyne and Orr, 2004).

A major challenge in delimiting the boundaries of genetic species is the continuous nature of evolution, which itself is far from reaching a final and steady state of overall species fidelity, but instead presenting persistent varying degrees of gene flow within and between groups and species (Cutter, 2013). Thus, delimiting species boundaries still remains a challenging task in many groups of organisms (Ellis *et al.*, 2006).

Species delimitation is already central to many areas of all biological research. Despite their importance, there is no covenant on the standard for delimitation of species; this is mostly due to differences in the point view of the various biological fields (Duminil and Michele, 2009). Systematists detect, formally depict and classify the species that serve as the basis for all biological research (Schlick-Steiner et al., 2010). All officially described species have scientific names, signs of groups of organisms that are identified according to the concept of the particular species. These scientific names have thus become the tools that have enabled biologists across various disciplines to communicate effectively about their research topics and to ensure the consistent application of names to special groups of organisms (Patterson et al., 2010 and Hardisty et al., 2013). Two main sets of diagnostic characteristics are commonly used to

distinguish species, these are the molecular ones and another group, traditional morphological groups (Duminil and Michele, 2009). The characterization of species by systematists has a significant impact on our knowledge of species conservation, biodiversity, environmental sustainability and resource management (Hardisti *et al*., 2013 and Costello *et al*., 2013). Species delimitation is a part of the taxonomy process in which a systematist needs to think about how to treat a group of organisms as distinguished species. Inferring species boundaries depends on the concept of selected species, and this has long been the subject of intense discussion (Baum and Donoghue, 1995; Mayden, 1997 and de Queiroz, 2007). The concept of biological species is the concept of species that is most common, especially among conservationists, ecologists and some evolutionary biologists (Mayr, 1942, 1963, 1970).

Plant species delimitation is of centric value in many areas of biology, such as ecology, conservation biodiversity, population genetics, biogeography and evolution phylogenetic systematics (Sites & Crandall, 1997; Schluter, 2001; Hedren, 2004 and Rodriguez *et al*., 2007)

The identification of plant species has always been depended on morphological features. Plant morphology is highly phenotypic and polymorphic features that may, in principle, permit the classification of plant species. However, different individuals of the same species may show differences in their morphology either naturally or with respect to local adaptations. This morphological variation within species could be the origin of the determining species delimitation (as Pratt and Clark, 2001). Alternatively, some species of the same genus can be morphologically very similar and therefore can be assigned to the same species in spite of the fact that they appear to have separate taxonomic entities (Chan *et al*., 2002; Whittall *et al.,* 2004; Duminil and Michele, 2009).

Over the past few decades, the use of molecular markers as a tool for species delimitation has increased dramatically. In general, it can be used in plant genomes (mitochondrial, chloroplastic, and nuclear) for species identification. However, in plants, nuclear and chloroplastic genomes are therefore the most generally used, but the mitochondrial genome at all events is not sufficiently polymorphic between individuals to be useful (Duminil *et al*., 2002).

The correct delimitation of species is therefore of essential importance for all studies with evolutionary, ecological and conservation aims. This is particularly true for species that are morphologically indistinguishable by definition (Fritz *et al*., 2005; Kaliontzopoulou *et al*., 2011). The use of genetic data can contribute to the study of species that are morphologically identical, thereby providing additional valuable information about the processes related to speciation and species identification, compared to traditional morphological traits (Hey, 2010). In this way, morphologically efficient, molecular markers can identify species as not being the same.

1.4.2. Species Problems and the Taxonomic background of *Pimpinella saxifraga*

Delimitation of species boundaries is one of the two main goals of systematics (Wiens, 2007). Since "there is no single definition as yet that satisfies all natural scientists, and yet every scientist knows slightly what he means when he speaks of a species", Darwin 1859), Charles Darwin elevated eruptive issues in his attempt to define what a species is and what we should take into account when making this definition.

The *Pimpinella saxifraga* complex is already well-known for its complicated morphological variation styles and problematic taxonomy. It has been the subject of intensive studies of variation patterns in several expert groups. In spite of this, however, no agreement on the taxonomy and the species circumscription within the complex has yet been reached. In this study, two hypotheses of the relationship between *Pimpinella saxifraga* individuals were explored using morphological and molecular marker data sets. These were the following: Hypothesis 1: to study the differentiated leaflet shapes between individuals in different geographical regions according to the altitude; Hypothesis 2: to study molecular variation that depends on the SSR markers according different geographical regions.

1.5. Variation of taxa- and taxonomic history of *Pimpinella saxifraga*.

"Taxonomy is the science of depicting, classifying and ordering organisms dependent on shared biological characters (Schu, et al., 2009) Species form the basic entities in this system and are then aggregated to higher categories, such as genera, families or orders depending on characteristics that reflect common ancestry. Each category in this system is referred to as a

taxon. Biological systematic uses taxonomy as a tool to reconstruct the evolutionary history of all taxa"(Simpson, 2010).

The most recent and complete taxonomic classification of the species *P. saxifraga*, has been taken as the basis of *P. saxifraga*. taxonomy in this study (Linnaeus, 1753).

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: (Apiales)

Family: Apiaceae

Genus: *Pimpinella*

Species: ***Pimpinella*** *saxifraga* L.

There is no record of *P. saxifraga* in the early postglacial history. The earliest botanical description was given in data collected in the botanical Renaissance, which took place in the16th century. It was first recorded by Rembert Dodoens (1583, 1616) in his *Stirpium historiae* and *Remberti Dodonaei mechilensis stirpium historiae pemptades sex*. Gerarde (1597, 1633) provided an illustrated account of *P. saxifraga* in the *Herball or Generall Historie of Plantes* and described its growth in the dry pasture and meadows of London. In 1688 John Ray recorded the occurrence of the species in the forests and fields of Cambridge shire and Bedfordshire, as well as elsewhere in England. *P. saxifraga* was already well-known to the ancient Greeks and is in fact still found in excess throughout all of central Europe (Miloradovich, 1986).

In the first edition of *Species Plantarum*, Linnaeus (1753) described *P. saxifraga* as *P. saxifraga* minor and *P. saxifraga* major (p 264 in Species Plantarum and later in Scopoli and in Flora Carniolica, 1771 p. 209). The Botanical Exchange Club also reported from the British Isles between 1908 and 1933), and *Pimpinella saxifraga* L. were then described as variety *poteriifolia* Wallr. and as *forma nana*.

Thellung (1926) described three subspecies, namely, *P. saxifraga* subsp. *nigra*, *P. saxifraga* subsp. *alpestris* (Spreng), and *P. saxifraga* subsp. *alpine* (Host) Nyman. Weide (1962) treated *P.*

nigra as a separate species differentiated by root colour, chemistry, the number of umbellules in the umbels, leaflet shape and serration margin.

Hegi (1965) in *Flora of Middle Europe* divided *P. saxifraga* into the following three subsp.: *P. eu-saxifraga* Thellung, *P. alpestris* (Spreng) Schultes, and *P. nigra* (Miller) Gaudin. Tutin (1968) in *Flora Europaea* agreed about the subspecies *P. alpestris* (Spreng) Schulte's but then added *P. dissecta* Retz. and *P. laconic* Halacsy.

Matthews (1972) has long disagreed on the delimitation of species and subspecies groups within *P. saxifraga.* Matthews insisted on dividing it into three subspecies: *P. saxifraga* subsp. *rotundifolia* Scop., *P. saxifraga* subsp. *calvertii* Boiss., and *P. saxifraga* subsp. *eusaxifraga* Thell., and one variety *P. saxifraga* var. *dissectifolia* Boiss.

Reduron (2008) accepted the treatment of Thellung that divided *P. saxifrga* into three different subspecies, and also detected a new variety of *P. saxifraga*, called *P. saxifraga var. tevennensis* Buord.

More recently, Sell and Murrell (2009) revised the classification of *P. saxifraga* and divided it into the following three sub-species: *P. saxifraga* subsp. *alpestris* (Spreng.) Vollm., *P. saxifraga* subsp. *nigra* (Mill) Gaudin, and *P. saxifraga* subsp. *saxifraga.* In the latter subspecies he included the following different varieties: var. *dissecta* with, var. *nana* (Druce) P.D.Sell, var. *intercedens* Thell, and var. *ovate* Spreng.

However, modern site floras, such as the Plant List (http://www.theplantlist.org/), (Euro+Med 2006) and The International Plant Names Index (IPNI; www.ipni.org), list various subspecies names within *P. saxifraga*, only most of these, however, are synonyms and unresolved except for two subspecies that are accepted (*P. saxifraga* subsp. *saxifraga*, *P. saxifraga* subsp. *nigra*). However, the scientific debate is still open, with some scientists recognizing different subspecies but others not distinguishing between subspecies.

1.6. Assessing diversity using molecular markers

1.6.1. Genetic diversity and environmental variability

Genetic diversity is an important component of biodiversity and an essential characteristic of species, groups, and ecosystems, whereby genetic diversity perform the evolutionary ability of a species to survive and be appropriate to environmental changes. High genetic diversity can contribute to maintaining a healthy population by included alleles that may be worthy in resisting disease, pests, and any other pressures to come (Ahuja and Jain, 2015). The evolution of genetic diversity is a structured, non-random way that occurs over time at organism and molecular levels and across all geographic scales (Nevo 2001; Ahuja and Jain, 2015). Overall, genetic diversity emerges from different biological processes, where the gene flow and mutations have a positive impact on genetic diversity, while direct selection and genetic drift negatively affect it (Eriksson and Ekberg 2001).What is more, many other factors, such as barriers of dispersion, geographic and environmental variability (Wu *et al*., 2016), demography (Hensen and Oberprieler, 2005), Population dispersal due to human activities (Thompson, 1999) can indeed amplify and influence these processes.

Genetic diversity is present within and between different populations of plants and its structure and level can play a useful role in the effective use of plants (Cole, 2003). Molecular markers, however, play the most important role in genetic diversity estimates to measure genetic variation, linkage mapping and cultivar identification because of the relative ease of their generation and elimination. The evolutionary background, the process of gene flow, the population density, and the mating system are important factors used to discover the structure and the level of these variations (Hamrick, 1989). Genetic diversity is controlled by the effect of the following four different biological processes: gene flow, natural selection, mutation and genetic drift (Figure 1.5). The genetic diversity among the population is increased when you are affected by biological processes, such as natural selection, genetic drift, and mutation, but is decreased when you are affect by gene flow. On the other hand, genetic diversity within a population increases the effect from biological processes, like gene flow and mutation, while decreasing diversity is affected by natural selection, genetic drift and inbreeding (Eriksson and Ekberg, 2001).

Molecular markers avert complications in the environmental effects on characters and represent a credible and potentially quick method for characterizing diversity for variations between species (Idrees and Irshad, 2014). The evolution of genetic diversity is an orderly, non-random process that occurs overtime at the organismal and molecular level across all environmental scales (Ahuja and Jain, 2015). On the other hand, uncovering the role of environmental variability, as well as of ecological variegation among habitats, The importance of maintaining biodiversity among populations has a role in understanding the evolutionary forces that make up the differentiation and genetic structure of local populations (Linhart and Grant, 1996 ; Gram and Sork, 2001).

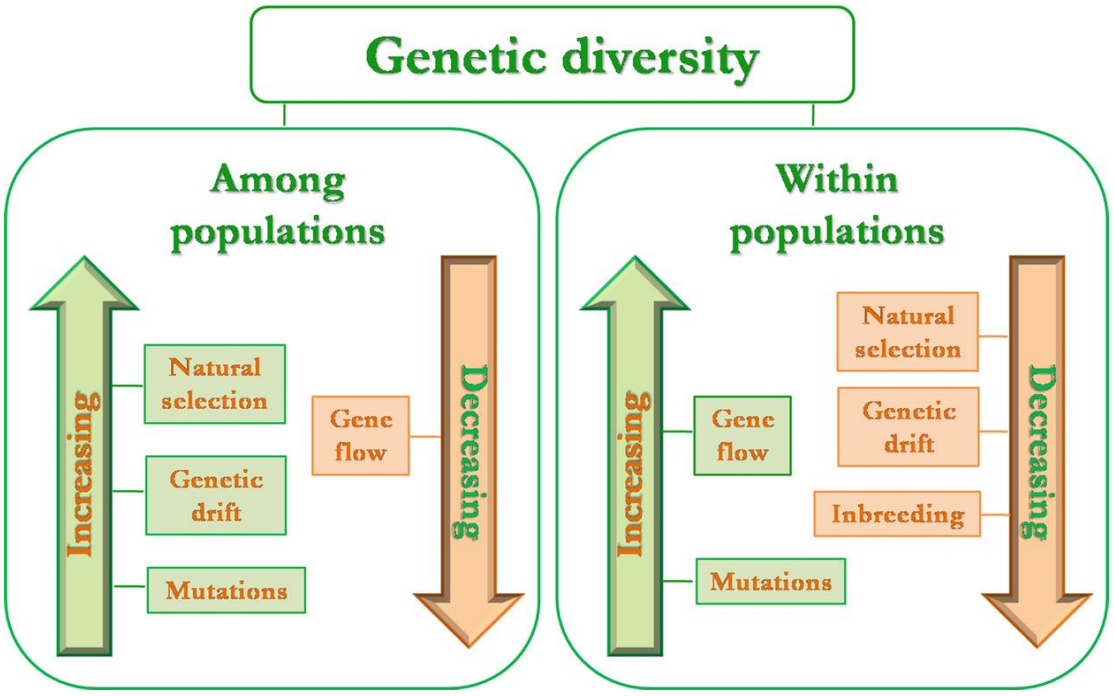


Figure 1.5: Graphic clarification of processes that affect genetic diversity among and within populations (Nonić and Šijačić-Nikolić, 2021).

A positive correlation between genetic diversity with environmental characteristics (environmental heterogeneity, areas and connectivity) could be detected at different levels of diversity, according to other research on different species (Nevo, 2001 and Kahilainen *et al.*, 2014). It can create a disruptive selection and thus support different genotypes across different populations (Via and Lande, 1985). However, increased environmental heterogeneity can also

lead to reduced population sizes and thus to genetic drift, which can lead to loss of alleles or reduced genetic diversity (Kahilainen *et al*., 2014).

Therefore, when the genetic geneflow in plant populations is spatially restricted due to environmental heterogeneity, natural selection or closed gene flow, genetic drift, and inbreeding can be expected (Linhart and Grant, 1996). The evaluation of the influence of environmental factors on the genetic diversity of a population is then one of the primary reasons for population genetic studies (Wu *et al*., 2016).

External influences, such as environmental conditions, can act on three different genomes in plants; these are the mitochondrial, chloroplast and nuclear genomes, all of which differ in size, with the mitochondrial and chloroplast ones being the foremost maternal inherited genomes (Palmer, 1987). In this study the nuclear genomes of *P. saxifraga* are used; they are inherited from parents and have large numbers of genes and copies.Gene arranging in the nuclear genome is Known to be stable, at least within species, and may also be constant within groups of species (Judd *et al.,* 2002 and Xu, 2005). This characteristic is a valuable instrument for classification and examination of related fields in many disciplines, like plant taxonomy (Roalson *et al.,* 2004; Bekele *et al,.* 2006 and Oumar *et al.,* 2008), plant evolution (Fehrer *et al,.* 2007), plant identification (Benedetti *et al.,* 2000; Lee *et al*., 2004), and plant genetic diversity (Missaoui *et al.,* 2006).

1.6.2. Type of markers

Genetic markers are involved in many fields of biological study. Several genetic markers associated with key genes are responsible for the important characteristics and the genes selected for study .There are the following three different types of genetic markers: biochemical markers, which cover allelic variants of proteins; morphological markers, which are phenotypic traits that have been used as a source of taxonomic evidence since the inception of molecular plant classification, and molecular markers which detect neutral sites of contrast at the level of the DNA sequence (Kumar 1999; Judd *et al.,* 2002 and Simagen *et al.,* 2006). In the end, all these genetic markers perform the varaition in DNA sequences between individuals.

DNA-based traits derived from DNA fingerprinting and DNA sequences were found to be appropriate molecular markers for investigating genetic variation. Molecular characters have

several advantages over morphological traits. It is stable and reveals the ability in all tissues without being directly influenced by the environment. Because of their inherited nature, they can often supply a detailed picture of relationships between organisms than those dependent on morphological forms and, at a lower range, physiological characteristics. Molecular markers are more plentiful than morphological traits (Semagn *et al*., 2006 and Kumar *et al.*, 2009). Although the molecular technique has been developed to be more accurate, quicker and cheapee to estimate genetic variation, there is still no single molecular technique to solve the many questions in genomic research at once. For all that, there are no molecular markers obtainable that meet all the demands that researchers need (Semagn *et al.*, 2005 and Kumar *et al*., 2009).

A molecular marker is a small fraction of the DNA sequence, which is located at a known placement on a chromosome or gene whose phenotypic expression can be easily distinguished and used to detect an individual, or can be used as a probe to distinguish a chromosome, nucleus or locus (Schulman, 2007). The markers exhibit polymorphisms, which may arise due to the alteration of nucleotides or, for example, through by mutation in the genome loci (Hartl and Clark, 1997), and make it possible to identify genetic differences between individual organisms or taxonomic groups (Collard *et al*., 2005).

Molecular markers can be classified into the following three groups: (1) Biochemical molecular markers, such as Isoenzyme, (2) Non-PCR-DNA markers, such as restriction fragment length polymorphism (RFLP) (Tanksley *et al*., 1988), which is based on the hybridization of a labeled probe to the fragments of genomic DNA, following digestion with a restriction enzyme, (3) A polymerase chain reaction (PCR) based on DNA markers, such as RAPD (Williams *et al*., 1990), AFLP (Vos *et al.*, 1995), ISSR (Wolfe *et al.*, 1998), EST (Udall *et al*., 2006), and SSR (Tautz *et al.*, 1989) (Figure 1.6).

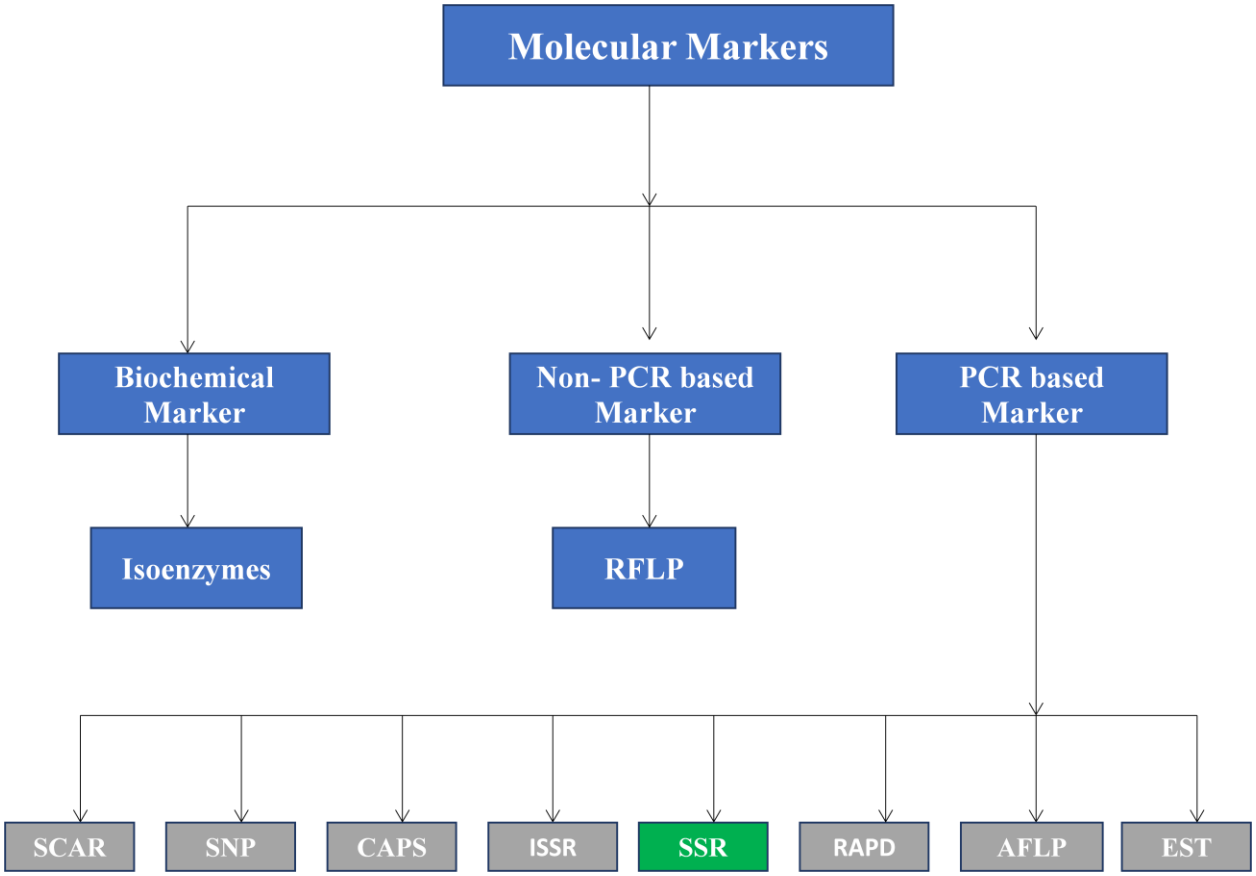


Figure 1.6: A flow chart categorizing the different genetic markers (Fiaz Ahmad *et al.*, 2017)

Science has rapidly increased the knowledge about the sequencing of the plant genome and the molecular part of various plant genes, thereby revolutionizing molecular genetics and its efficiency in the systemic study of the plant (Li and Harkess, 2018 ; Chen *et al*., 2020). Different types of molecular markers are available (Gous *et al*., 2013) and more effective tools for assigning known and unknown plant taxa have been developed to answer evolutionary and taxonomic questions (Arif *et al*., 2010).

1.6.3. Definition and evolution of SSR microsatellites

SSRs are co-dominant markers for evaluating genetic variation and relationships in plant species with high reproducibility. DNA sequence motifs are also most numerous in various genomes and have been widely used in genetic studies and as molecular markers (Han *et al.*, 2015) The first, described by Litt and Luty, (1989), was for the study of neurological diseases in humans and subsequently enabled substantial applications in several molecular fields to be made. They are

also known as Simple Sequence Length Polymorphism (SSLP) (Tautz, 1989), Short Tandem Repeats (STRs) (Edwards *et al.,* 1991), or Simple Sequence Repeats (SSR's) (Jacob *et al.,* 1991), Microsatellites are co-dominant inherited markers which are highly polymorphic and normally randomly interspersed throughout the eukaryotic genomes (Weber and May, 1989; Johansson *et al.,* 1992). They are sequences of DNA (nucleotides: thymine T, adenine-A, cytosine-C, guanine-G) that make repeated short motifs with one to six base pairs (bp) length with repetitive patterning units side-by-side (Toth *et al*., 2000 and Wheeler *et al.,* 2014).

Microsatellites can be found everywhere in the genome; in coding and non-coding regions they may also differ in their composition. Due to their high mutability, SSR's are thought to play an important role in genome evolution by making and maintaining a quantitative genetic variation (Tautz *et al*., 1986; Kashi *et al*., 1997). Tautz *et al*. (1986) reported that tri- and hexanucleotide repeats were present in both coding and non-coding regions and are distributed through the nuclear genome, but other repeat types, such as di- or tetra-, were much less frequent in coding regions but are also found in the prokaryotes' organism (Zane *et al*., 2002 ; Phumichai *et al*. 2015).

In plant genomes, AT repeat motifs are prevalent (Morgante and Olivier, 1993), while in animals AC or TG repeats are the most common ones (Powell *et al*., 1996). SSR's have been detected in animals and plant genomes as well in the chloroplast genome (Valle, 1993; Kaila *et al*.,2007). Many microsatellite markers are now available for completely sequenced plant genomes, such as rice (Sasaki, 2005) and *Arabidopsis thaliana* (Arabidopsis Genome Initiative 2000). They are also to be found in all completely sequenced plant chloroplast genomes (Provan *et al*., 2001; Chung *et al*., 2006) as well as in plant and animal mitochondrial genomes (Rajendrakumar *et al*., 2007). In plant genomes, microsatellite frequency appears to be higher in the transcribed regions than in the other regions of the genome (Morgante *et al*., 2002).

1.6.4. Classification of microsatellite markers

Microsatellites are classified according to their size, repeat motif and their position throughout the genome. It has a variable length of repeat motifs from just a one base to thousands of bases; microsatellites can be classified according to the number of bases, i.e., short repeats (10–30 bases) classified as minisatellites and with longer repeats (between 10-100 bases) they are then

called macrosatellites. Satellites with even shorter repeat motifs are called microsatellites (Figure 1.7). They based on the length of the repeat units, and SSR's are classified into the following three groups: Class one more than a 20 base pair, Class two between 11 and 20 base pairs, and Class three minimal than an 11 base pair; scattered repetitive elements are specified at the flanking sites of the SSR's (Temnykh *et al*., 2001; Varshney *et al*., 2002 and Saeed *et al.,* 2016).

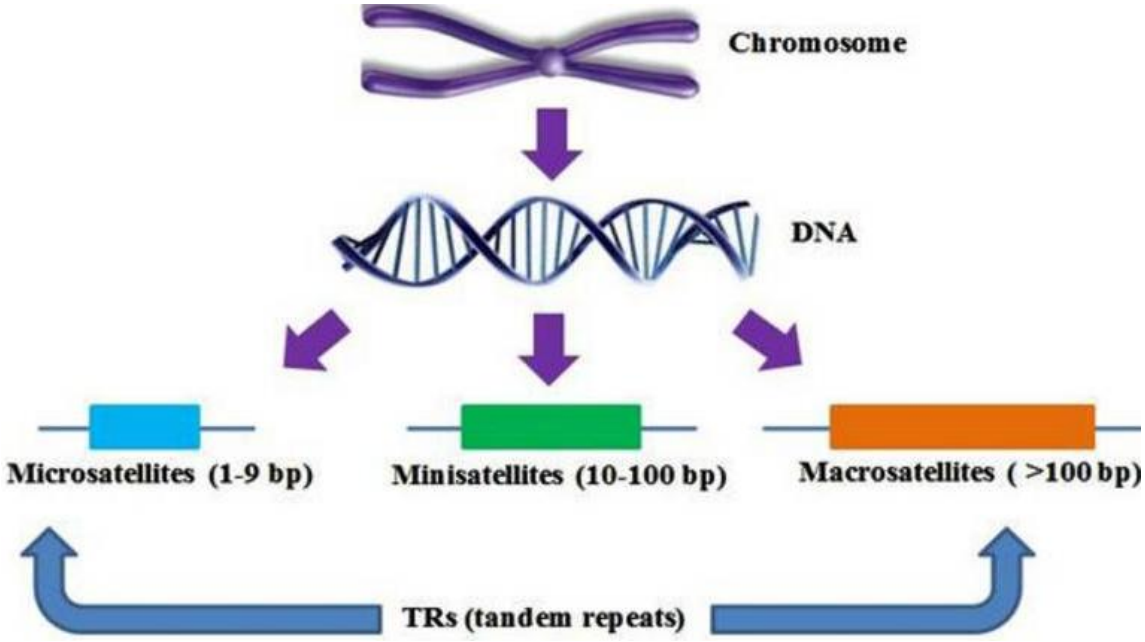


Figure 1.7: A graph clarifying the various types of tandem repeats (TRs) (Saeed *et al*., 2016).

The number of nucleotides per repeat unit are the so-called motifs (Abdelkrim *et al*., 2009). They can be classified as mononucleotides: (A)n, dinucleotides: (AT)n, trinucleotides: (GCC)n, tetranucleotides: (CTTA)n, pentanucleotides: (CTAAG)n, and hexanucleotides: (TAGGCC)n, depending on the arrangement of nucleotides inside the repeat motifs; the number of repeat times such a motif appears (n) is recognized as the repeat frequency (Scribner and Pearce, 2000; Rabello *et al*., 2005; Merritt *et al*., 2015). According to Weber (1990) (Figure 1.8), they are also classified into three different types depending on the type of arrangements based on the repeat pattern or purity length. For example, i) perfect repeats (pure repeats) of the SSR sequence without any cut-out in the runs of the repeats (Buschiazzo and Gemmell, 2006; Bhargava and Fuentes, 2010), ii) imperfect repeats (interrupted repeats) when the SSR sequence contains no repeat sequence between two or more compound or perfect repeats (Scribner and Pearce, 2000), and iii) compound repeats of an SSR sequence composed of two or more sequent groups of perfect repeats (Scribner and Pearce, 2000; Temnykh *et al*., 2001; Selkoe and Toonen, 2006).

Based on the tandem repeated motifs are found through i) nuclear genome (*nu* SSR) (Jarne and Lagoda,1996). Also, in organelle genomes ii) chloroplast (*cp* SSRs) (Powell *et al*., 1995) and iii) mitochondria (*mt* SSRs) that were most prevalent in primitive microbial world (Soranzo *et al*., 1999).

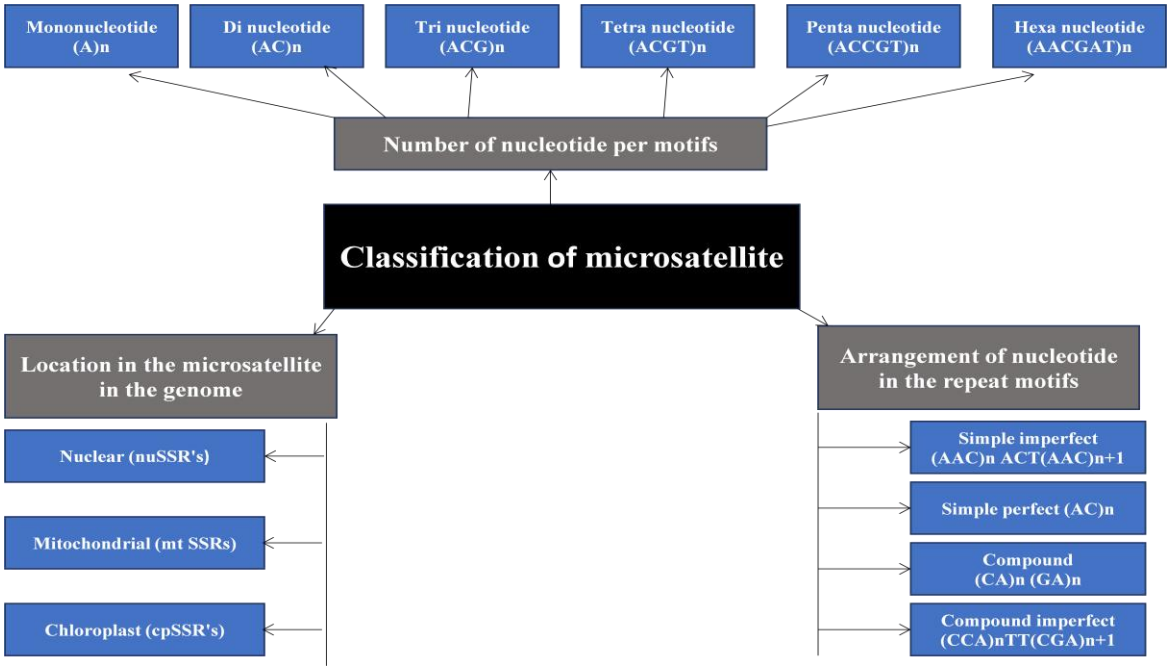


Figure 1.8: Classification of microsatellite markers (Weber, 1990).

1.6.5. The advantages of microsatellite (SSR) markers.

There are microsatellite (SSR) markers that characterize satellites with important qualities that are very useful and desirable molecular markers:

Hypervariability: microsatellite SSR's are highly polymorphic, with inclusive allele diversity in repeat numbers (Litt and Luty, 1989).

PCR-based: low quantities of template DNA (10-100 ng per reaction) are required due to the use of long PCR primers. Highly degraded or old DNA can be successfully used as a template (Morgante and Olivieri, 1993).

Locus specificity: the DNA sequence varies, or there is amplification of specific alleles At the same SSR site. The design of long primers (with >20 bp) in the region surrounding the microsatellite provides tag specificity and allows for selective amplification of a specific SSR

locus in contrast to multi-locus tags, such as RAPDs or microsatellite tags. (Morgante and Olivieri, 1993; Powell *et al*., 1996).

Diversity: microsatellite SSR markers have been the most used molecular markers to address genetic diversity. They depend on many repeat motifs and occur in various functional domains (Hancock, 1990).

Across species transferability: SSR's marker is more informative than molecular markers. Primers designed for particular species could be successfully applied for a study to be carried out on the genetic relationship among the species (Mnejja *et al*., 2010). Effective cross-species amplification of SSR primers has been widely depicted in many species in such studies as those by Kijas *et al*., 1995, Pandey *et al*., 2004 ; and Barbara *et al*., 2007. But, there can be some problems, such as when null alleles may show when microsatellite primers are being transferred to other species.

Co-dominant Mendelian inheritance: The Microsatellites that have been widely used for individual studies are single markers where alleles of an individual can be distinguished heterozygous from homozygous, which is not the case for dominant markers, such as AFLPs and RAPDs (Muneer *et al*., 2009).

Widespread distribution: Microsatellites are found in the genomes of all the species studied. They can easily interchange among researches because of their locus specificity. They are dispensed in the genome and many are in both the coding and non-coding regions of many higher plants and animals (Tautz *et al.*, 1986; Hokanson *et al*., 1998; Temnykh *et al*., 2000).

1.6.6. Disadvantages of microsatellite markers

Development: Microsatellite primer design is work-intensive, expensive and time-consuming. Technically the isolation of microsatellite demands and often results in the low efficiency of microsatellite discovery (Zane *et al*., 2002).

Null alleles: The presence of microsatellite null alleles has been listed in PCR primer characterization and in population genetics studies (Dakin and Avise, 2004), showing any alleles where microsatellite locus-specific primers cause disorder to amplify the detected level of the

polymerase chain reaction (PCR) products. Null alleles are generally referred to as mutations within the binding site of a DNA primer, preventing the binding and leading to the loss of PCR product (Nascimento *et al.*, 2005). It is recorded in studies of many organisms of, for example, humans (Callen *et al*., 1993), voles (Ishibashi *et al*., 1996), deer (Pemberton *et al*., 1995), pupfish (Jones *et al*., 1998), and different plant species (Fisher *et al*., 1998; Sefc *et al*., 1999; Stachel *et al*., 2000; Devey *et al.*, 2002; Pastorelli *et al*., 2003 and Vornam *et al*., 2004). The presence of null alleles will result in decreases in the estimated frequency of heterozygotes (Pemberton *et al*., 1995).

Stutter bands: Through the amplification of microsatellite sequences, insertion-deletion mutations in a minor product appear that differ in size from those in a major product to produce 'stutter bands' or 'shadow bands' that arise during PCR and may then complicate the accurate scoring of SSR polymorphism (Viguera *et al.*, 2001 and Shinde *et al*., 2003).

Homoplasy: Alleles of the same length are identical in state but not identical in origin. This occurs when characters are similar but are not derived from a common ancestor but instead resulting from convergence or parallelism (Estoup *et al*., 1995). In SSR markers, microsatellite divergence is detected by electrophoresis of PCR products. Allele classes differ according to the length of the amplified fragments (bp). Two PCR products of the same length may not be transcribed without mutation of the same inherited sequence, providing the possibility of size homoplasy. A substantial amount of size homoplasy is predictable at most SSR loci (Estoup and Cournet, 1999).

1.6.7. The application of microsatellite SSR's markers

Microsatellites are very popular and multilateral genetic markers with various applications in conservation biology, evolutionary biology, and population genetics. They are sources of highly polymorphic and evenly distributed eukaryotic genomes. Genetic variation arises between individuals, leading to differentiation at the level of the population, the species, and the higher-order taxonomic groups. The development of microsatellite markers has a strong impact enabled genetic variations between individuals, populations, or species to be exposed.Mendelian inheritance enabled microsatellite to be used as co-dominant markers and they were found to be informative in several species and very suitable for the study of population structure and

pedigree analysis (Taylor *et al*., 1994). Microsatellite markers, along with new statistical advances, have revolutionized the analytical intensity needed to search genetic diversity and have now the markers of choice due to their broad extent of application in transferability and comparative mapping, gene tagging, population genetics, conservation, and evolutionary biology (Figure 1.9).

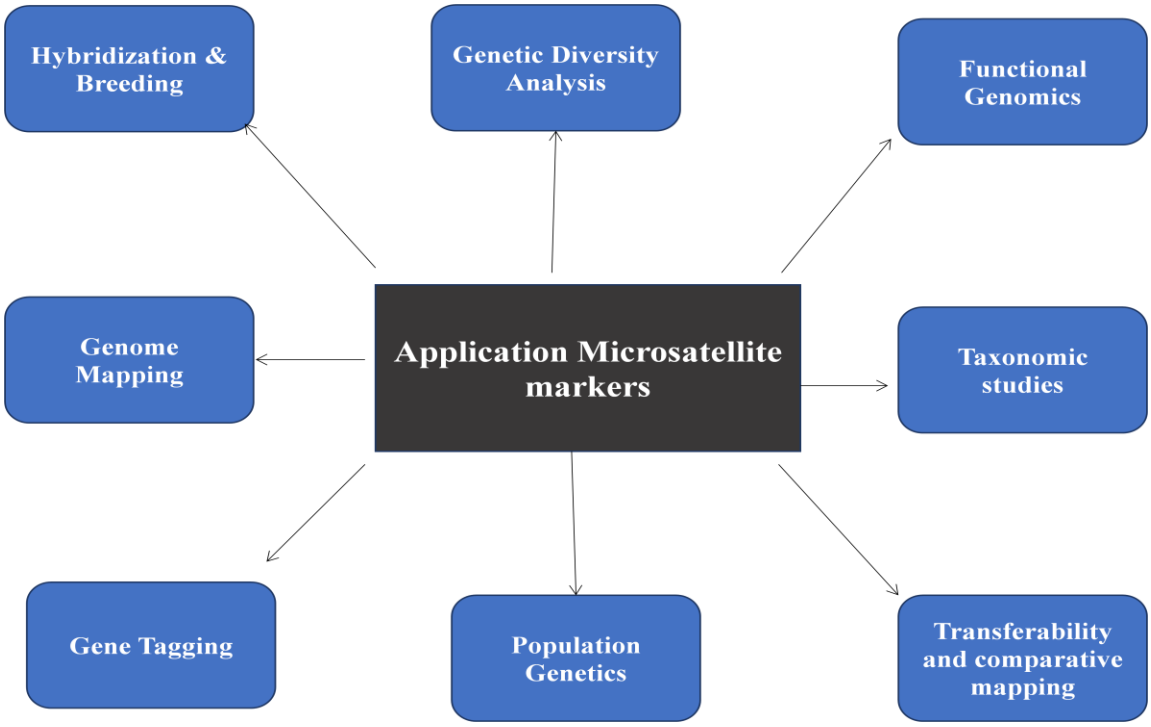


Figure 1.9: Different applications of microsatellite markers in plant species (Varshney *et al.*, 2005).

1.6.8. Genetic diversity parameters

The genetic diversity of species can be investigated using various techniques, such as an inclusive analysis of morphological traits (Sinkovi *et al*., 2019), biochemical characteristics (Kottawa-Archchi *et al*., 2014), isozymes (Jain *et al.*, 2006), and molecular markers (Halasz, 2019).

Genetic diversity is measured at the level of molecular markers depending on the frequencies of the genotypes and a llele. Genetic diversity is an important part of the dynamics of populations, as it is immediately related to the evolutionary potential of the population (Hughes *et al*., 2008). Nonetheless, there are several types of measures of genetic diversity, the most prominent being

one that depends on heterozygosity and another based on allelic richness. These measures differ in their formulations and in their ecological and evolutionary interpretations, as well as in the mathematical frameworks within which they can be applied.

Heterozygosity is one of the most exceedingly applied parameters used to measure diversity within a population. Observed and expected heterozygosity are often compared. These measurements are particularly critical to differences in allele frequencies. A reduction in the observed heterozygosity can be a hint for a decrease in the average fitness in a population (Szulkin *et al*., 2010). The most widely used parameter to measure diversity within populations is the expected heterozygosity; it has been shown to reveal a higher allele variety in a population. These parameters are significant elements in conservation strategies (Caballero *et al.,* 2010).

Another second standard to measure diversity is allelic richness. It is calculated as the average number of alleles per locus (Hughes *et al*., 2008) and provides a measurement of the genetic diversity of a population and its long-term potential for persistence and adaptability (Greenbaum *et al*., 2014). Allelic richness is insensitive to allele frequencies and thus tends to favour rare alleles. Whilst heterozygosity quantifies the effective number of alleles, allelic richness determines the real number of alleles (Petit *et al*., 1998). Allelic richness is a strong indicator of the evolutionary potential of a population (Caballero and Garcia, 2013), and it has also been suggested that this measurement is of key significance in population management and conservation (Foulley and Ollivier, 2006).

A third locus-level biodiversity measure for biological prospecting and for community ecology is Shannon's index (Shannon; 1948), also known as the Shannon-Wiener index (Spellerberg and Fedor, 2003). It has been often used to estimate genetic diversity at various levels from genes and populations to whole species and ecosystems (Gaggiotti *et al*., 2018; Sherwin, 2018). Sherwin *et al*. (2017) also presented its potential advantage in genomic studies. However, the index was confirmed to be negatively biased when it was dealing with small sample sizes. It was proposed that modifications to the original Shannon equation should be made to obtain an unbiased estimator (Konopiński, 2020). The Shannon index provides more information than allelic richness does and is also more sentient to the loss of rare variants than heterozygosity is (Sherwin *et al*., 2017).

1.6.9. Population structure instruments

STRUCTURE is a freely available program for population analysis, first proposed by Pritchard *et al*. (2000). STRUCTURE analyses distinctions in the distribution of genetic variants among populations, using a refined Bayesian algorithm by placing samples into groups whose members share similar types of variance.

The structure defines both populations of the data and assigns individuals to the population they are best suited to according to the existing variation patterns. It is therefore important to assess whether the allocation of individuals to populations with non-genetic criteria is consistent with the genotypes detected among the populations. (Pritchard *et al.,* 2000; Jobling *et al,* 2014).

It also deduces relationships between individuals, genetic variances and similarities within and between groups of genotypes, as well as their evolutionary history. Ways of reconstructing a population structure include methods for distance-based evolution (Pickrell and Pritchard, 2012). A popular technique for the estimation of ancestry is the model-based clustering approach (Alexander *et al.,* 2009; Pritchard *et al.,* 2000) and a multivariate unanimity exemplification of genetic relationships between populations (Price *et al*., 2006). Different coefficients, such as Jaccards, Dice (Nei and Li), and Squared Euclidean distance, are utilized for genetic dissimilarity or similarity (Kosman and Leonard, 2005). In addition, analysis of molecular variance (AMOVA) may be utilized to partition variance in order to deduce the population structure (Excoffier *et al.,* 1992). The option of appropriate coefficients and methods is specified by the marker system and has to be validated with appropriate statistical methods, such as, for example. bootstrap.

1.6.10. Cross- species SSR transferability

The translocation of highly protected gene regions is known to provide a cost-effective source of markers for related species, which is especially important for taxa with the lower microsatellite frequencies or a form that is itself difficult to isolate. The developed microsatellite (SSR) is an informative molecular marker for Apiaceae and species from the same family were effectively differentiated by polymorphism among the selected plants, namely, *Angelica archangelica*, *Daucus carota*, *Centella asiatica, Petroselinum crispum*, *Anethum graveolens, Apium*

graveolens, Coriandrum sativum, Foeniculum vulgare. and These species are known to be important and economical types. (SSR) markers that were developed for these species are known to be powerful tools for basic and applied research into the Apiaceae plant species to assess the genetic variation within and between populations.

Accordingly, in the present study, we investigated the transferability of 45 SSR microsatellite markers that had been primarily developed as *Daucus carota* markers by Cavagnaro *et al*., 2011, for use in *Pimpinella saxifraga* from Germany. Genomic information from this species is still sparse, and so the possibility to carry out cross-amplification using the markers developed in a randomly selected but related species from the common interspecific SSR markers was shownto be a valid method to initiate the analysis of the *P. saxifraga* species without sequence analysis and without the need to design *de novo* primers.

1.7. Working Hypothesis

Based on previous experiments with *P. saxifraga* at the Botanical Institute Giessen, Systematic Botany Group we hypothesize, that

1) Tested the inter-generic transferability microsatellite markers that were originally developed from *Daucus carrota* in *Pimpinella saxifraga*

2) molecular data separate individuals from biogeographic regions

3) morphological features discriminate taxa from Germany

4) molecular and morphological data are congruent in their information content about diversity of *Pimpinella saxifraga* in Germany

1.8. Objectives of this study

Using molecular markers (nuSSR markers) and morphological triats data, the current study aims to contribute to the taxonomic treatment of *Pimpinella saxifraga*.

More specifically, it aims:

1-The development of a larger set of genomic microsatellite markers, the determination of their frequency and comparative distribution and the evaluation of their cross-transferability, their polymorphic potential, and an efficient assessment of the molecular diversity in *P. saixfraga.*

2-An analysis of the morphological variation in *P. saxifraga*, using measurements and their statistic evaluation of morphological characters from herbarium plants. We will compare the morphological diversity results with the biogeographic region to reveal any site- dependent morphological features.

3-A justification of the taxonomic status and affiliation of the herbal group using morphological data and molecular analysis of DNA extraction from our herbarium sample.

CHAPTER II

MATERIALS & METHODS

2. Materials and Methods

2.1. Plant materials

The selection of *P. saxifraga* samples was according to biogeographical regions and the morphological parameter of leaf shape. Specimen were selected from eight herbaria in Germany (Figure 2.1). Individuals were divided into four groups according to their biogeographical regions in Germany: North lowland, Central upland, South, and Alpine regions. For this study a total of 78 individuals was sampled from thirteen provinces in Germany (Figure 2.2;Table 2. 1).

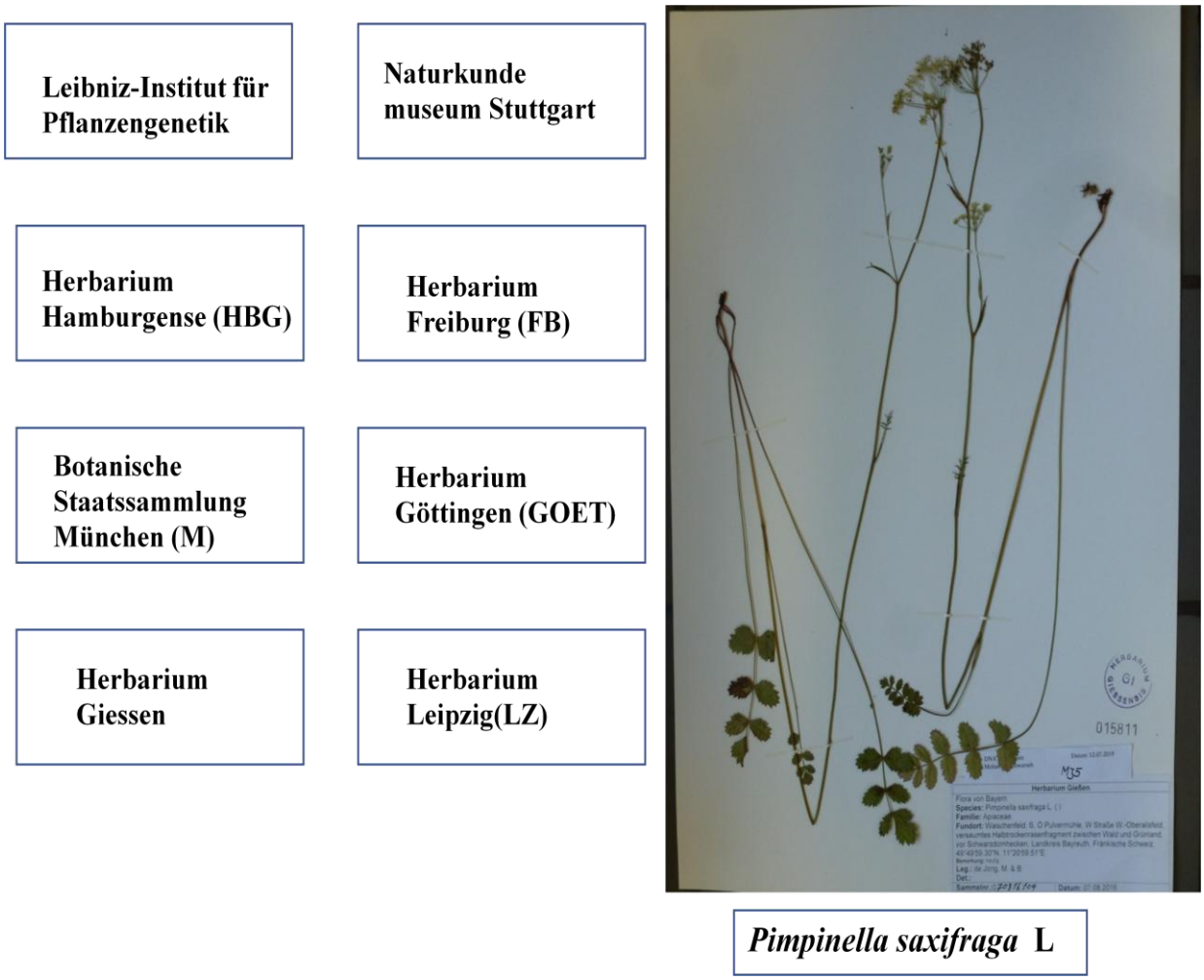


Figure 2.1: Eight herbaria in Germany where the *Pimpinella saxifraga* L. samples come from for this study.

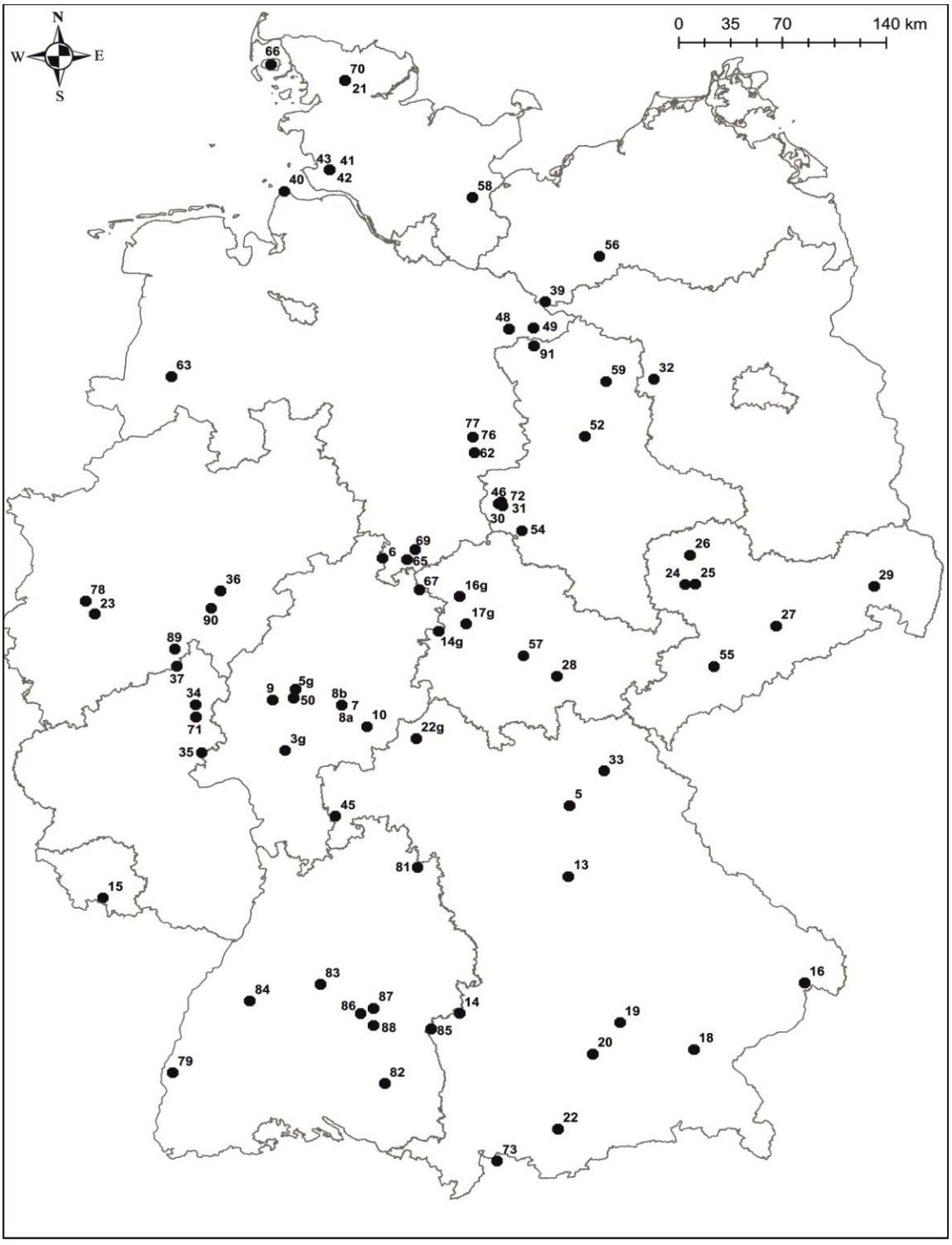


Figure 2.2: Map of geographic distribution from which Pimpinella saxifraga specimens were collected for this study in Germany (North lowland, Central upland, South, and Alpine region) (see also Table 2.1). Map was prepared with ArcGIS Desktop 10.2.2., Esri.

Table 2.1: Collection data for analyzed *Pimpinella. saxifraga* samples. Sample code (ID. No) from herbarium in Germany examined with SSR.

ID. No	Collecting regions	Provinace	Latitude (N)	Longitude (E)	Altitude (m)	Geographical Regions
MJ21	Großjörl (Flensburg)	Schleswig-Holstein	54.6126487	9.2720084	18	North lowland
MJ27	Falkenberg	Berlin	50.95259	13.39270	365	North lowland
MJ32	Glüpe, Rathenow	Sachsen-Anhalt	52.60639	12.33826	25-30	North lowland
MJ39	Ludwigslust Dömitz-Hafen	Mecklenburg-Vorpmmern	53.13926	11.27942	15-30	North lowland
MJ40	Duhnen, Cuxhaven	Niedersachsen	53.88554	8.64289	5-10	North lowland
MJ41	Gudendorf	Schleswig-Holstein	54.02537	9.10771	15-20	North lowland
MJ42	Gudendorf	Schleswig-Holstein	54.02537	9.10770	15-20	North lowland
MJ43	Gudendorf	Scheswig-Holstein	54.02537	9.10769	15-20	North lowland
MJ48	Lüchow-Dannenberg-Reddereitz	Niedersachsen	52.9668	10.90958	100-105	North lowland
MJ52	Wolmirstedt	Sachsen- Anhalt	52.24849	11.62674	50	North lowland
MJ56	Parchim	Mecklenburg-Vorpmmern	53.42585	11.84752	50-60	North lowland
MJ58	Salzwiese, Lübeck	Mecklenburg- Vorpmmern	53.83464	10.57218	10-15	North lowland
MJ59	Stendal	Sachsen- Anhalt	52.60513	11.85932	30-40	North lowland
MJ63	Hasealtarem	Niedersachsen	52.66237	7.52777	20	North lowland
MJ66	Norfriesiche Inseln	Schleswig-Holstein	54.717350	8.49576	2-10	North lowland
MJ70	Grossjorl	Schleswig-Holstein	54.61265	9.27201	18	North lowland
MJ76	Braunschweig	Niedersachsen	52.26466	10.52361	75-90	North lowland
MJ77	Braunschweig	Niedersachsen	52.26466	10.52361	75-90	North lowland
MJ91	Salzwedel	Sachsen- Anhalt	52.85285	11.15397	25-30	North lowland
MJ26	Eilenburg, Wölperner	Sachsen	51.44491	12.60394	105	North lowland
MJ6	Reinhardshagen-Vaake	Niedersachsen	51.478	9.61654	125-135	Central upland
MJ30	Wernigerode	Sachsen-Anhalt	51.83442	10.78625	240	Central upland
MJ31	Scharfenstein Wernigerode	Sachsen-Anhalt	51.80981	10.79822	245-260	Central upland
MJ46	Wernigerode, Hasserode	Sachsen-Anhalt	51.82489	10.75703	260	Central upland
MJ54	Guntersberge	Sachsen-Anhalt	51.64292	10.97663	405-420	Central upland
MJ67	Allendorf/werra	Hessen	51.26954	9.97200	150	Central upland
MJ65	Fürstenberg, Amstat	Thuringen	50.82430	10.95760	310-330	Central upland
MJ34	Helferskirchen	Rhienland-Pfalz	50.51255	7.82429	330-350	Central upland
MJ35	Segelflugplatz Nastätten	Rhienland-Pfalz	50.198281	7.8897555	360	Central upland
MJ36	Westfalen Sauerland	Nordrhein-westfalen	51.26098	8.04755	500	Central upland
MJ37	Sieg Bahn au Alten	Nordrhein-westfalen	50.7646471	7.6397533	180-200	Central upland
MJ50	Busek	Hessen	50.61921	8.77524	220-280	Central upland
MJ69	Göttengen/ Sieboldshausen	Niedersachsen	51.53276	9.93521	185	Central upland
MJ62	Wolfenbüttel	Niedersachsen	52.16253	10.53482	85-100	Central upland
MJ65	Kries Göttengen/volkerode	Niedersachsen	51.46790	9.85647	280	Central upland
MJ29	Kleinförstchen, Bahndamm	Sachsen	51.17131	14.35893	224	Central upland
MJ24	Belgershainer	Sachsen	51.25388	12.53965	160	Central upland
MJ25	Großsteinberg –Naunhof	Sachsen	12.63854	12.63854	145	Central upland
MJ28	Thüringen Bad Blankenburg	Thuringen	50.68299	11.27263	250	Central upland
MJ15	Saabruken	Sarrland	49.23436	6.99638	190-200	Central upland
MJ23	Dormagen	Nordrhein-westfalen	51.0941656	6.8407931	45-50	Central upland
MJ72	Wernigerode	Sachsen- Anhalt	51.8344172	10.7862526	245	Central upland
MJ71	Montabaur	Rhienland-Pfalz	50.432219	7.8302494	235	Central upland
MJ89	Waldbröl	Nordrhein-westfalen	50,8758	7.61595	270-290	Central upland
MJ90	Biggetal, Haggen	Nordrhein-westfalen	51.14648	7.95906	280	Central upland
MJ55	Stollberg	Sachsen	50.70877	12.77626	440	Central upland
MJ78	Grimlinghausen, Neus	Nordrhein-westfalen	51.17602	6.74710	40-50	Central upland
MJ7	Breungeshain Schotten	Hessen	50.51667	9.21667	690-700	Central upland
MJ8a	Breungeshain Schotten	Hessen	50.51667	9.21667	690-700	Central upland
MJ8b	Breungeshain Schotten	Hessen	50.51667	9.21667	690-700	Central upland

Table 2.1 (continued).

MJ9	Wetzlar	Hessen	50.54922	8.55731	200-250	Central upland
MJ10	Steinau-Schmidtmuhle	Hessen	50.37437	9.45416	280-300	Central upland
5g	Fernwald	Hessen	50.561315	8.757745	250	Central upland
22g	Schwarzer Berg.	Thuringen	50.292924	9.920461	680	Central upland
17g	Berka v. d. Hainich	Thuringen	51.039571	10.415881	375	Central upland
16g	Eichsfeld	Thuringen	51.220721	10.358348	370	Central upland
3g	Ober Erlenbach 1a	Hessen	50.219039	8.679331	145	Central upland
14g	Herleshausen 1	Thuringen	50.995496	10.153041	200	Central upland
MJ14	Langenau, Kreis Ulm(Donaurid)	Bayern	48.48616	10.28301	440-460	South land
MJ19	Freising Oberbayren	Bayern	48.39995	11.74499	475	South land
MJ20	München, Allacher Loche	Bayern	48.19958	11.48556	500-510	South land
MJ45	Erlenbach/Main/Miltenberg	Bayern	49.78746	9.15419	125-140	South land
MJ81	Tauber-Gebiet	Baden Württenmburg	49.44892	9.91549	360-380	South land
MJ83	Böblingen	Baden Württenmburg	48.68497	9.01136	430-455	South land
MJ85	Schwäbisch-Frankish waldberge	Baden Württenmburg	48,38856	10.02111	460--480	South land
MJ86	Hohen urach (Bad urach)	Baden Württenmburg	48.49234	9.3796	660---680	South land
MJ87	Schlattstall	Baden Württenmburg	48,52525	9.49797	520---530	South land
MJ88	Munsingen	Baden Württenmburg	48.41286	9.49479	700-720	South land
MJ5	Waischenfeld	Bayern	49.83306	11.34972	380-390	South land
MJ13	Nurnberg	Bayern	49.36800	11.31837	300-420	South land
MJ73	Tannheimer Berge	Bayern	47.51365	10.59496	175-1800	Alpine & Alps
MJ33	Bad Berneck	Bayern	50.05312	11.68686	545-560	Alpine & Alps
MJ16	Passau, Oberilzmühle	Bayern	48.60760	13.44684	320-340	Alpine & Alps
MJ18	Waldkraiburg	Bayern	48.20588	12.40442	435-445	Alpine & Alps
MJ22	Kreis Garmish-Partenkirchen	Bayern	47.7144384	11.1506437	660	Alpine & Alps
MJ79	Kaiserstuhl	Baden Württenmburg	48.09677	7.67622	370-390	Alpine & Alps
MJ82	Braunenweiler. Riedwirsen	Baden Württenmburg	48.03178	9.59696	635-640	Alpine & Alps
MJ84	Schwarzwald Besenfeld	Baden Württenmburg	48.57245	8.36790	770-810	Alpine & Alps

2.2. Molecular analysis

2.2.1. Genomic DNA extraction

Dry leaf material from *Pimpinella saxifraga* was collected from the Herbarium sheets into the laboratory, total genomic DNA was extracted of each sample. Leaf material was crushed into soft powder in a 2 ml Eppendorf tube, in a retsch mill (Tissue layser II Retsch, Germany). The genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2.2. DNA quality and concentration analysis

The concentration of the extracted DNA and purity was checked by the OD260/OD280 ratio using a NanoPhotometer TM (ImplenGmBH, München, Germany). Quality check of DNA (ds) was done by using 1µl of each extract and scanning its absorbance range. Furthermore, an additional quality check of the DNA was taken by visualization on a gel electrophoresis using quality of the DNA was taken 1µl of each DNA sample mixed with 1µl of SYBR® Gold fluorescence dye (Invitrogen ™) and 2µl loading buffer. Each mixture was loaded into a well prepared 1.0% (w/v) agarose gel (Roth). Electrophoresis in TAE buffer was carried for 1 h at 80V and DNA sample band densities were compared with DNA standard marker Lambda DNA/HindIII (Thermo scientific). The gel was visualized under UV rays in the gel documentation machine (E-BOX, VILBERLOURMAT, France). The DNA long time storage took place in TAE buffer at -20°C for later sequencing and PCR analysis or stored at 4 °C to use within a few days. DNA of each individual for PCR amplification was then diluted to final concentration between 10 ng/µl to 50 ng/µl with ddH_2O.

2.2.3. SSR markers selection

After screening sequences of *P. saxifraga* and other taxa of the Apiaceae family in GenBank and the possibilities for using microsatellites developed for taxa closely related to *P. saxifraga*, microsatellite markers developed for *Daucus carota* were chosen. We selected 44 SSR primer pairs with di-, tri-, tetra-, and compound nucleotide repeats, based on the published carrot microsatellite loci developed by Cavagnaro *et al*. (2011). For cross-amplification in *P. saxifraga*. Initially, conditions used for SSR markers amplification were those described for the source species (carrot) (Cavagnaro *et al* .2011) Nine microsatellite primers with a successful amplification were used for final PCR amplification (Table 2.2). Of these markers, eight microsatellites were developed by Cavagnaro *et al*. (2011) for its use in *D. carota*, one microsatellite was developed by Henry *et al*. (2008) for its use in *Heracleum mantegazzianum*. The other 35 sets of SSR markers failed to amplify or showed very weak amplification.

Table 2.2: SSR markers of *Pimpinella saxifraga* including their repeat motif, sequences of microsatellite primers (forward and reverse), annealing temperature (Ta) used in PCR amplification, fragment length (bp), fluorescent dye and NCBI accession number.

SSR Marker	Repeat Motif	Primer Sequences 5'-------3'	Ta (°C)	Fragment length (bp)	GC content	NCBI number.	Fluorescent dye at 5'	Ref
BSSR76	(AAG)4	F:GGAGAGAAAAAGCCAGCAGAGAA R: ATA TCA GGG TGG ACT TGC TTC AAC	58°C	172-205	40.74	FJ 148203	HEX	a
BSSR 99	(TTTGTTGA)2at(TTTGTTGA)	F:ATTGTTGGTTATTGGTTTGAATGAG R:CAAGAGGGTTCAAGAATATGAAGAA	54°C	196-206	34.00	FJ147926	FAM	a
BSSR53	(AT)8	F:GCTTTAGAACTTCTTCTAGTCGTCCA R:CTCATGAGCTCACTTCATCTAACTCC	51°C	171-205	44.23	FJ148355	HEX	a
GSSR10	(GACA)7	F:CTTAGTAGTAGCACACACCAGACG R: GAGCTGAACGAGTCAGAAAGG	55°C	235-254	51.11	FJ816120	FAM	a
GSSR154	(TC)11	F:CTTATATGTGATGGCGTCGAAA R: GACTGCACCGCTCCTAACTC	53°C	248-268	45.65	FJ816262	HEX	a
GSSR111	(ATAC)3atccatc(CATA)9tat(CA)20	F:GAGGAAGGGTAGATCCAGTCA R: ATGGGATGTCTTTCCCCTCTAT	54°C	229-323	48.84	FJ816220	HEX	a
GSSR97	(GA)8 (AG)7aagtattcca(AG)6 (GA)7	F:GGCAAAGAAACAGATTTGGAGA R: CTGCCCTAGCATCAAAACAAAC	52°C	147-260	43.18	FJ816206	FAM	a
ESSR80	(CA)9	F:ACAGCCAGATGAGCAGGACT R: GAGATTTGGCAATGTGGGAT	51°C	183-197	50.00	No information	FAM	b
GSSR 107	(ATAC)8 (ACAT)4	F:TTCTGGTCTTTTGACATGAAGG R: CGGATTTGAGGTGAGTTGAATA	51°C	191-196	40.91	FJ816216	FAM	a

The reference for each SSR marker is indicated with "a" for Cavagnaro *et al*. (2011) and "b" Henry *et al*. (2008).

2.2.4. Polymerase Chain Reaction (PCR)

A PCR is used to replicate DNA using a DNA polymerase. In this chapter, the PCR is briefly explained because it serves as the basis for the methods of the SSR microsatellite. A traditional PCR (Mülhardt, 2009), consists of three steps: denaturation, annealing, and elongation (Figure 2.3). The denaturation step is needed in order to separate the two strands of the starting DNA (denaturation is executed at 94^{o}C). This is followed by the annealing step (temperature varies according to bases and length of the primer sequence), in which the primers used to attach the separated DNA strands. In the third step, elongation, the temperature is increased to 72^{o}C so that the primer sequences are extended by the Taq polymerase and two identical DNA double strands are present (Mülhardt, 2009). The sequence of these three steps comprises one cycle. The higher the number of cycles, the higher the exponential multiplication.

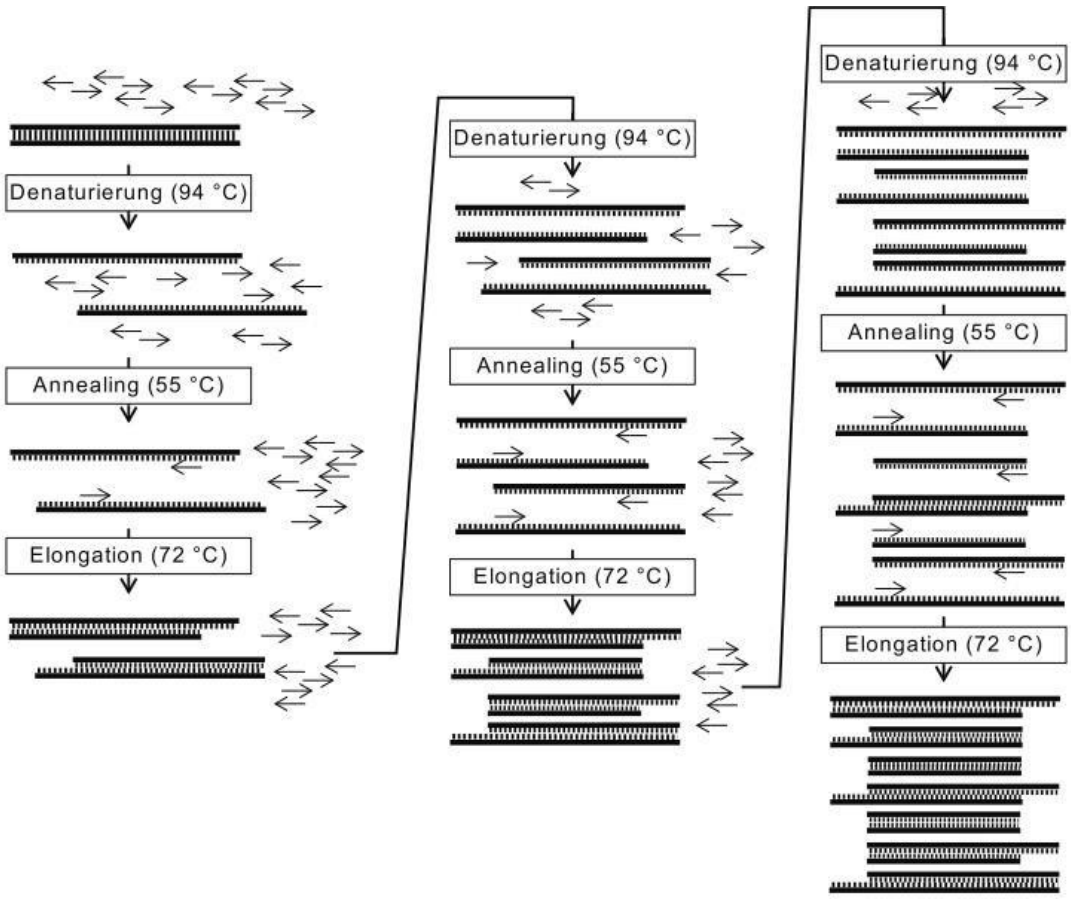


Figure 2.3: Graphic representation of three PCR cycles. Denaturation. This is followed by the annealing step, and the third step, elongation, the increased number of cycles, leads to high exponential multiplication (Mülhardt 2009).

2.2.5. SSR marker Amplification

PCR condition were optimized by increasing or decreasing the annealing temperature and add some chemical additives to PCR reaction (like PVP, BSA, DMSO and Betain). SSR markers were selected depending on the high level of polymorphism recognition ability and the quality of band scoring from former studies for different species of Apiaceae family (Piya and Nepal, 2013; Jutta *et al*. 2015). PCR reactions were performed in a final volume of 20 µl in a thermocycler (Eppendorf- Mastercycler). To check the suitability of primer at first a screening, was executed using four test samples of *P. saxifraga*, selected random from the sampled biogeographical region and one *D. carota* as a positive control. For sequencing, samples were shipped to LGC Forensics (Colonge, Germany). Out of 44 tested primers nine primers amplified successfully products of expected size range across *P.saxifraga*. The other primers produced no products at various annealing temperature or get no specific bands.

All samples used fluorescent labeled forward primers with different fluorescence dyes shown in Table 2.3. For this project 6-FAM and HEX were used because their spectra were not overlapping, so the PCR products were mixed to permit multiplexing of markers during fragment analysis.

Table 2.3: Fluorophores of microsatellite excitation and emission spectra.

Dye	Excitation(nm)	Emission(nm)	Anaylsis color
6-FAM	495	520	Blue
HEX	535	553	Green
Rox	575	607	Red
NED	546	575	Yellow
TET	521	536	Yellow
TAMRA	557	583	Yellow
VIC	538	554	Green
LIZ	638	655	Orange

For microsatellite analyses, *P. saxifraga* individuals were amplified using two different methods. The first method directly labeled microsatellites. Each PCR reaction in 20µl contained: 1µl genomic DNA (ca. 30 ng/µl), 2µl of 10x Dream Taq PCR buffer including $MgCl_2$ (Thermo Fisher Scientific), 1µl forward and reverse primers (respectively 5 pmol/µl) (Sigma–Aldrich®), 2µl dNTPs (Ferment's, Thermo Fisher Scientific) 1µl of 2% PVP, 0.4 µl of DreamTaq polymerase (5U/µl) (Thermo Fisher Scientific), and 11.6 µl ddH2O (Rotipuran® Ultra Roth®).

The PCR reaction run under amplification conditions as follows: initial denaturation at 95°C for 3 minutes, 38 cycles at 95°C for 30 second, optimized primer annealing temperature (Ta, see Table 2.2) for 30 second, primer elongation at 72 °C for 30 second. A final extension was performed at 72 °C for 10 minutes. PCR was carried out in an Eppendorf-Mastercycler.

The second method for the remaining primer GSSR10 and GSSR111 using M13 tail to test the primer (Schuelke, 2000) each microsatellite then amplified for visualized on the capillary sequencer using three primers added to the same Polymerase Chain Reaction (PCR): (i) forward tail primer that is tagged with specific DNA sequence attached to the 5` end (ii) a labeled tail primer consisting of the same tail sequence but fluorescent label attached on the 5` end; and (iii) reverse primer (Figure 2.4; Table 2.2). The fragment in the first set of PCR cycles is amplified using a forward tail primer and a reverse primer, thus incorporating the tail sequence. In the next set of PCR cycles, anneal the labeled tail sequence, along with the reverse primer, amplify the fragment and in doing so incorporate the desired fluorescent label. Two elements of this protocol have been recommended by Schuelke (2000) as necessary to ensure that the forward tailed primer is incorporated before the labeled tail. PCR reaction was carried out in 20µl containing: 1µl of template DNA (ca. 30 ng/µl),), 2 µl of 10x Dream Taq PCR buffer including $MgCl_2$ (Thermo Fisher Scientific), 0.64 µl of reverse primer (5 pmol/µl) and 0.16 µl of forward primer (5 pmol/µl) tagged with M13 tail, 0.32µl of M13 tail primer (10 pmol/µl) (5′-TGTAAAACGACGGCCAGT-3′) labeled with one of the FAM or HEX dyes, 2µl dNTPs (Fermentas, Thermo Fisher Scientific), 1µl DMSO (99%), 0.5µl Dream Taq polymerase (5 U/µl) (Ferments, Thermo Fisher Scientific), and 12.38µl ddH_2O (Rotipuran® Ultra Roth®). The conditions used for the PCR amplification were as follows: initial denaturation at 94°C for 3 minutes, followed by 30-32 cycles at 94°C for 45 second, optimized primer annealing temperature (Ta, see Table 2.2) for 45 second enhance binding to forward tailed primer, primer elongation at 72 °C for 40 second. The Followed by 8 cycles 94°C (45s) 53°C (45s) for the second set of cycles to facilitate annealing of the larger labeled tail 72 °C (40s).

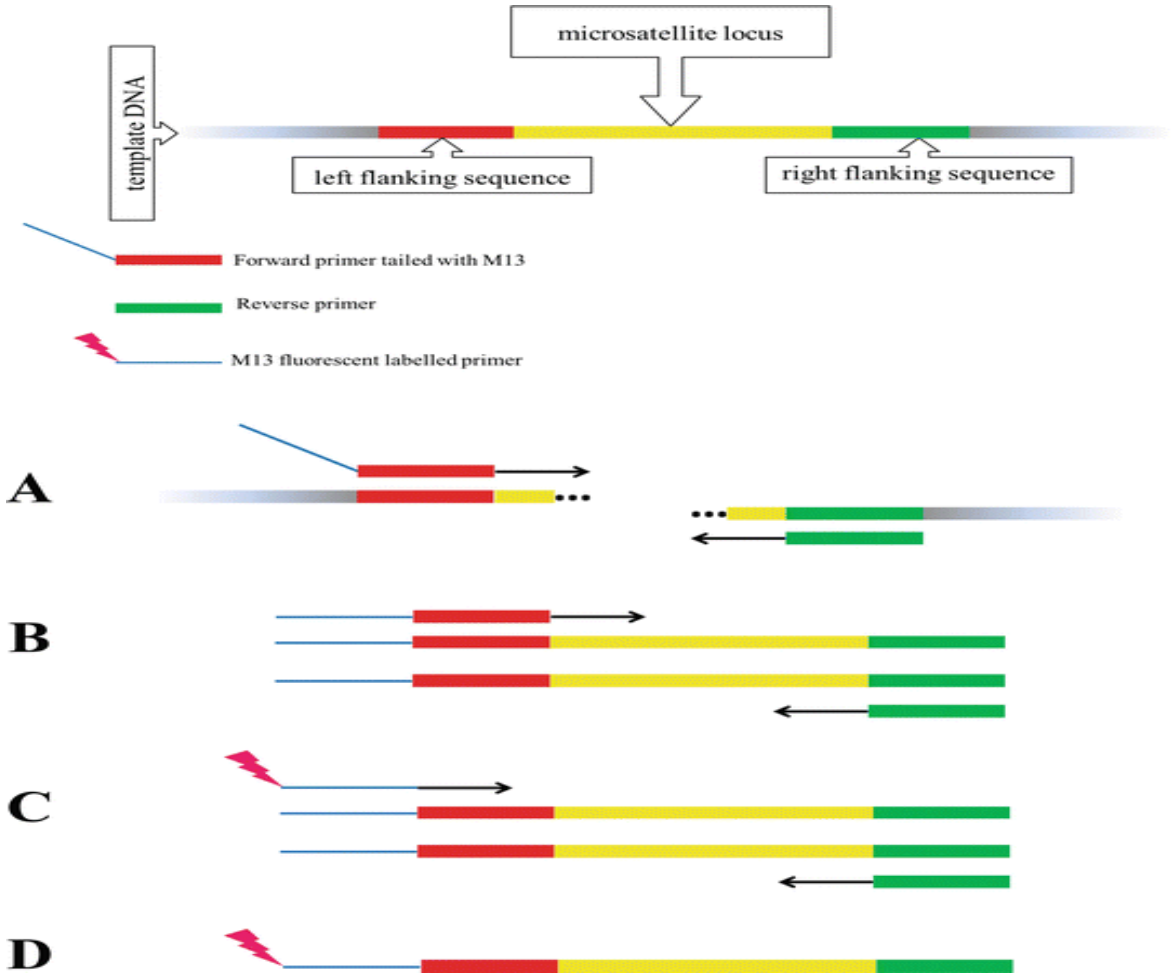


Figure 2.4: M13-tailed primer PCR. They are three different primers for the M13- tailed PCR reaction: forward primer with the M13 tail, reverse primer, and M13 primer labeled with a fluorescent dye (Onyśk and Boczkowska 2017) This diagram is based on a figure from (Schuelke 2000).

A final extension was performed at 72 °C for 10 min. For control of successful amplification, 4µl PCR products were mixed with 2µl SYBR® Gold (Invitrogen ™) and loaded in 2% agarose gel in TAE buffer. As size standard 5µl Gene Ruler ™ 100 bp Plus DNA Ladder (Ferments, Life Science) was loaded onto the gel. Electrophoresis at 80 V for 42 min. For each microsatellite locus, size of the alleles was estimated by comparing with standard DNA Ladder (100 bp). Visualization was under a UV ray using gel documentation machine (E-BOX, VILBERLOURMAT, France). The PCR products were shipped for analyze on a Genetic Analyzer at LGC Forensics (Cologne, Germany).

2.2.6. Scoring of DNA fragments

PCR products were processed on a genetic analyzer at LGC Forensics (Cologne, Germany). Fragment size products among various individuals were specified relative to an internal standard DNA (Gene Scan 500 Rox), used to conceive and score the SSR fragment by Peak Scanner™ version.1.0 (Applied Biosystems). Scoring the highest peaks dropout irregular peaks and stutter for SSR's.

2.2.7. Molecular data statistical analysis

For statistical data analysis five programs were used: "GenAlEx", "POLYGENE", "DARwin" "PopART" and "STRUCTURE".

GenAlEx is an "add on" for Excel and contains a number of population genetic options, such as frequency-based (F-statistics, heterozygosity, population assignments) and distance-based analyzes (AMOVA, PCoA, Mantel test) (Peakall and Smouse 2012).

Polygene software is an A population simulator can be used to simulate population genetic drift and to generate the input data. The simulation parameters, including the ploidy levels, population sizes, locus numbers and the allele frequency at each locus. The input the phenotypic/genotypic data for all individuals in a population and at each locus. (Hung *et al*. 2020).

DARwin is a software package developed for diversity and phylogenetic analysis based on evolutionary dissimilarities. Standard methods for tree and factorial representation have been proposed, augmented by original and specific methods, particularly addressing the issue of sensitivity to data accuracy. Data that can be inherited from other sections or imported directly from other sources. Standard data formats are available as input and output (Perrier and Jacquemoud-Collet 2016).

STRCUTRE it contains a program structure that is a free software package for using multi-locus genotype data to examine population structure. Its uses include inferring the existence of distinct populations, assigning individuals to populations, studying hybrid zones, identifying migrants and admixed individuals, and estimating population allele frequencies in situations where many individuals are immigrant or admixed (Pritchard *et al.* 2000).

PopART (Population Analysis with Reticulate Trees) is population genetics software that was advanced as part of the Allan Wilson Centre Imaging Evolution Initiative. To develop the best software to understood evolutionary relation between populations (Leigh *et al*. 2015).

3.3.1.1. Genetic diversity and heterozygosity

To test for variation among loci, sited and across the species as a whole, allelic frequencies at each locus were calculated for each site and for the whole species in Germany using GenALEx version 6.503 (Peakall and Smouse 2012). The following genetic diversity parameters were calculated to determine the level of genetic variation within region including number of different alleles per locus (*Na*), number of unique alleles, effective number of alleles (*Ne*), percentage of polymorphic loci (*P*), average expected heterozygosity (*He*), average observed heterozygosity (*Ho*), sample-size weighed expected heterozygosity (*uHe*), Shannon's information index (*I*) (Shannon's 1948) and inbreeding coefficient (*Fis*) for each locus were computed for each *P. saxifraga,* regions based on the obtained allele frequencies.

3.3.1.2. Polymorphism Information Content (PIC).

Polymorphism information content (PIC) was evaluated polymorphism of the SSR marker can be a relative amount ranging from 0 to 1; the higher numbers, the greater variety of alleles. It is calculated using POLYGENE version.1.3. (Hung *et al*., 2020).

3.3.1.3. Population differentiation

Analysis of molecular variance (AMOVA) depends on analyses of gene frequencies to determine population genetic structure. Also possible partitioning of genetic differentiation among individuals, among populations, within individuals, and within-population, can be detected by using Nei's genetic distance GenAlEx 6.503 (Peakall and Smouse, 2012). The comparison between samples in pairs, the analysis of variance allows the genetic variations to be partitioned, to calculate F-statistics which is useful to analyse inbreeding coefficient within individuals F_{IS}. F_{IT} is the inbreeding coefficient within individuals relative to the total and F_{ST} reflects genetic differentiation (Wright, 1978). The calculated F_{IS}, F_{IT}, and F_{ST} values are always in the range from 0 to 1, with 0 showing no genetic differences between individuals or no different haplotypes and 1 showing the highest degree of differentiation (Wright 1978). The significance

level of 0.05 was determined using 1000 permutations. Hardy–Weinberg equilibrium (HWE) were calculated using GenAlEx version 6.503.

3.3.1.4. Principle coordinate analysis (PCoA)

Principal Coordinates Analysis was used to determine the percentage of variations between previously determined groups, and genetic region relationships between the genotypes. For this purpose, all samples were divided into four biogeographical regions (Table 2.1). The resulting allelic matrices of the SSR methods, served as a basis. PCoA was based on standardized covariance of computed genetic distances for codominant markers. Nei´s distance (Nei, 1972) matrices between all pairwise individuals were computed using GenAlEx software version 6.503 was performed with 999 permutation.(Peakall and Smouse, 2012).

3.3.1.5. Mantel test

Mantel test is the most commonly utilized method to estimate the correlation between geographic distance (GGD) matrix and genetic distance (GD) among individuals of species, here *P. saxifraga* (Mantel, 1967). Mantel test is implemented in GenAlEx version 6.503. (Peakall & Smouse, 2012). and was performed with 1000 permutations.

3.3.1.6. Individuals and regions clustering

To explore population genetic structure the Bayesian clustering analysis approach was performed in STRUCTURE v.2.3.3 (Pritchard *et al.*, 2000). We explored individuals' genetic affiliation to genetic clusters on the basis of the genotype without a previously specified assignment to regions or population by applying the admixture model, with 100 000 Markov chain Monte Carlo (MCMC) replicates, with a burn-in period of 50 000 and 10 repeats per run for each chosen cluster number (i.e. K = 1–20), Ploidy = 2, and Recessive alleles = 0. For all other settings the default options were used. The delta K (Evanno *et al.*, 2005) was employed to select the most appropriate K value and maximum number of genetic cluster by using STRUCTURE HARVESTER (Earl and vonholdt, 2012). To verify the most probable cluster membership coefficient among the 10 runs of STRUCTURE and STRUCTURE HARVESTER we used CLUMPP v.1.1.2. (Jakobsson and Rosenberg, 2007). Corresponding graphs were constructed with DISTRUCT (Rosenberg, 2004).

An unweighted pair group method with arithmetic mean (UPGMA) based on genetic dissimilarity matrix with 1000 bootstrap. A neighbor-joining (NJ) growing tree and hierarchical clustering method was assessed from cluster analysis with 1000 bootstrap replication using DARwin version 6 (Perrier and Jacquemoud-Collet, 2006) on the depend of Nei genetic distance, which was used to estimate the genetic connection among individuals.

3.3.1.7. Relatedness Analyses

To estimate the pattern of relatedness within and among regions we calculated the average pairwise relatedness in GenAlEx version 6.503 (Peakall and Smouse, 2012). First, individual pairwise relatedness (r) was calculated using the Queller and Goodnight (1989) estimator. Then, pairwise values were averaged using "Pops mean" analysis of the individual pairwise outcomes. Significance was examined using 999 permutations and 1000 bootstrap re samplings to estimate 95% confidence intervals. the mean within pairwise values of the region was compared with the lower and upper confidence limits estimated from the null hypothesis of no difference between regions.

2.3. Morphological analysis

The study used morphology of herbarium individuals, 72 individuals were assessed (Table 2.1), those were collected from eight herbaria (Figure 2.10). On each individual 5 quantitative and 6 qualitative traits (Table 2.4) were examined. These morphological traits the following floras included all major species were used Flora European (Tutin *et.al.*, 1968), Flora von Mitteleuropa (Hegi, 1965), Flora of Great Britain and Ireland (Sell & Murrell, 2009), Flora of Turkey (Davis, 1972) and online atlas of the British and Irish flora .

Table 2.4: Morphological traits of *Pimpinella saxifraga* for morphometric analysis.

Number	Traits	Types
1	Shape of leaflet	Qualitative
2	Tooth leaflet	Qualitative
3	Margin leaflets	Qualitative
4	Hair leaflets	Qualitative
5	Terminal leaves	Qualitative
6	Stem hair	Qualitative
7	Number of leaflets in basal leaf	Quantitative
8	Plants length	Quantitative
9	Rays number	Quantitative
10	Pedicals number	Quantitative
11	Basal leaf length	Quantitative

2.3.1. Quantitative and qualitative Data analysis

The median data of four quantitative traits of each individual were used for data analysis of variance ANOVA and Kruskal-Wallis tests. The determination of the variation of parameters between morphological traits was performed with the aid of the program “Origin” (OriginPro 2020, OriginLab Corporation, Northampton, MA, USA). The Mann-Whitney U test was used to compare the two groups, such as e.g. plant length versus plant shape. The results were considered statistically significant when the *P*-value was smaller than 0.05. Data were expressed as median and range. Data were analyzed using non-parametric correlation (Spearman's test). Correlations between morphological traits were also performed with Origin (OriginPro 2020, Origin Lab Corporation, Northampton, MA, USA). The significance of correlation (r) between morphological traits were computed by using a t-statistic test.

Six qualitative morphological traits were used to get a binary code as 1 for presence 0 for absence in each individual (e.g., shape leaflet: presence (1) or absence (0); margin leaflet presence (1) or absence (0)). To build Neighbor-joining (NJ) trees using quantitative and qualitative traits using the program DARwin 6.0 software (Perrier and Jacquemoud-Collet, 2006). Bootstrapping with 1000 replicates was also performed with DARwin. Relationship among the morphological traits were analyzed by Factorial analysis also using DARwin 6.0 software (Perrier and Jacquemoud-Collet, 2006). Principal Coordinates Analysis (PCoA) was used by GenAlEx version 6.503. (Peakall and Smouse, 2012) to project on the axis the analyzed individuals. Principal Coordinate Analyses of the morphological data set were used to test morphologically distinct *Pimpinella saxifraga*. PCoA is an coordination method derived from Principal Component Analysis (PCA; Gower 1966). Correspondences are techniques that enable the examination of the relationships of multidimensional data points along two or three axes that explain most of the differences between these data . PCoA is one of the most common methods for exploring genetic and morphometric distances (such as. Dufresne *et al.,* 2014 and Shepherd *et al*., 2015) Also, we estimated networks for morphological trait by computing absolute pairwise distances with Median Joining Network (Bandelt *et al*., 1999) implemented in PopART v. 1.7 (Population Analysis with Reticulate Trees) (Leigh *et al.,* 2015).

CHAPTER III

RESULTS

3. Result

3.1. Development and validation of identified SSR markers

3.1.1. Amplification, polymorphism and transferability of SSR markers

Out of the 44 microsatellite primer pairs (forward and reverse) which were selected and used to amplify on *P. saxifraga* DNA by PCR, only nine (20.5%) of generated an amplification product in a small pre-study (Table 2.2) .That produced clear obvious bands of the expected size and many polymorphisms. Eleven displayed a multiband profile pointing to a generally low rate of transferability. Primer size ranged from 20 to 25bp, annealing temperature for nine nuclear microsatellite primers varied from 50°C to 58°C and GC content of primers between 34% and 52% (Table 2.2). Therefore, these primer pairs were chosen for subsequent analyses. SSR markers for which the primers displayed positive transferability were assorted 33% i.e. 3 SSRs were compound while 67% i.e. six SSRs were simple in nature (Figure 3.1).

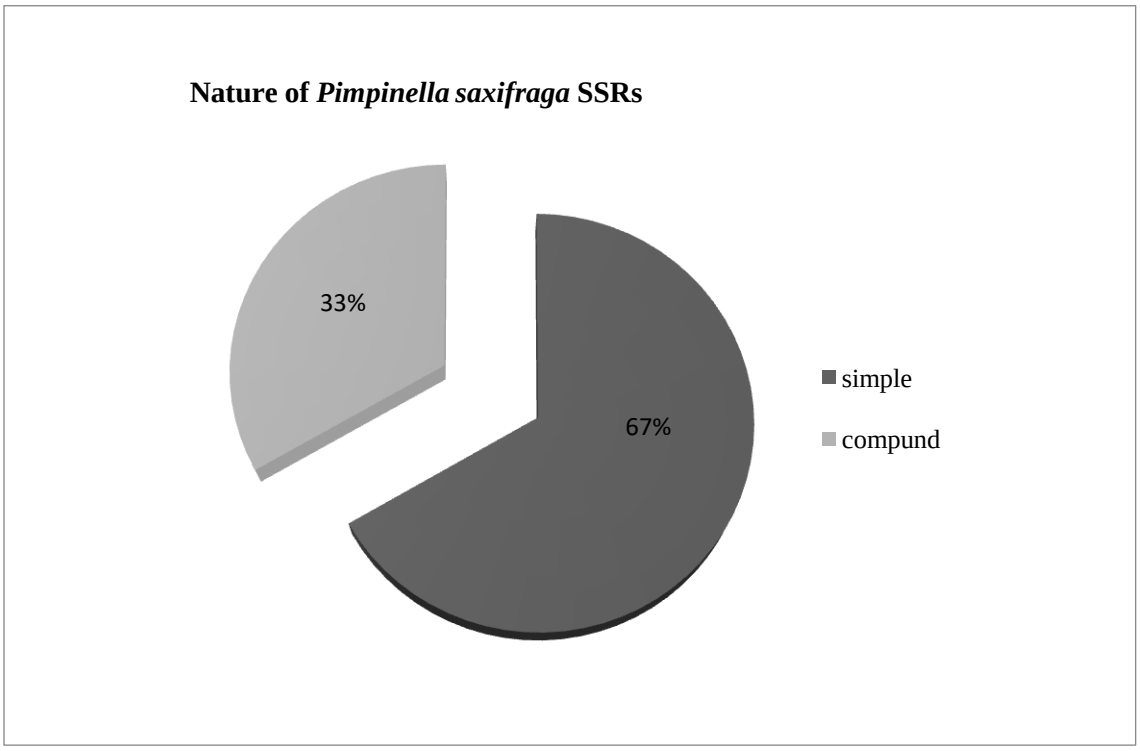


Figure 3.1: Nature of *Pimpinella saxifraga* SSR motifs derived.

The motifs for the nine SSR markers included dinucleoride (3, 33.34%) and compound (3, 33.34%), followed by tetranucleotide (2 , 22%), and trinucleotide (1, 11%) (Figure 3.2).

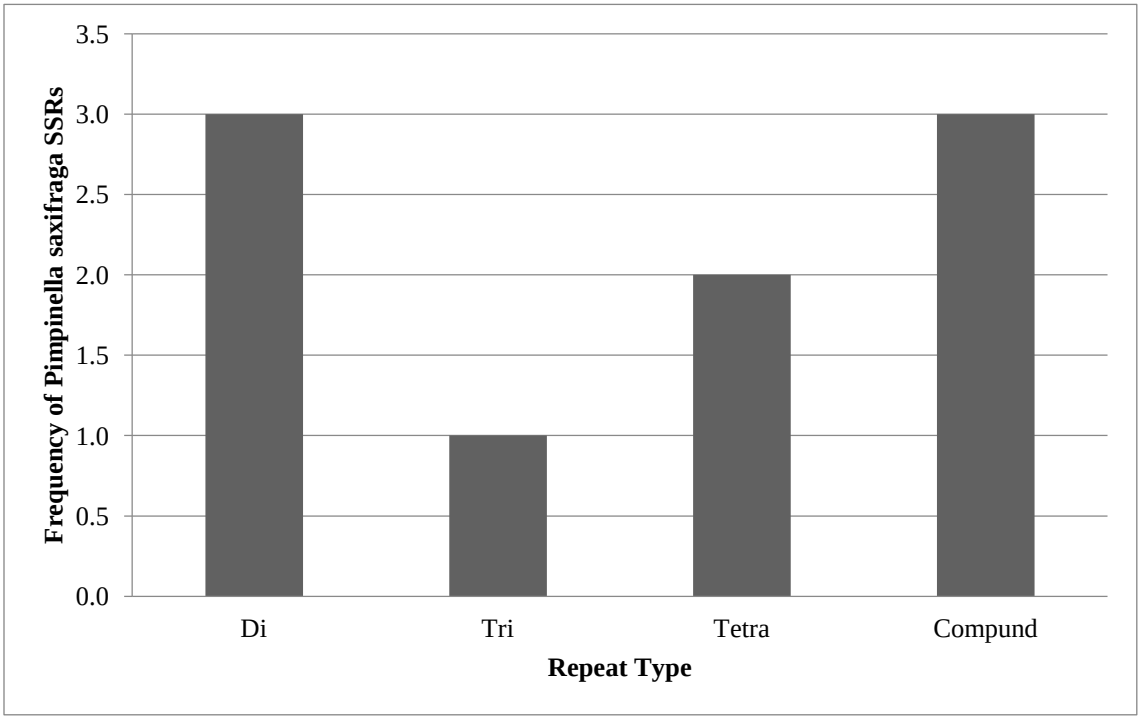


Figure 3.2: Frequencies of *Pimpinella saxifraga* SSR repeat types.

Our first step was to estimate the activity of SSR amplification by separating DNA fragments in a 2% agarose gel (Figure 3.3). Inclusively, we obtained good amplification of all *P. saxifraga* genomic DNA with all simple sequence repeats primers, and the extent of the band sizes matched within the required base pair size range compared to the 100bp DNA ladder to the extent obtained in former studies. The size of the amplified fragments ranged from 151 to 324 bp (Table 2.2).

The (PIC) value of each marker, which could be assessed on the basis of its alleles, ranged from 0.25 in GSSR154 to 0.85 in GSSR97 across all markers with a mean of 0.57 (Table 3.1, Figure 3.4). In accordance, the most polymorphic markers (six of nine markers) showed high levels of informativeness (PIC > 0.50). This means, that these markers can effectively measure genetic diversity in *P. saxifraga*, whereas the other three markers (GSSR154, BSSR76, and BSSR53) show moderate informativeness (0.50 > PIC > 0.25).

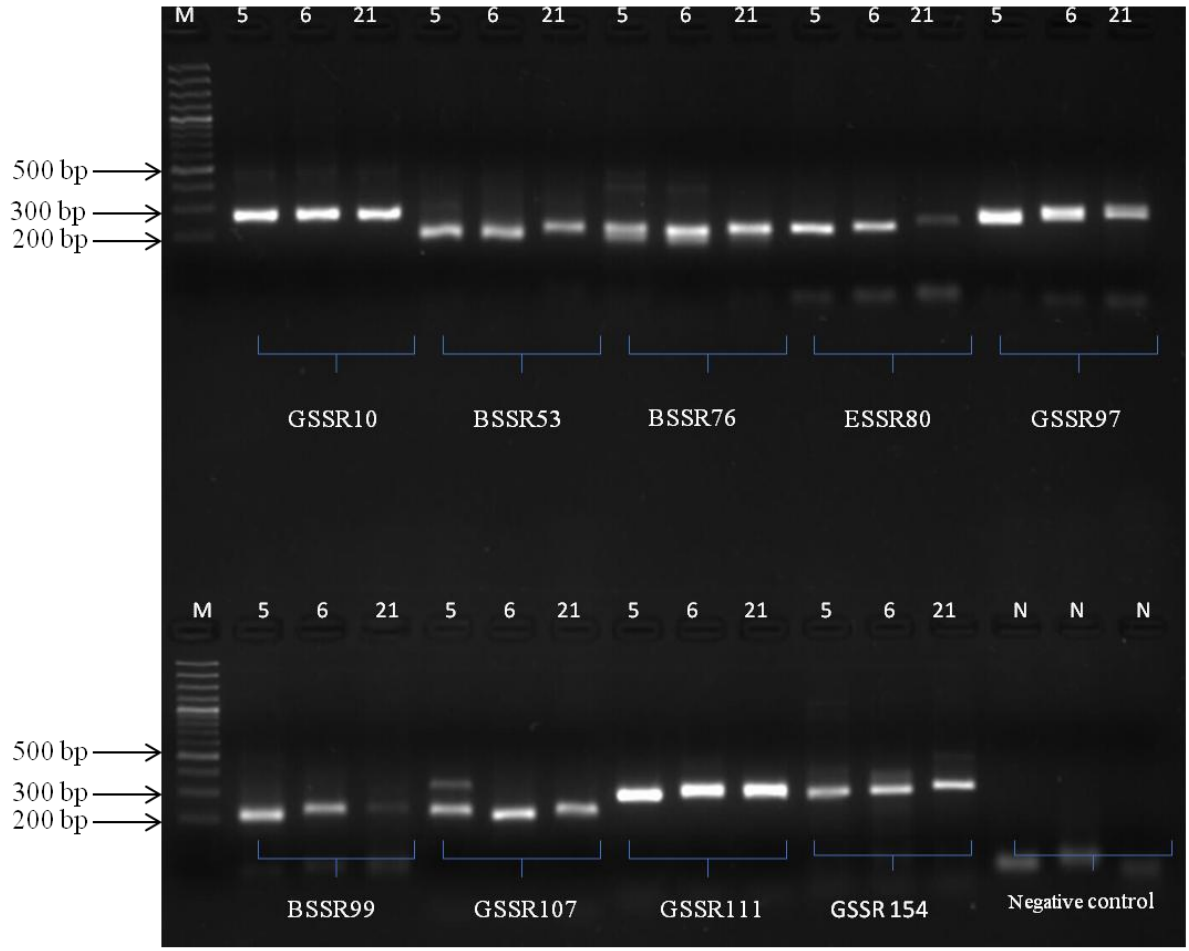


Figure 3.3: Representative PCR amplification profile products of three genotypes using GSSR10, BSSR53, BSSR76, ESSR80, GSSR97, BSSR99, GSSR107, GSSR111 and GSSR154 the markers amplified different size of alleles ranged from 150 bp to 350 bp across *Pimpinella saxifraga* genotypes. Lane M 100bp DNA ladder, Lane 2 - 21 per markers three individuals of *Pimpinella. saxifraga*.

All nine primers pairs were polymorphic among 78 *P. saxifraga* samples, taken from different herbaria of Germany. The here analyzed SSR markers produced 112 alleles. The highest number of alleles belonged to GSSR97 primer pairs with 26 polymorphic alleles by different sizes, whilst BSSR76 primer pairs had six alleles (Figure 3.5). Six of the nine markers (GSSR111, GSSR97, GSSR10, BSSR99, BSSR76, and ESSR80) showed highly significant deviation from Hardy-Weinberg equilibrium (HWE) over the entire biogeographic region. However, the other three marker (GSSR107, GSSR154, and BSSR53) featured no significant deviation from HWE.

Table 3.1: Microsatellite markers characteristics for 78 *Pimpinella saxifraga* samples. PIC: Polymorphic Information value, *P* HWE: loci significantly deviating from Hardy Weinberg equilibrium (HWE).

SSR marker	Expected allele size (bp)	Minimum allele size (bp)	Maximum allele size (bp)	Allele number	PIC	P_{HWE}
GSSR10	255	237	253	9	0.74	0.000***
GSSR97	265	194	269	26	0.85	0.000***
GSSR107	265	151	251	15	0.55	0.407^{ns}
GSSR111	355	224	324	12	0.62	0.000***
GSSR154	328	234	297	9	0.25	1.000^{ns}
BSSR53	190	170	199	10	0.47	0.971ns
BSSR76	209	173	205	6	0.43	0.000***
BSSR99	204	160	216	16	0.81	0.261ns
ESSR80	235	177	198	9	0.62	0.000***
Mean				12.4	0.57	

HWE correlation coefficient, *** highly significant, *P*<0.001 ; ns=not significant.

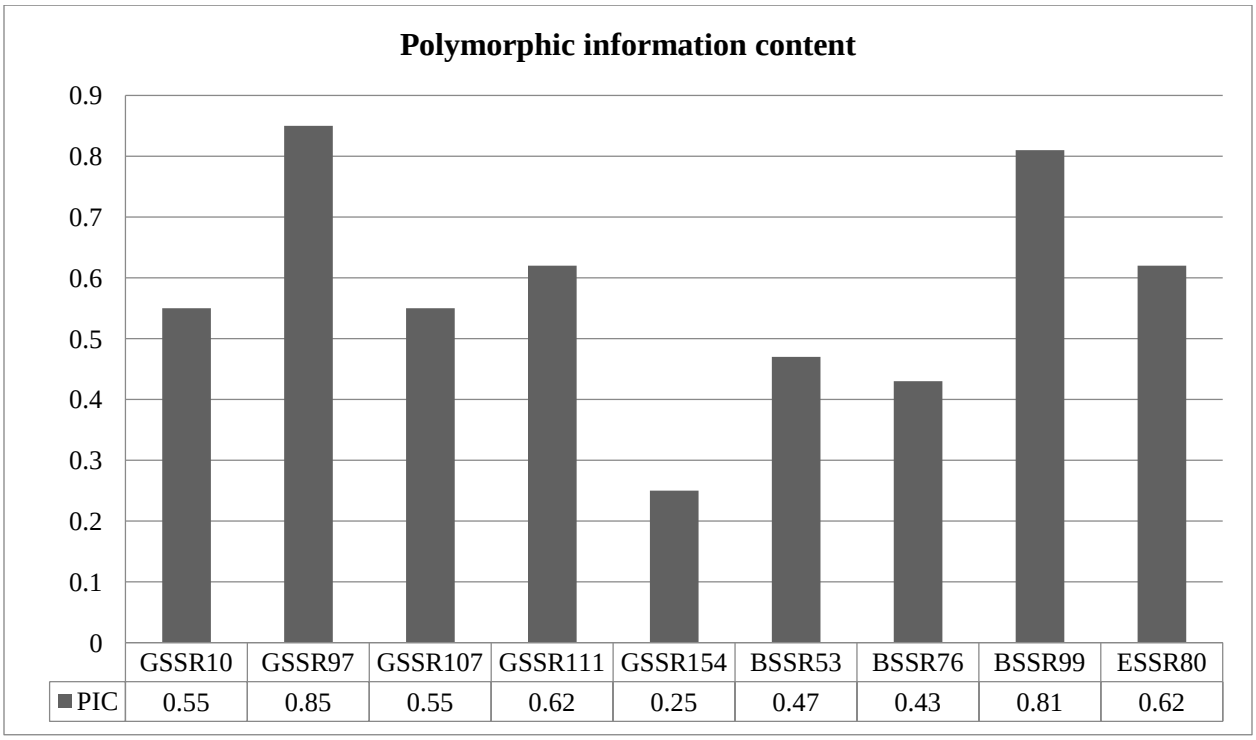


Figure 3.4: Polymorphic information content (PIC) values for nine microsatellite, markers (GSSR10, GSSR97, GSSR107, GSSR111, GSSR154, BSSR53, BSSR76, BSSR99, ESSR80).

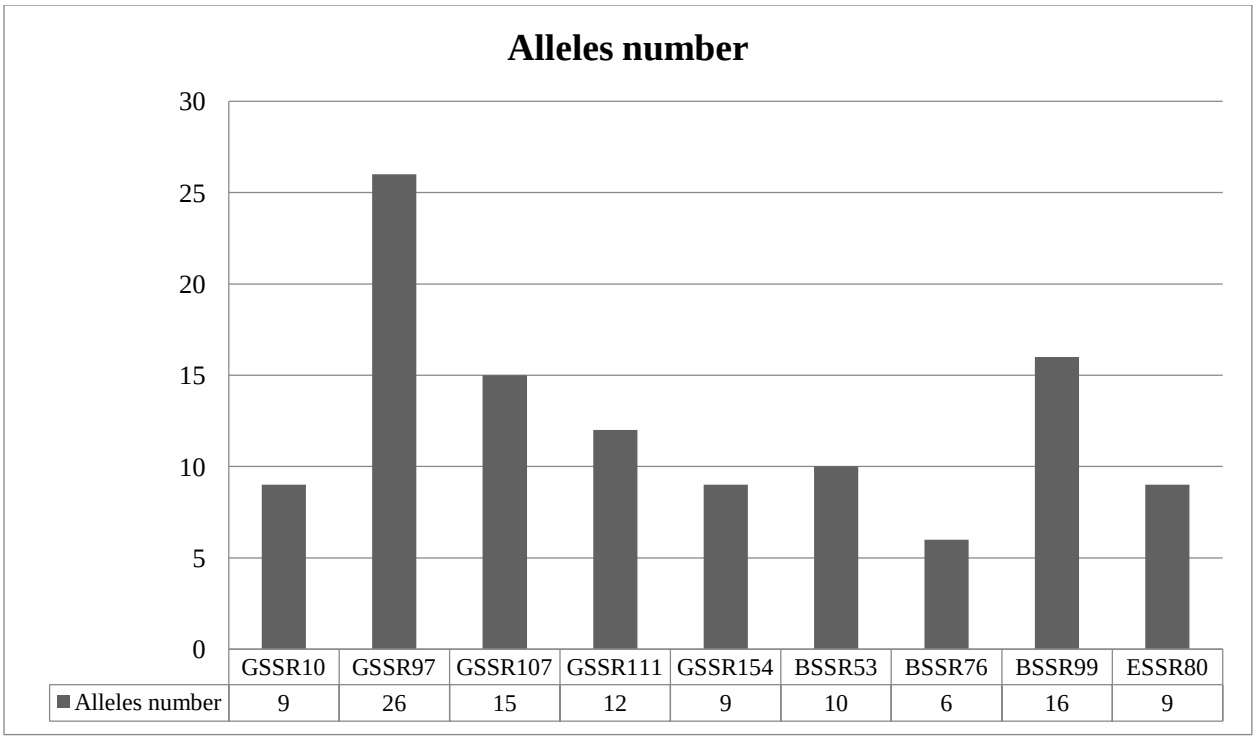


Figure 3.5: Number of alleles for the nine microsatellite markers (GSSR10, GSSR97, GSSR107, GSSR111, GSSR154,BSSR53, BSSR76, BSSR99, ESSR80).

3.1.2. Allelic diversity analysis by SSR markers

Within the 78 screened *P. saxifraga* individuals and by using nine microsatellite markers a total of 112 different alleles were found. The observed heterozygosity (*Ho*) ranged from 0.31 for GSSR154 to 0.95 for BSSR76 with an average of 0.74 (Figure 3.6). Whereas the expected heterozygosity (*He*) varied from 0.27 for GSSR154 to 0.82 for GSSR97 and BSSR99, with the overall average was 0.63 (Figure 3.7). Shannon's information index (*I*) varied between 0.54 (GSSR154) and 2.11 (GSSR97) with an average of 1.25 (Figure 3.8). The average number of alleles per marker (*Na*) was 6.33, range from 3.00 for BSSR76 to 13.25 for GSSR97 (Figure 3.9). However, the number of effective alleles per markers (*Ne*) range from 1.38 for GSSR154 to 6.18 for GSSR97 with an average of 3.2 per marker (Figure 3.10). The fixation index (F_{is}) vary from -0.728 for BSSR76 to 0.303 for GSSR97 (Figure 3.11).

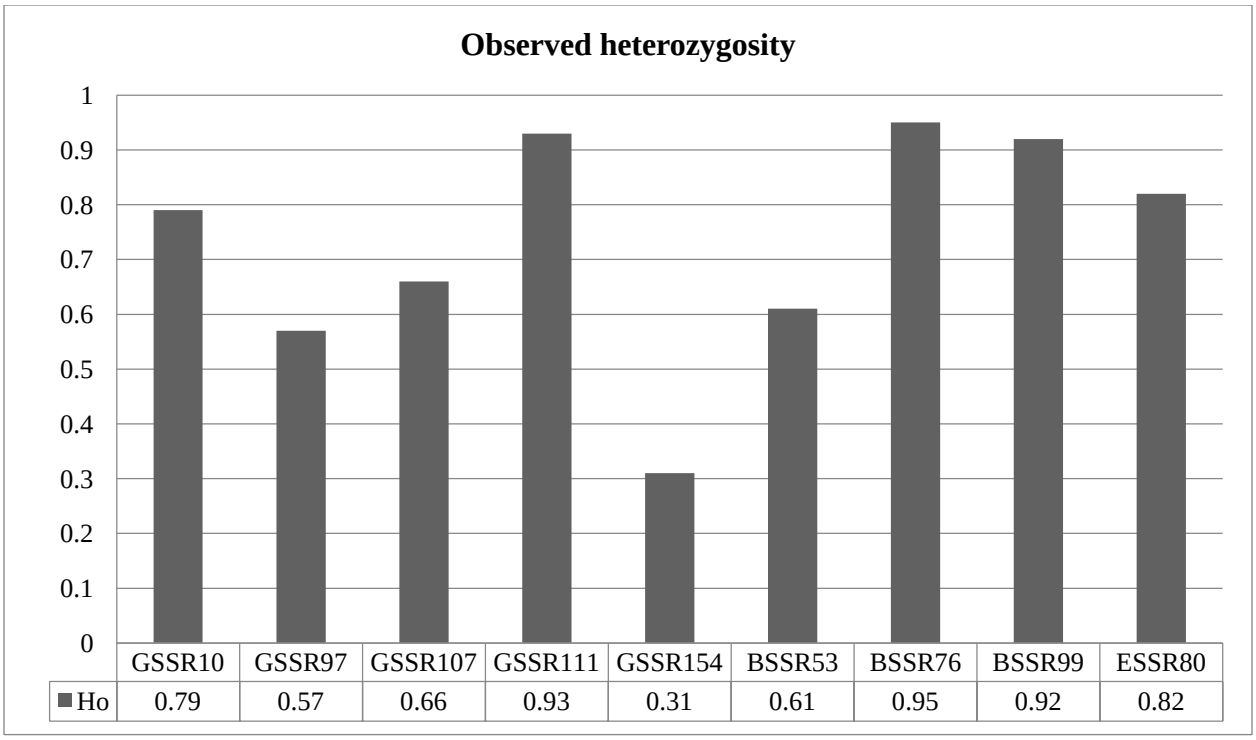


Figure 3.6: Statistical analysis of observed heterozygosity (Ho) among SSR Markers based on 78 individuals of *Pimpinella saxifraga*.

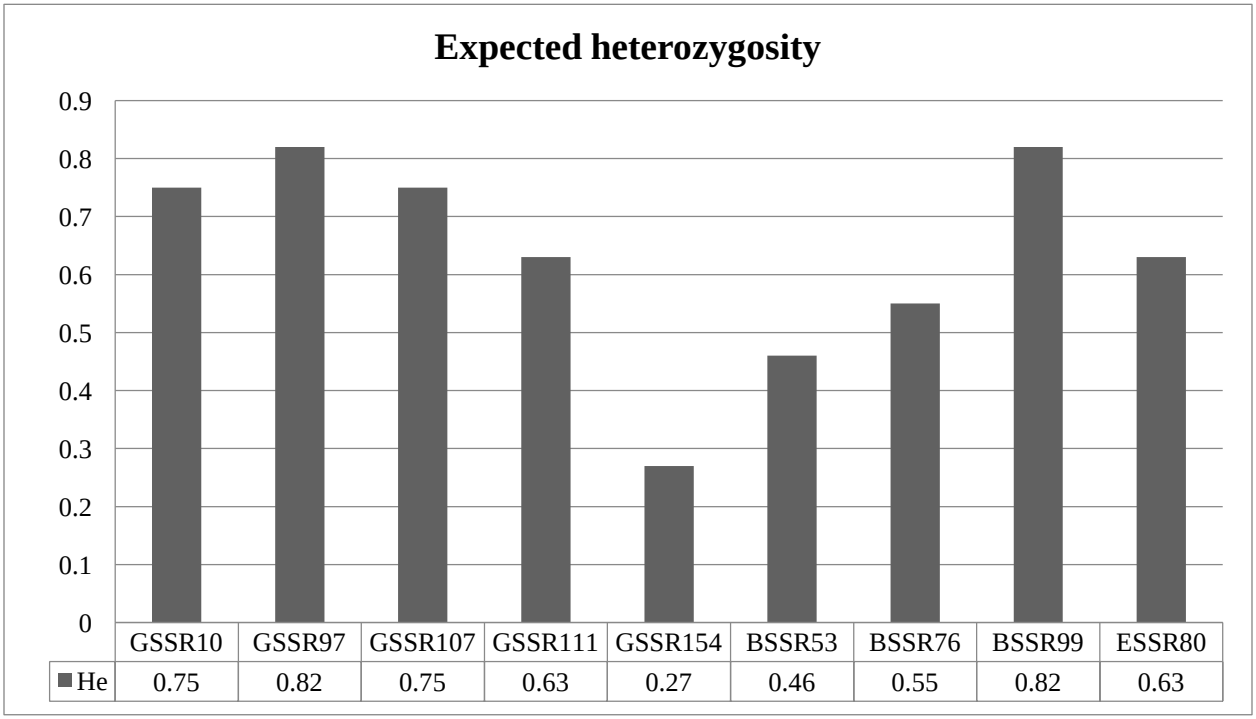


Figure 3.7: Statistical analysis of expected heterozygosity (He) among SSR Markers based on 78 individuals of *Pimpinella saxifraga*

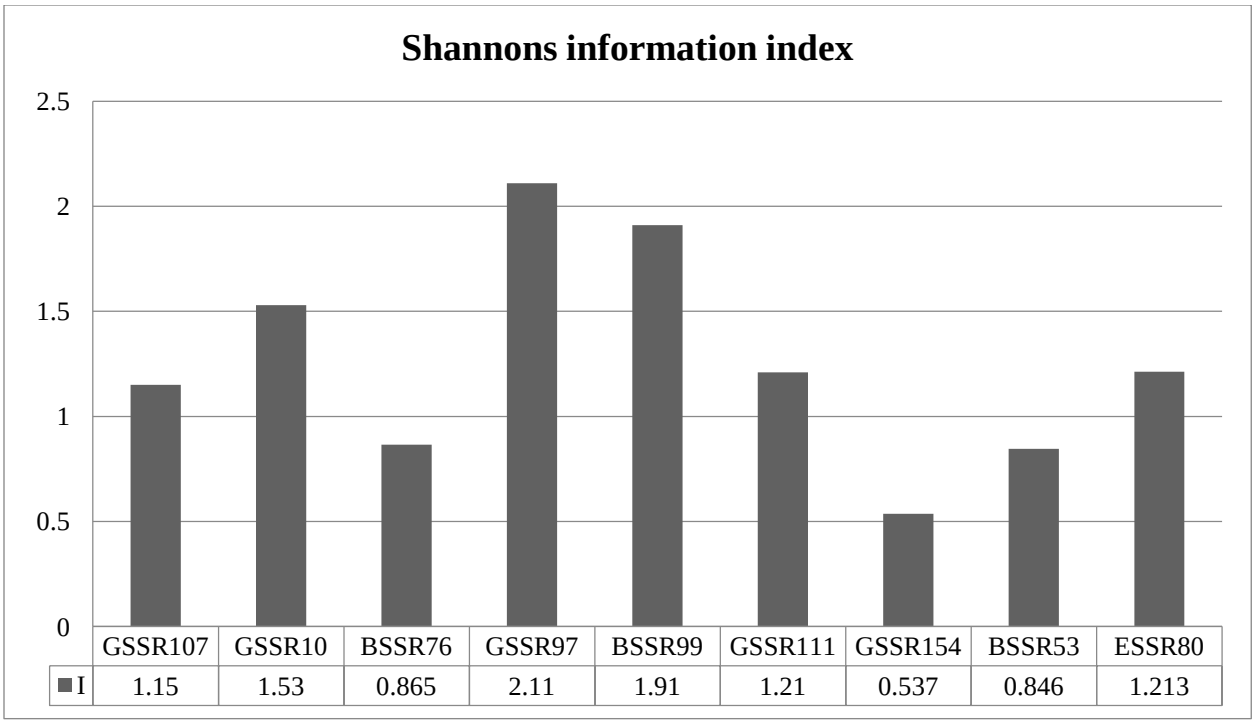


Figure 3.8: Statistical analysis of Shannon's information index (I) among SSR markers based on 78 individuals of *Pimpinella saxifraga*.

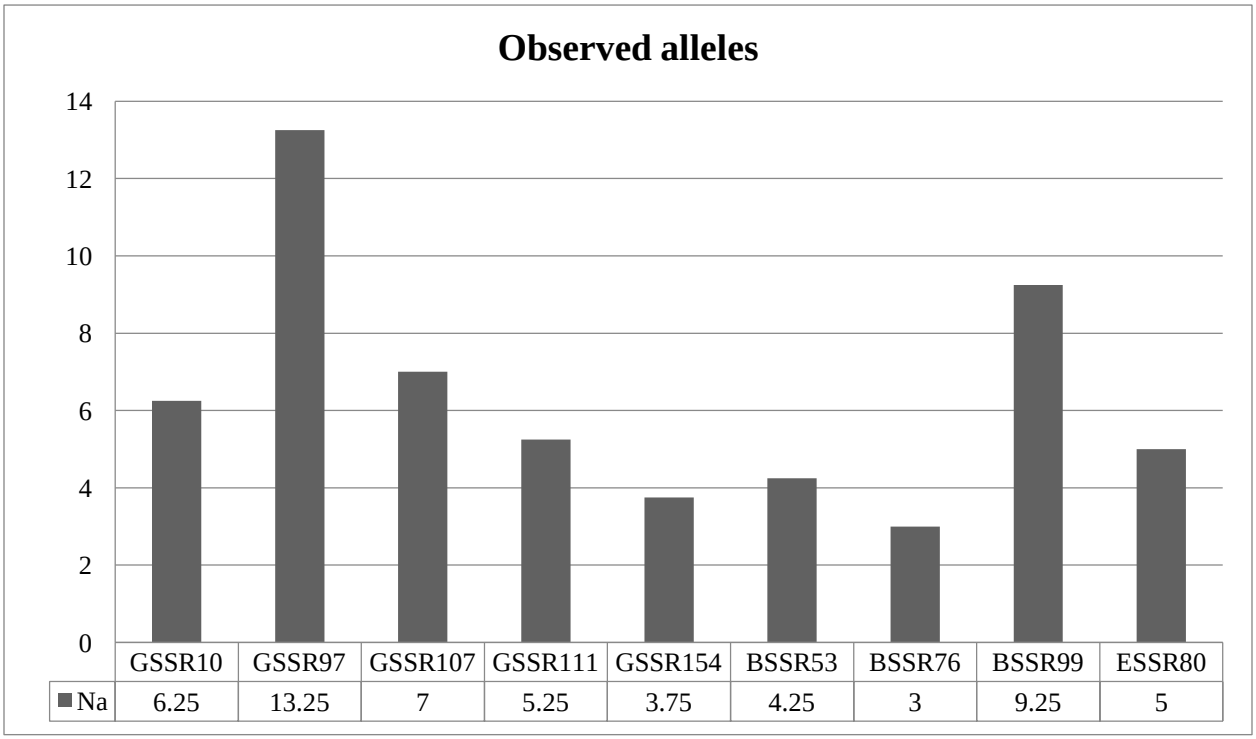


Figure 3.9: Statistical analysis number of observed alleles (Na) among SSR Markers based on 78 individuals of *Pimpinella saxifraga.*

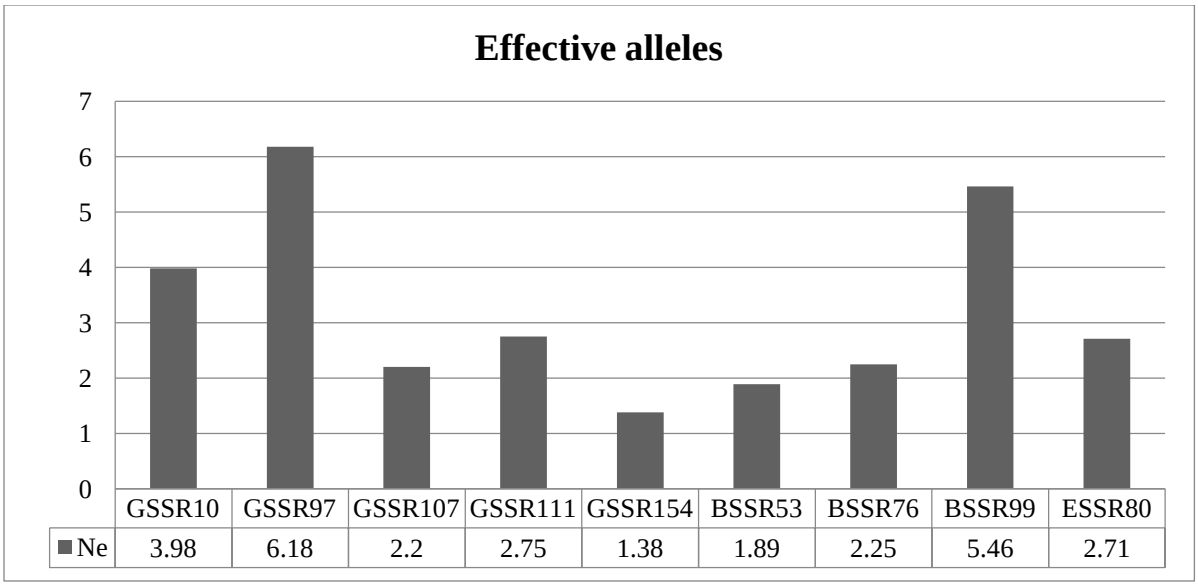


Figure 3.10: Statistical analysis number of effected alleles (Ne) among SSR Markers based on 78 individuals of *Pimpinella saxifraga*.

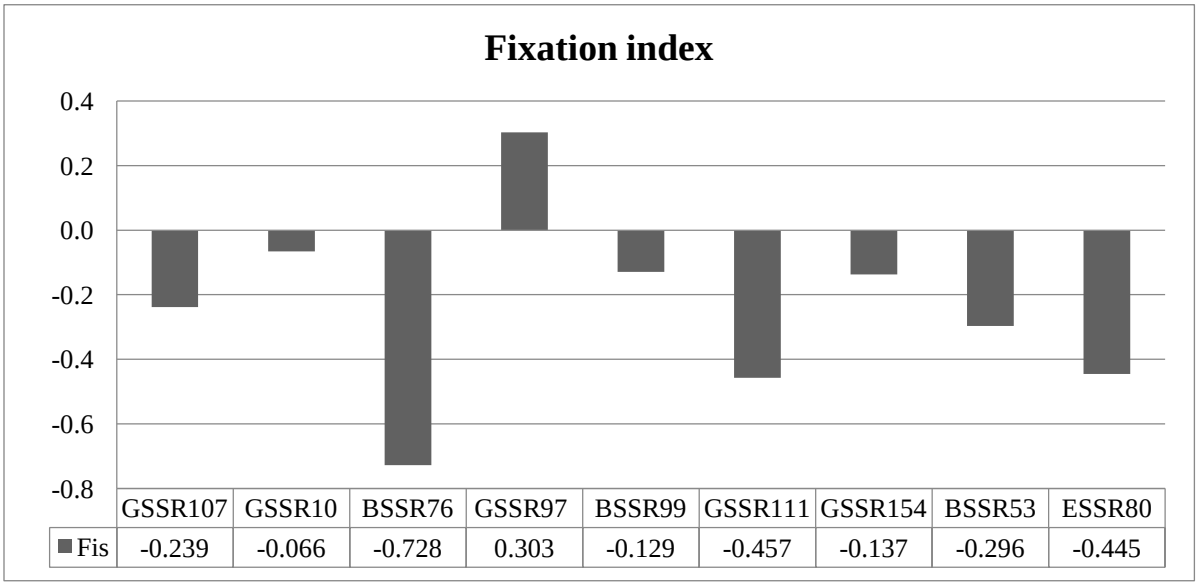


Figure 3.11: Fixation index (F_{is}) among the different SSR markers based on 78 *Pimpinella saxifraga* individuals.

For the nine SSR markers, an exploratory Analysis of Molecular Variance (AMOVA) confirmed microsatellite variation between sequences of four biogeographical regions based on genotype of *P. saxifraga*. We found the highest percentage of variation was found within the regions (98%), while low genetic differentiation among regions (2%). The significant according to the SSR markers only two markers (BSSR53 and BSSR76) are showing significant genetic differentiation among the regions with 8% (Table 3.6).

Table 3.2: AMOVA results for nine microsatellite markers in *Pimpinella saxifraga*.

Locus	Source	Df	SS	MS	Est.Var	%	*PhiPT*value	*P*<
GSSR10	Among Pop. Within Pop	3 74	2.626 55.515	0.875 0.750	0.007 0.750	1% 99%	0.010	0.283
GSSR97	Among Pop. Within Pop	3 74	3.831 84.875	1.277 1.147	0.008 1.147	1% 99%	0.007	0.298
GSSR107	Among Pop. Within Pop	3 74	1.718 34.067	0.573 0.460	0.007 0.466	1% 99%	0.014	0.204
GSSR111	Among Pop. Within Pop	3 74	1.270 28.409	0.423 0.384	0.002 0.384	1% 99%	0.006	0.333
GSSR154	Among Pop. Within Pop	3 74	0.294 18.117	0.098 0.245	0.000 0.254	0% 100%	-0.36	0.895
BSSR53	Among Pop. Within Pop	3 74	2.644 26.049	0.881 0.352	0.031 0.352	8% 92%	0.080	**0.027***
BSSR76	Among Pop. Within Pop	3 74	0.605 6.215	0.202 0.084	0.007 0.084	8% 92%	0.075	**0.024***
BSSR99	Among Pop. Within Pop	3 74	2.650 53.953	0.883 0.729	0.009 0.729	1% 99%	0.012	0.261
ESSR80	Among Pop. Within Pop	3 74	1.575 27.014	0.525 0.365	0.009 0.365	2% 98%	0.025	0.151
Total	Among Pop. Within Pop	3 74	18.687 341.210	6.229 4.611	0.094 4.611	2% 98%	0.020	0.032

Df : degrees of freedom ; SS : sum of sequence ; MS : mean squares ; Est. var : estimate of varaince
**P*- value significant P<0.05

3.1.3. Allele frequency of SSR markers

The highest rate of common allele frequency was recorded in GSSR154 marker with 0.846 while the lowest in BSSR99 marker (0.25) (Figure 3.12).

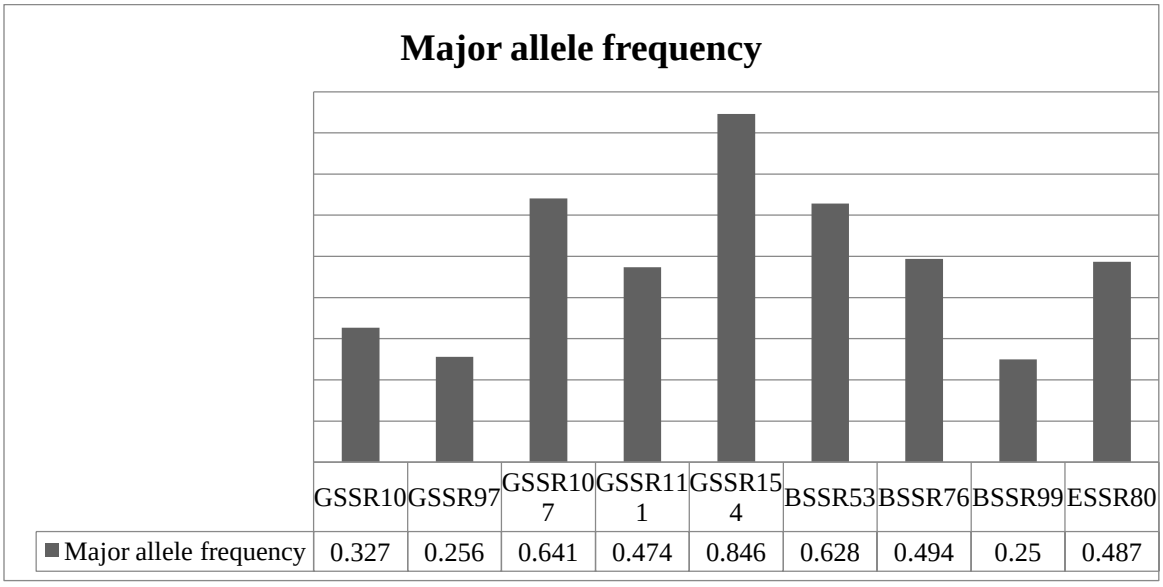
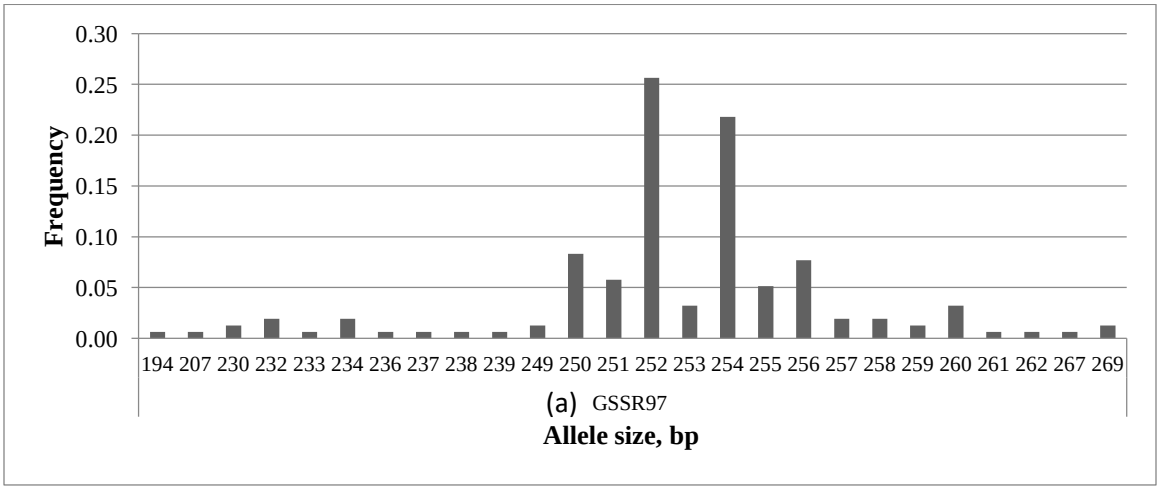
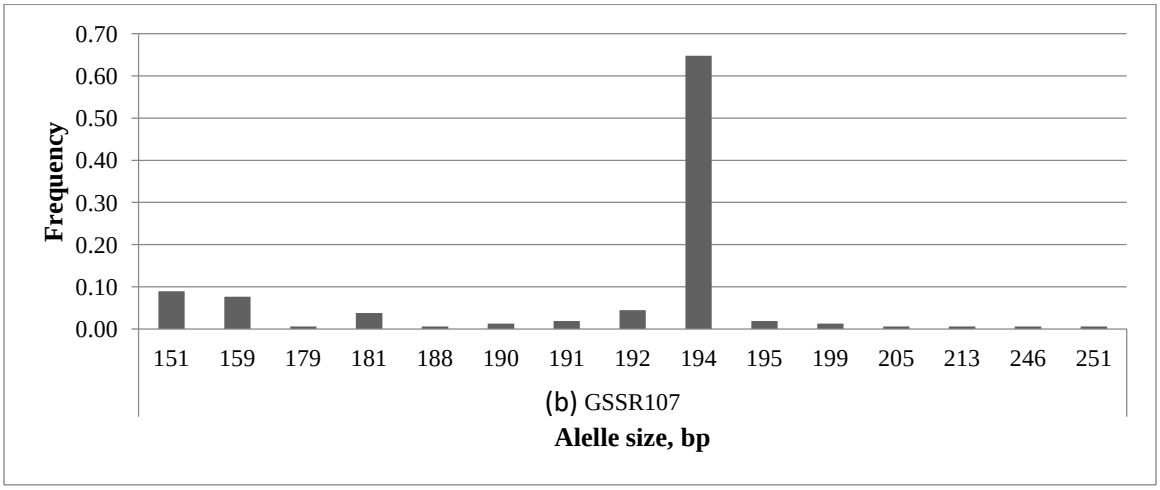
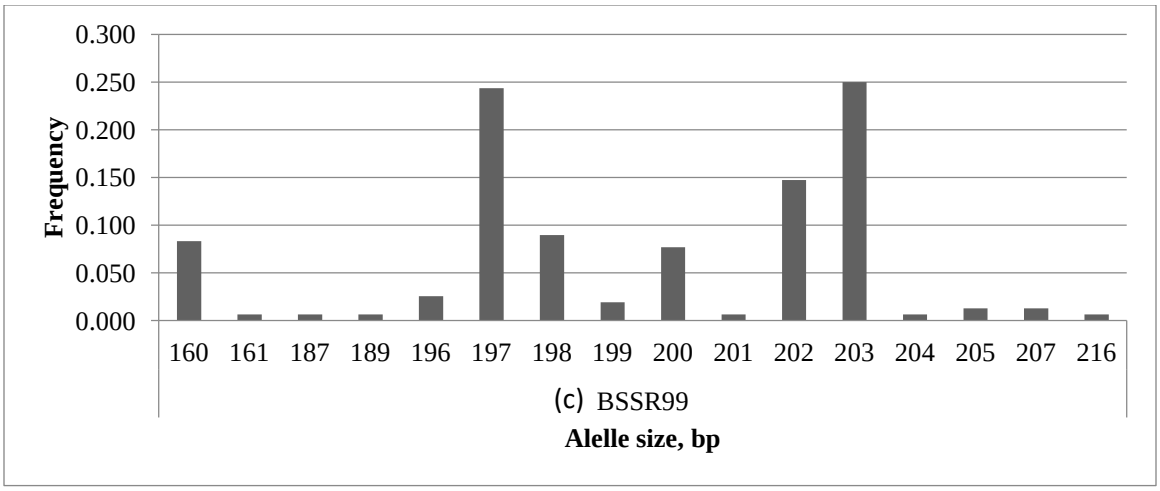


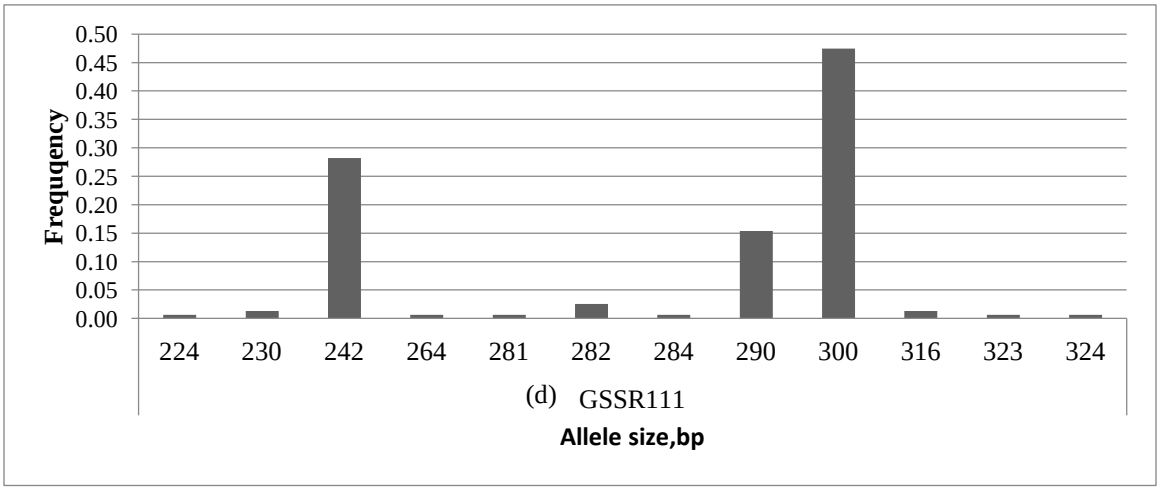
Figure 3.12: Major allele frequency of polymorphic SSR Markers in 78 individuals of *Pimpinella saxifraga.*from Germany

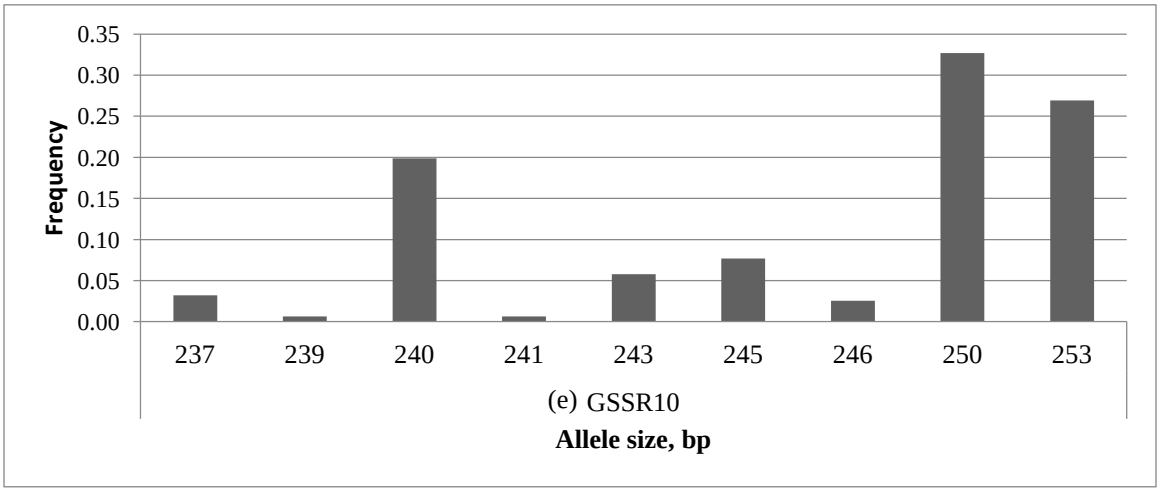
The frequencies of identified alleles in *P. saxifraga* from nine microsatellite are presented for each marker ranged from 0.006.to 0.846 (Figure 3.13(a-i)). The BSSR 53 (179 bp – 185 bp), BSSR76 (173 bp – 196 bp) and ESSR80 (196 bp – 197 bp) markers each had two alleles with high frequencies. The GSSR107 and GSSR154 markers each had one allele with higher frequency than other alleles (194 bp and 269 bp, respectively). The other markers GSSR111 (242bp – 300bp), GSSR97 (252bp – 254bp), GSSR10 (250bp -253bp) and BSSR99 (197bp-203bp), each had a minimum of two alleles with high frequency.






0.300
0.250
0.200
0.150
0.100
0.050
0.000
Frequency
160 161 187 189 196 197 198 199 200 201 202 203 204 205 207 216
(c) BSSR99
Alelle size, bp


0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frequqency
224 230 242 264 281 282 284 290 300 316 323 324
(d) GSSR111
Allele size,bp


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Frequency
237 239 240 241 243 245 246 250 253
(e) GSSR10
Allele size, bp

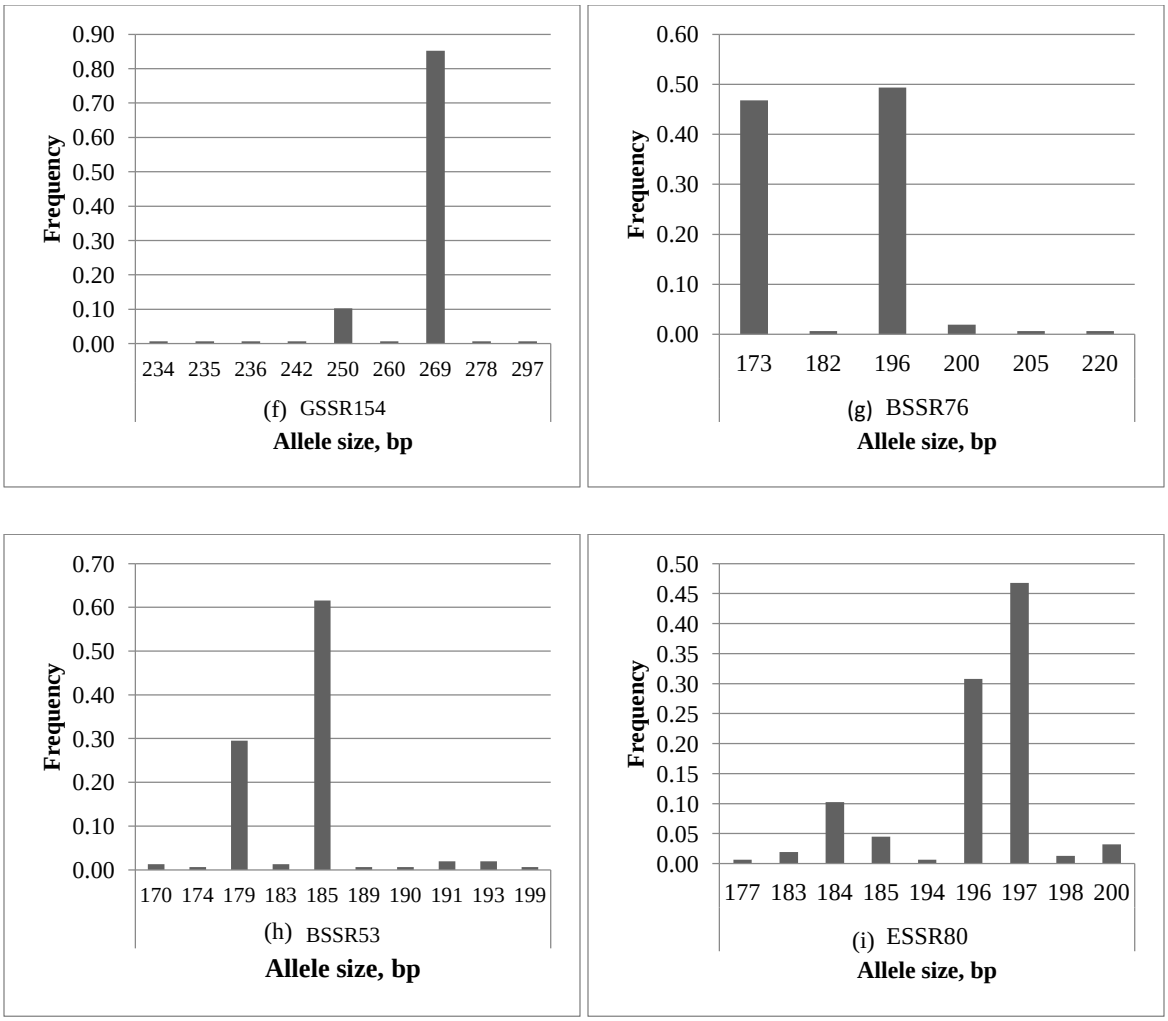


Figure 3.13: Allele frequency distributions of nine SSR markers.(a) GSSR97, (b) GSSR107, (c) BSSR99, (d) GSSR111, (e) GSSR10, (f) GSSR154 (g) BSSR76 (h) BSSR53, and (i) ESSR80. In graph, vertical axis allele frequency horizontal axis allele size (bp) for microsatellite marker.

3.2. Population genetic results

3.2.1. Population genetic diversity analysis

In the present study, a total of 78 individuals from four different geographical regions in Germany (Europe) was screened using nine SSR microsatellite-markers which resulted in 112 alleles. The allele sizes varied between 151–323 base pairs and all markers were 100% polymorphic. At region level, the genetic diversity indices (in terms N_a, N_e, I, H_e, H_o, F) varied across regions of 78 *P. saxifraga* individuals. The mean value of observed alleles (*Na*) and effective alleles (*Ne*) were 6.30 and 3.20 respectively, the southern region recorded the lowest

value of (*Na*) 4.89 and (*Ne*) 2.95. Similarly, the highest values of (*Na*) 9.22 and (*Ne*) 3.48 were recorded from the central upland.

The observed heterozygosity (*Ho*) ranged from 0.71 (alpine & alps) to 0.79 (central upland) and expected heterozygosity (*He*) ranged from 0.58 (south) to 0.65 (central upland) with an average of 0.74 and 0.61 respectively. For the four biogeographical regions, the *He* values were less than the *Ho* values, suggesting an excess of heterozygotes.The unbiased expected heterozygosity ranged from 0.60 (North lowland) to 0.65 (central upland and alpine alps) with an average of 0.63. The values of the fixation index (*F*) of each regions were negative ranged from -0.27 (north lowland and south) to -0.21 (alpine alps) with an average of -0.25 at the regional level revealed that four regions (north, central, south, and alpine & alps) are negative values presented an excess of heterozygotes, indicating out breeding. The private alleles (*Pa*) ranged from 5 (alpine & alps) to 38 (central upland) with an average of 12.5. In the central upland with the highest samples size the greatest genetic diversity was found, while the southern and alpine & alps region with the lowest samples size showed the smallest genetic diversity (Figure 3.14).

The polymorphic information content (*PIC*) value ranged from 0.54 in North lowland to 0.61 in Central upland with mean value of 0.57. The percentage of polymorphic loci in all regions was 100% (Table 3.3).

Table 3.3: Genetic diversity indices for *Pimpinella saxifraga* from different geographical regions based on nine microsatellite markers computed from GenAlEx (Peakall and Smouse 2012).

Regions	N	P(%)	Na	Ne	Pa	I	No	He	Ho	PIC	F
North lowland	20	100%	6.23	3.21	10	1.21	1.00	0.58	0.72	0.54	-0.27
Central upland	38	100%	9.22	3.48	38	1.41	3.67	0.65	0.79	0.61	-0.26
South	12	100%	4.89	2.95	7	1.17	0.78	0.59	0.74	0.55	-0.27
Alpine & Alps	8	100%	5.00	3.17	5	1.22	0.56	0.60	0.71	0.56	-0.21
Mean		100%	6.30	3.20	12.5	1.25		0.61	0.74	0.57	-0.25

Number of individuals (N), Number of different alleles (*Na*), Number of effective alleles (*Ne*), number of private alleles (*Pa*), Shannon's Information Index (*I*), expected Heterozygosity (*He*), unbiased expected heterozygosity (*uHe*), observed Heterozygosity (*Ho*), percentage of polymorphic loci (*P%).*and inbreeding coefficient (*F)*

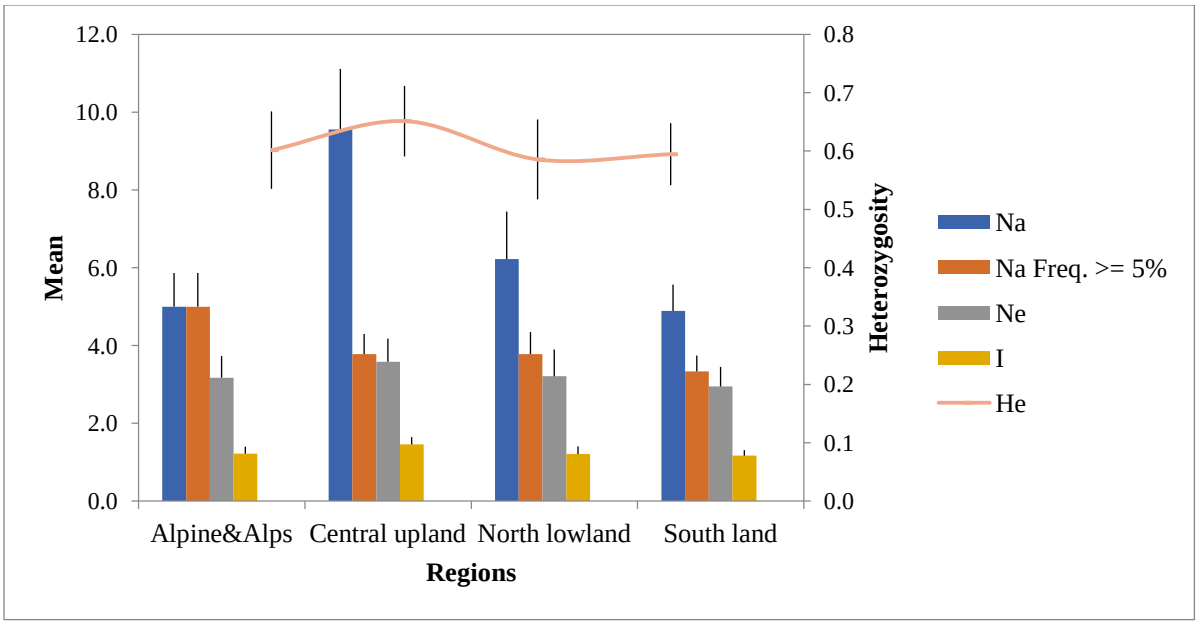


Figure 3.14: Allelic pattern of *Pimpinella saxifraga* across geographical regions in Germany. Number of different alleles (*Na*), Number of effected alleles (*Ne*), Shannon's Information Index (*I*), Observed Heterozygosity (*Ho*), expected Heterozygosity (*He*).

Shannon's information index (*I*) statistics partitioned by geographic and total areas, regions and the total, presented ranging from 1.17 south region to 1.41 central region with a mean of 1.25 (Table 3.3). Also, 15% overlap among the regions and 75% overlap within the regions (Figure 3.15). The overall genetic variability of the individuals studied, represented by Shannon's indexes, was high genetic distance with the a mean of 1.25. A high value of Shannon's information index represents the effectiveness of microsatellite markers to detect the variation.

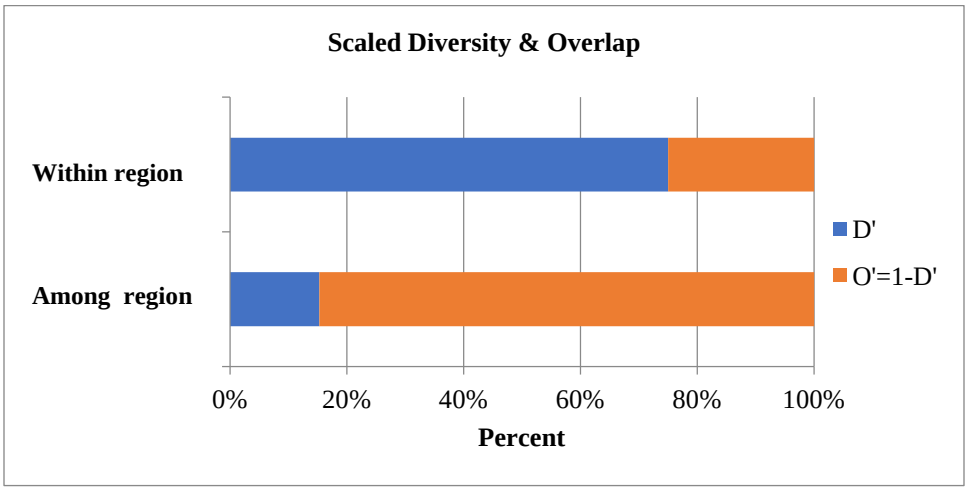


Figure 3.15: Scaled Diversity overlap within and among regions of *Pimpinella saxifraga* in Germany.

Hardy- Weinberg equilibrium (HWE) showed deviation of allele frequencies through markers for four geographical regions in which overall 78 individuals. Chi square test of statistical differences from zero for these regions were presented in Figure 3.16. Significant deviation from Hardy–Weinberg equilibrium was detected in the four biogeogrphical regions in Germany: Central upland (five markers), North lowland (six markers), south land(three markers), and Alpine & Alps (three markers) Several regions did not fully satisfy, as the majority of markers were in accord with the Hardy- Weinberg Equilibrium (HWE).

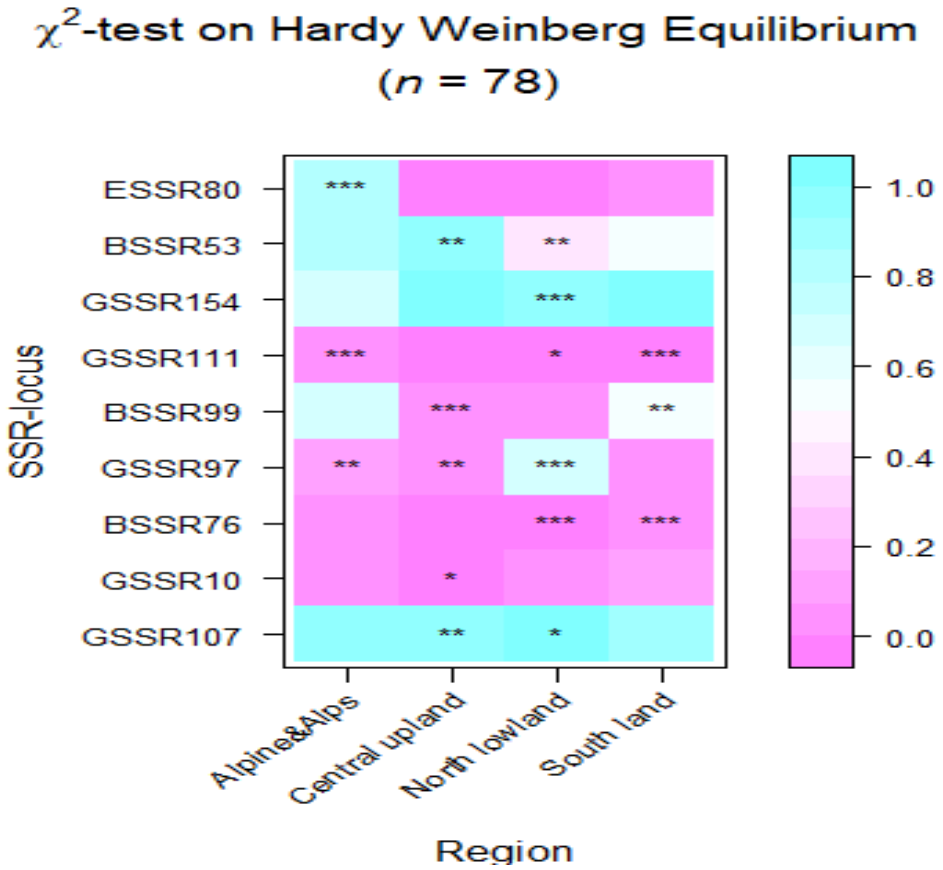


Figure 3.16: Summary of Chi-Square Tests for Hardy-Weinberg Equilibrium in different individuals of four regions. HWE markers showing a significant departure from Hardy-Weinberg equilibrium with a global test at 0.05 level (*$P < 0.05$. **$P < 0.01$. *** $P < 0.001$).

3.2.2. Population Genetic Differentiation *Pimpinella saxifraga*.

The Analysis of molecular variance (AMOVA) on genetic differentiation among and within the region of *P. saxifraga* based on the molecular data, revealed that 98% of total variations were contributed by differences within regions, which was notably and significantly higher than that

among regions whereas 2% of total genetic variance was a virtue of differences among regions (Table 3.4). *PhiPT*, were 0.020 ($P < 0.05$), this displays a low genetic differentiation.

Table 3.4: Analysis of molecular variance (AMOVA) from nine SSR microsatellite data collected from 78 *Pimpinella saxifraga* individuals from four biogeography regions in Germany.

Source of variation	DF	SS	MS	Est Var.	%	PhiPT	Nm
Among region	3	18.687	6.229	0.097	2%		
Within region	74	341.210	4.611	4.611	98%		
Total	77	359.897		4.705	100%	0.020	12.275

Df = Degree of freedom; *SS* = Sums of squares; *MS* = Mean squares; *Est. var* = Estimate of variance; Nm = gene flow the probability level after 999 permutations across the full data set .*$P < 0.05$ significant.

The F_{st} value ranged from 0 to 1, with 0 representing no differentiation and 1 representing complete differentiation. An F_{st} value in the range of 0.00 to 0.05 shows little differentiation, an F_{st} value in the range of 0.05- 0.15 a moderate differentiation and an F_{st} value> 0.15 indicates a high level differentiation (Holsinger and Weir, 2009). The difference in genetic differentiation (F_{st}) between regions varying from 0.014 (between north lowland and central upland also, north lowland and alpine) to 0.020 (between central upland and alpine), with an average value of 0.016 (Table 3.5, measured across four regions based on 9 markers).

Table 3.5: Pairwise F_{st} values among *Pimpinella saxifraga* occurring in four regions in Germany.

	North lowland	Central upland	South	Alpine & Alps
North lowland	0.000			
Central upland	0.014	0.000		
South land	0.015	0.015	0.000	
Alpine & Alps	0.014	0.020	0.016	0.000

Nei's genetic distance (GD) was calculated for each pairwise comparison between the four regions in Germany (Table 3.6). The GD of *P. saxifraga* ranged from 0.045 (between north lowland and central upland also, north lowland and alpine) to 0.072 (between central upland and alpine) based on SSR markers with an average value of 0.052.

Table 3.6: Nei's genetic distance value (GD) between 78 *Pimpinella saxifraga* genotypes in four geographical regions

	North lowland	Central upland	South	Alpine, Alps
North lowland				
Central upland	0.045			
South	0.051	0.050		
Alpine & Alps	0.045	0.072	0.051	

3.2.3. Correlation of geographical and genetic (SSR) distance

The Mantel test for correlation between genetic distance and geographic distance (Figure 3.17), revealed that the genetic distance was not significantly correlated with the corresponding matrix of geographical distance (r = 0.096, *P* =0.094). this mean that genetic distance among individuals decease with geographical distance.

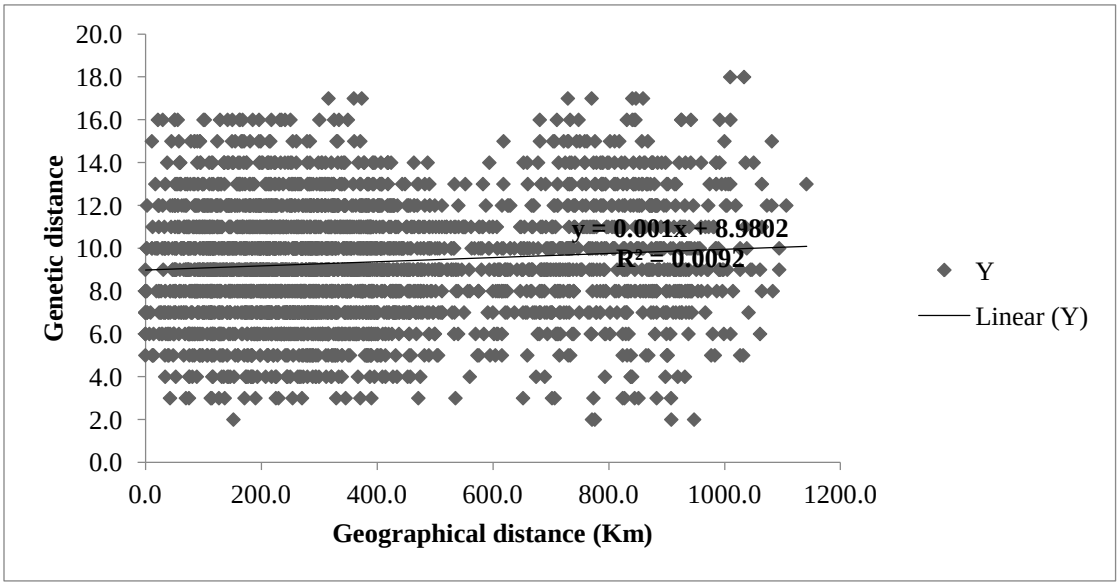


Figure 3.17: The relationship between pairwise geographic (Km) and pairwise genetic distances among regions of *Pimpinella saxifraga* from Germany (based on 999 permutation).

3.2.4. Population genetic structure and genetic relationships

An assortment of methods were applied to reveal genetic diversity and population structure. It is suitable to join three effective techniques, and so that the collection of structure, PCoA, and UPGMA analysis is able to produce reliable results.

3.3.1.1. Principle coordinate analysis (PCoA).

The Principal Coordinate Analysis (PCoA) from GenALEx with SSR markers provides a spatial representation of the relationship between regions (Figure 3.18). The results from PCoA showed no clear structuring or differentiation over the samples of different regions. The analysis only depicted an overall cumulative percentage of variation on the first three axes of 31.45% of the total variation. In PCoA all individuals were labeled with different colors based on their different

geographical regions. According to the PCoA a distribution of the samples from four regions can be seen without any differentiation.

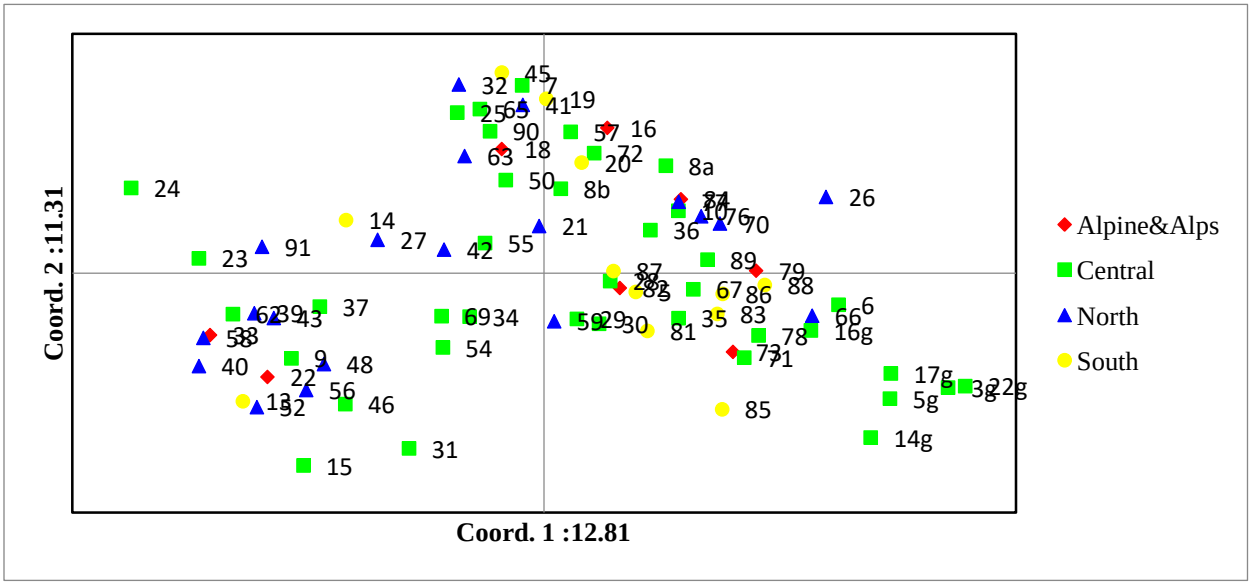


Figure 3.18: Principal Coordinate Analysis (PCoA) plot of SSR markers of 78 *Pimpinella. saxifraga* individuals color coded for four geographic regions in Germany.

The factorial analysis based on dissimilarity distance matrix using DARwin 6.0.010 software (Figure 3.19) confirmed the results of the PCoA, individuals from different regions are mixed and no clear pattern was found.

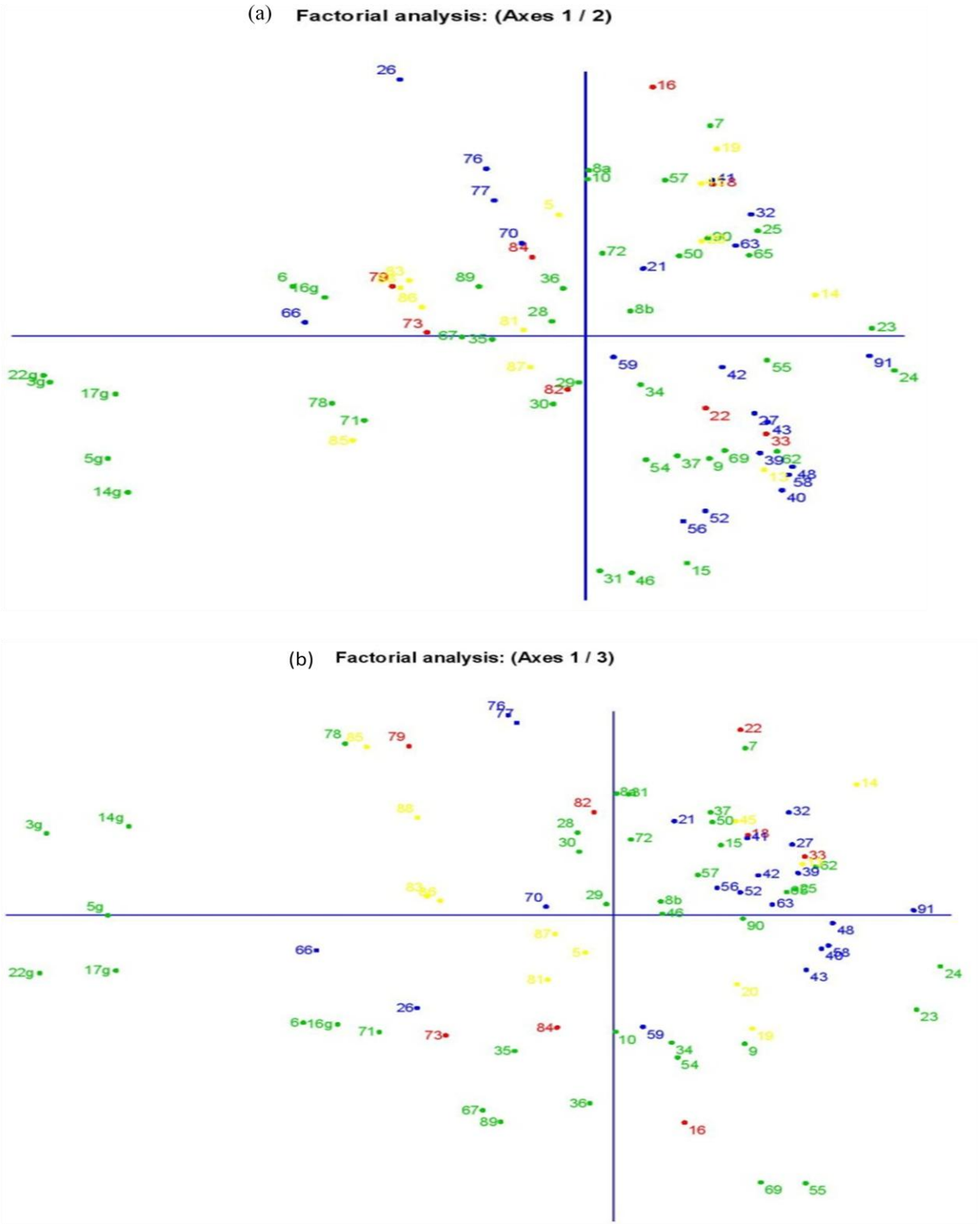


Figure 3.19: Factorial analysis using 78 individuals of *Pimpinella saxifraga* based on 9 SSR microsatellite markers color coded in accordance to the geographical origin of individuals: red Alpine & Alps region, blue North region, green Central region and yellow South region. First and second components (**a**) and first and third components (**b**) of the factorial analysis.

3.3.1.2. Population structure analysis

The population genetic structure analysis of the 78 individuals analyzed by Bayesian clustering STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000), was to study the regional structure and genetic reaction among the 78 *P. saxifraga* individuals. The optimal number of genetic cluster was 2 when ΔK was at its maximum for K= 2 (Figure 3.20). Here it becomes obvious that neither a geographic affiliation is visible, nor differentiation between potential subspecies, e.g. *P. saxifraga* or other taxa can be observed, however, there is a gradual differentiation among the here analyzed data (Figures 3.21 and 3.22).

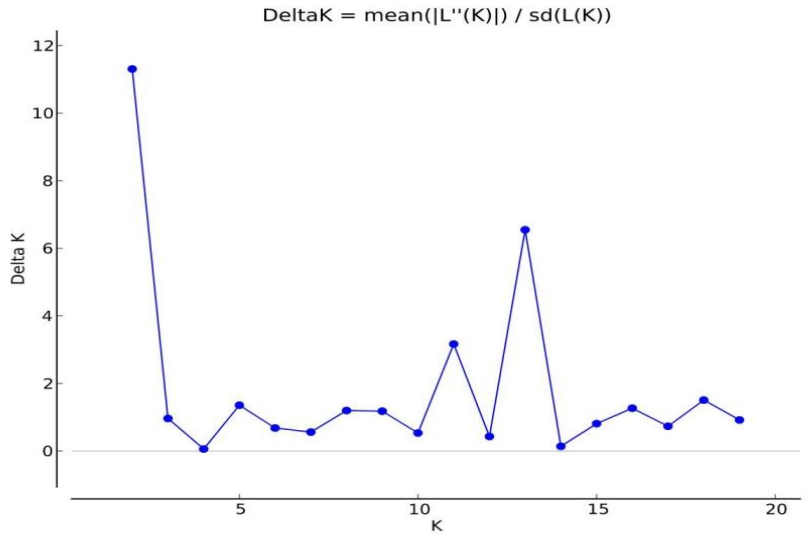


Figure 3.20: The distribution of ΔK over K= 1-20 the best value of K =2.

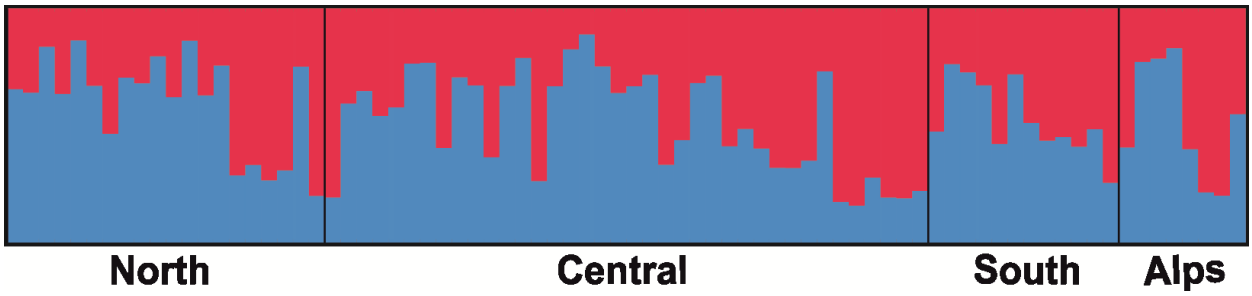


Figure 3.21: SSR results estimated in a STRUCTURE analysis for the 78 individuals from geographical regions in Germany of *Pimpinella saxifraga.*

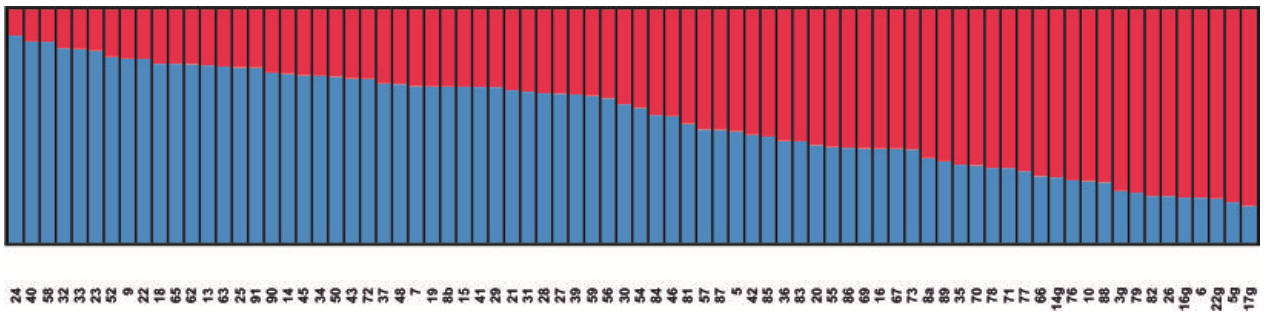


Figure 3.22: Result of a STRUCUTRE analysis of 78 individuals of *P. saxifraga* when K= 2 each single vertical line is represent an individuals.

Furthermore, the Neighbour – joining (NJ) tree based on Nei's genetic distance clustered the 78 *P. saxifraga* individuals from the four biogeographical regions into clusters but no clear structuring or differences between the individuals and geograpghical regions. Also, the pattern clustering of Unweighted neighbor–joining was similar to the PCoA and factorial analysis (Figures 3.23 and 3.24).

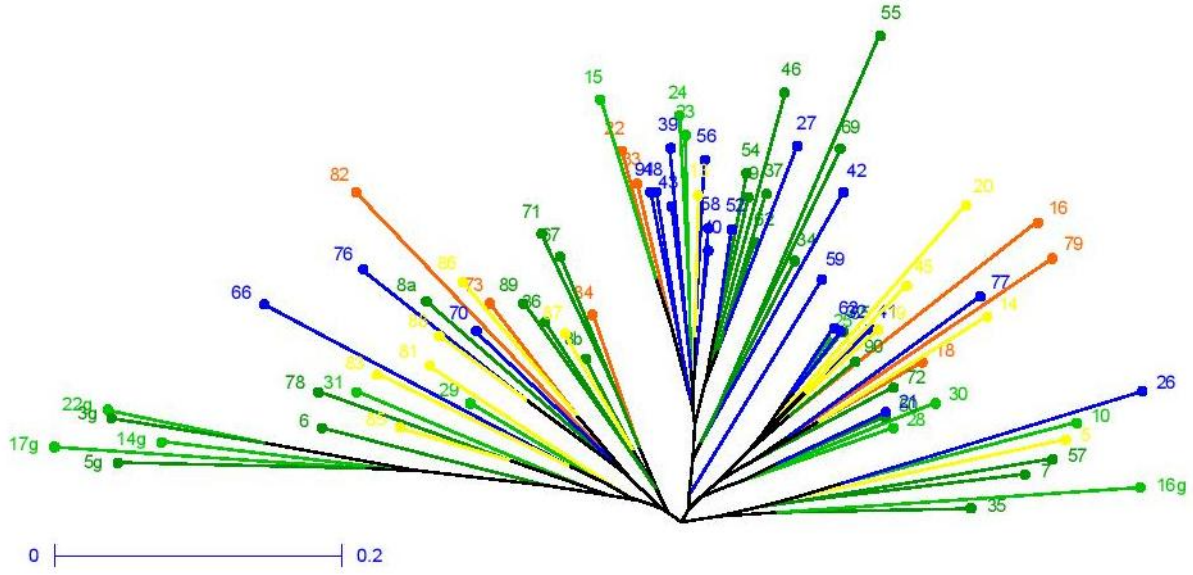


Figure 3.23: Hierarchical neighbor-joining tree drawn in Darwin 6.0 of the 78 *P. saxifraga* individuals based on 9 SSR microsatellite markers colour coded in accordance to the geographical origin of individuals: red Alpine & Alps region, blue North region, green Central region and yellow South region.

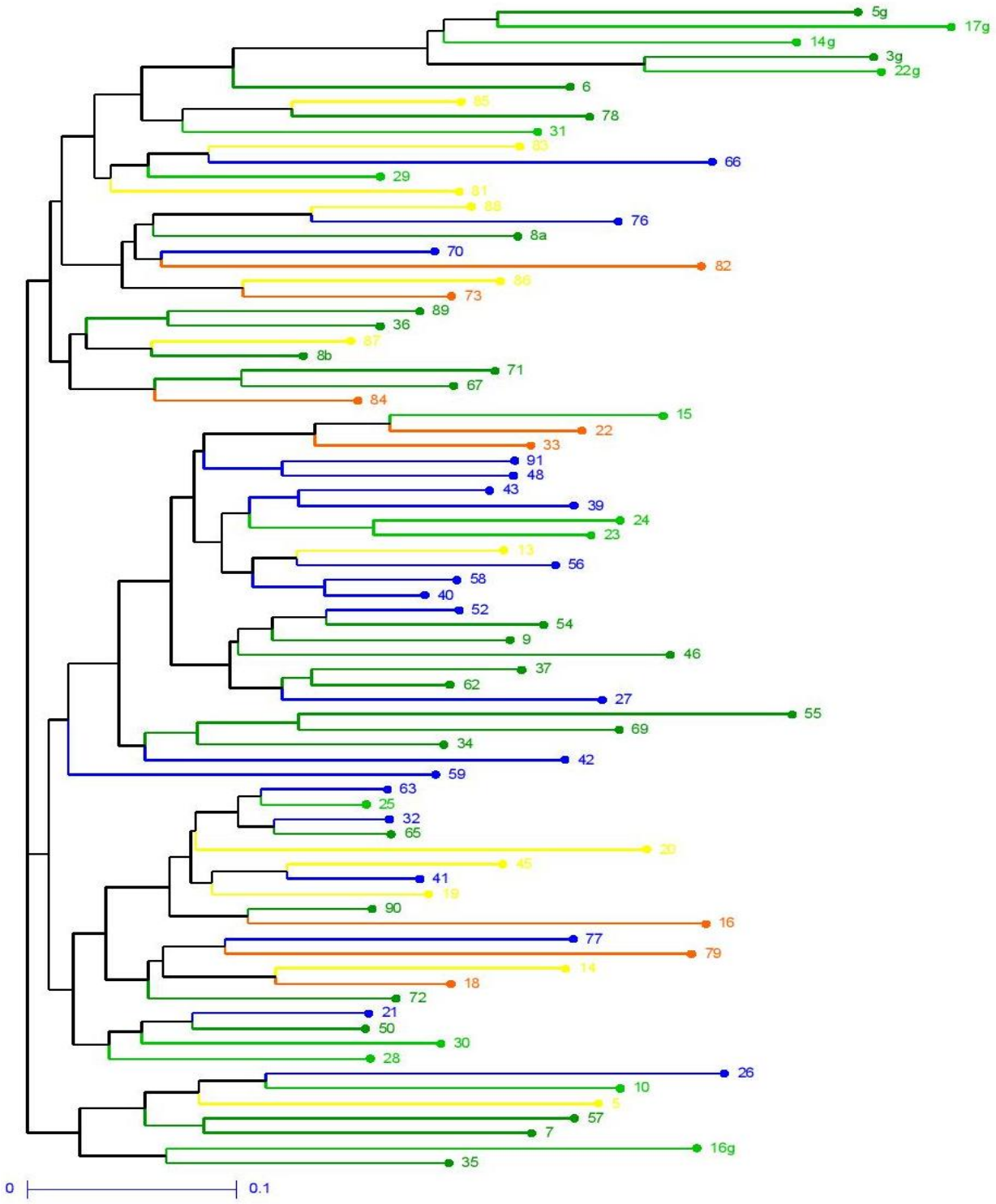


Figure 3.24: Hierarchical neighbor-joining tree drawn in Darwin 6.0 of the 78 *Pimipnella saxifraga* individuals based on 9 SSR microsatellite markers, colour coded in accordance to the geographical origin of individuals: red Alpine & Alps region, blue North region, green Central region and yellow South region.

3.3.1.3. Genetic Relatedness

The mean pairwise relatedness between and within the four regions showed values greater than zero, which would be indicative for random mating or random distribution by man. These results indicate non-significant differentiations between all four regions. The mean relatedness values in the Alpine & Alps region featured mating (mean r = 10.18, confidence interval (CI) = 7.43-10.89), while North lowland region had the lowest values of mating (mean r = 8.50, CI. = 8.12-10.10), Central and south region values mating (9.15 and 8.80 respectively) in between these values (Table 3.7; Figure 3.25). All relatedness values feel between the 95% confidence limits depend on the permutations of null hypothesis indicate the individuals less related.

Table 3.7: Mean pairwise relatedness (r) values within geographical regions.

Region	North lowland	Central upland	South land	Alpine & Alps
Mean	8.505	9.15	8.80	10.18
Upper value	10.10	9.70	10.47	10.89
Lower value	8.12	8.60	7.77	7.43

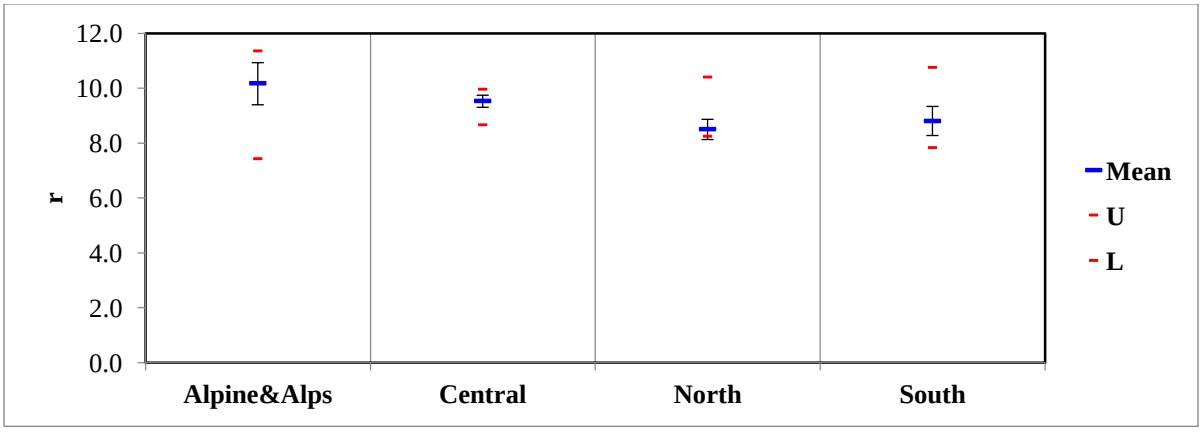


Figure 3.25: Mean relatedness (r) within-region relatedness (R) Upper (U) and Lower (L) values boxplot (blue) represent the observed mean relatedness and standard error red lines represent 95% confidence interval about zero which determined by individuals size.

3.3. Morphological characterization of *Pimpinella saxifraga*

3.3.1. Variation of Morphological Traits

The morphological traits were divided into quantitative and qualitative traits. Out of the 78 herbarium samples for molecular analysis seventy-one individuals were tested as well for morphological variation using eleven morphological traits (six qualitative and five quantitative

ones). Samples were investigated and assigned to four geographical regions in Germany (Figure 2.2), allowing to tests for differentiation of these artificial populations, as no real populations were sampled.

3.3.1.1. Morphological traits across four biogeographical regions.

Morphological trait's diversity displayed by the species *Pimpinella saxifraga* was observed in almost all qualitative traits of the leaflet such as: leaflets shape, leaflets teeth, leaflet margin, hairs on the surface of the leaflet, terminal leaves, and hairs (indument) along the main axis (Table 3.8). Four of six qualitative traits showed significant differences between individuals according to biogeographical regions in Germany. The other two traits (terminal leaves and hair along the main axis) were not significant between individuals.

Table 3.8: Analysis of variance of 6 qualitative morphological characters for *P. saxifraga* individuals within and between regions in Germany: leaflets shape, leaflets teeth, leaflets margin, hairs on the surface of the leaflets, terminal leaves and hairs.

	Source	Df	SS	MS	Est.Var	%	PhiPT Value	P<
Leaflet shape	Between region Within region	3 67	2.135 14.344	0.712 0.214	0.031 0.214	12% 88%	0.116	**0.027***
Leaflets teeth	Between region. Within region	3 67	2.135 14.344	0.712 0.214	0.031 0.214	12% 88%	0.125	**0.025***
Leaflet margin	Between region. Within region	3 67	1.805 14.927	0.602 0.223	0.023 0.223	9% 91%	0.94	**0.048***
Hairs on the surface of the leaflets	Between region. Within region	3 67	1.978 20.467	0.659 0.301	0.022 0.301	7% 93%	0.068	**0.043***
Terminal leaves	Between region. Within region.	3 67	0.190 17.31	0.063 0.255	0.000 0.255	0% 100%	-0.048	0.863
Hairs along the main axis	Between region. Within region	3 67	0.788 20.448	0.263 0.301	0.000 0.301	0% 100%	-0.007	0.461

Df = Degree of freedom; *SS* = Sums of squares; *MS* = Mean squares; *Est. var* = Estimate of variance; (999 permutations)
**P*<0.05 significant.

Kruskal-Wallis test was used to compare the variation pattern of quantitative morphological traits of *P. saxifraga* across the four different biogeographical regions. Out of five quantitative morphological traits, two traits (plant length and basal leaf length) showed significant differences with $P = 0.019$ and $P = 0.018$ (Table3.9). The other three traits (rays number, pedicel number and number of leaflets in the basal leaf) showed no significant differences among individuals in the four biogeographical regions.

Table 3.9: Results on Kruskal-Wallis Rank test for five quantitative morphological traits of *P. saxifraga* across four biogeographical regions in Germany.

Morphological traits	North lowland		Central upland		South land		Alpine & Alps		*P*-value Kruskal-Wallis
	Median	SD	Median	SD	Median	SD	Median	SD	
Plant length (cm)	69	20.3	55	18.2	58.5	13.3	57.5	15.5	**0.0189**
Rays number	15	2.82	14	3.53	13	1.68	14.5	2.49	0.3729
Pedicels number	14.5	3.83	15	2.85	14.5	1.4	15	2.71	0.8608
Basal leaf length	21	7.08	16	8.43	15.5	5.87	15.5	6.55	**0.01759**
Leaflets number in basal leaf	5	1.08	5	1.19	5	0.79	4.5	0.53	0.1879

*P<0.05 significant

According to Table 3.9 the plant length showed the largest differences among the four geographical region. The most prominent length with 69 cm occurs in the North lowland, which is greater than the overall median rank of the other biogeographical regions, while in Central upland the lowest length with 55 cm (Figure 3.26) occurs. It showed the most significant difference between four geographical regions among north lowland with central upland *P-value*=0.005 and north lowland with south land *P-value* =0.019.

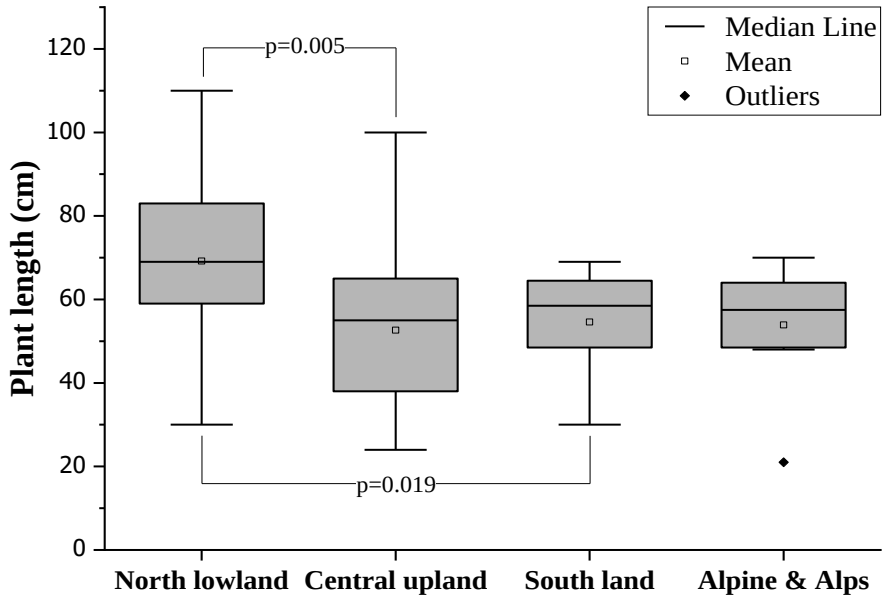


Figure 3.26: Kruskal Wallis rank test applied on morphological traits showed that there is a significant difference in plant length trait (cm) in *Pimpinella saxifraga* across four geographical regions in Germany. level of significance $P < 0.05$.

However, the basal leaf length showed differences as well among the four geographical regions. The basal leaf length with 21 cm occurs in the North lowland, which is greater than the overall

median rank of the other biogeographical region. The lowest length (15.5 cm) was found in south and alpine & alps region (Figure 3.27).

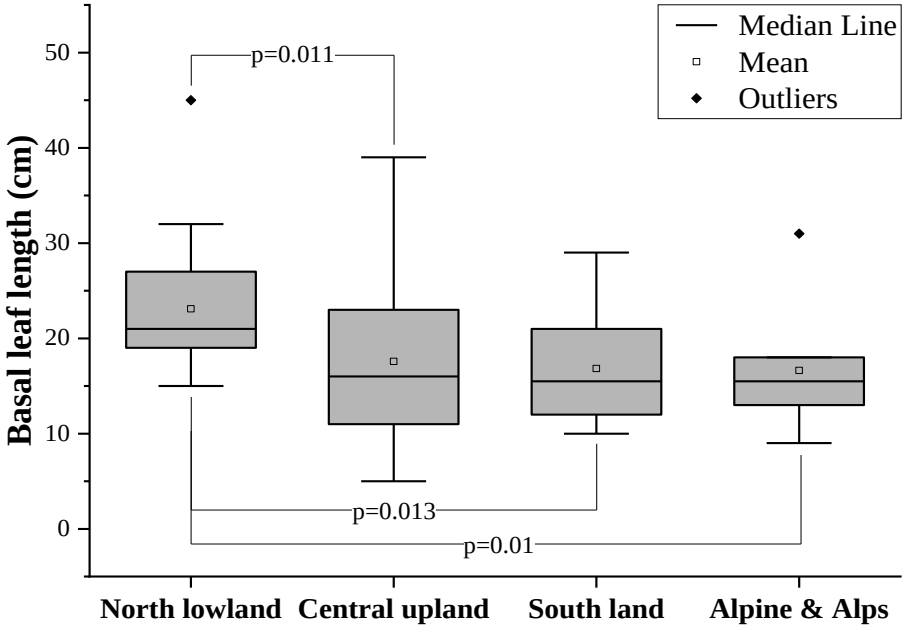


Figure 3.27: Kruskal Wallis rank test applied on morphological traits, showed that there is a significant difference in basal leaf length trait (cm) in *Pimpnella saxifraga* across four geographical regions in Germany. Level of significance $P < 0.05$.

3.3.1.2. Correlation analysis of the investigated morphological traits.

Among all quantitative morphological traits the Spearman's correlation coefficient indicated by Table 3.10 reveals a significant positive and negative correlation between five quantitative traits.

Table 3.10: Spearman's correlation coefficient (with *P value*) between quantities morphological traits of 71samples of *P. saxifraga* across Germany

	Plant length (cm)	Basal leaf length (cm)	Leaflets number in basal leaf	Rays number	Pedicels number
Plant length (cm)					
Basal leaf length (cm)	0.425***				
leaflets number in basal leaf	0.038^{ns}	0.264*			
Rays number	0.37***	0.424***	0.058^{ns}		
Pedicels number	0.347**	0.235^{*}	-0.270*	0.557***	

*,**,*** Correlation significant at the P < 0.05;P < 0.01; P < 0.001 respectively, ns: not significant.

The results by Table 3.10, Figure 3.28 show that there is a strong positive correlation between plant length with basal leaf length, rays number and pedicels number ((r = 0.425, *P* =0.001), (r = 0.37, *P* =0.001), and (r = 0.347, *P* =0.01)) respectively. Also, between basal leaf length with rays number (r = 0.425, *P* =0.001), and rays number with pedicels number (r = 0.557, *P* =0.001).

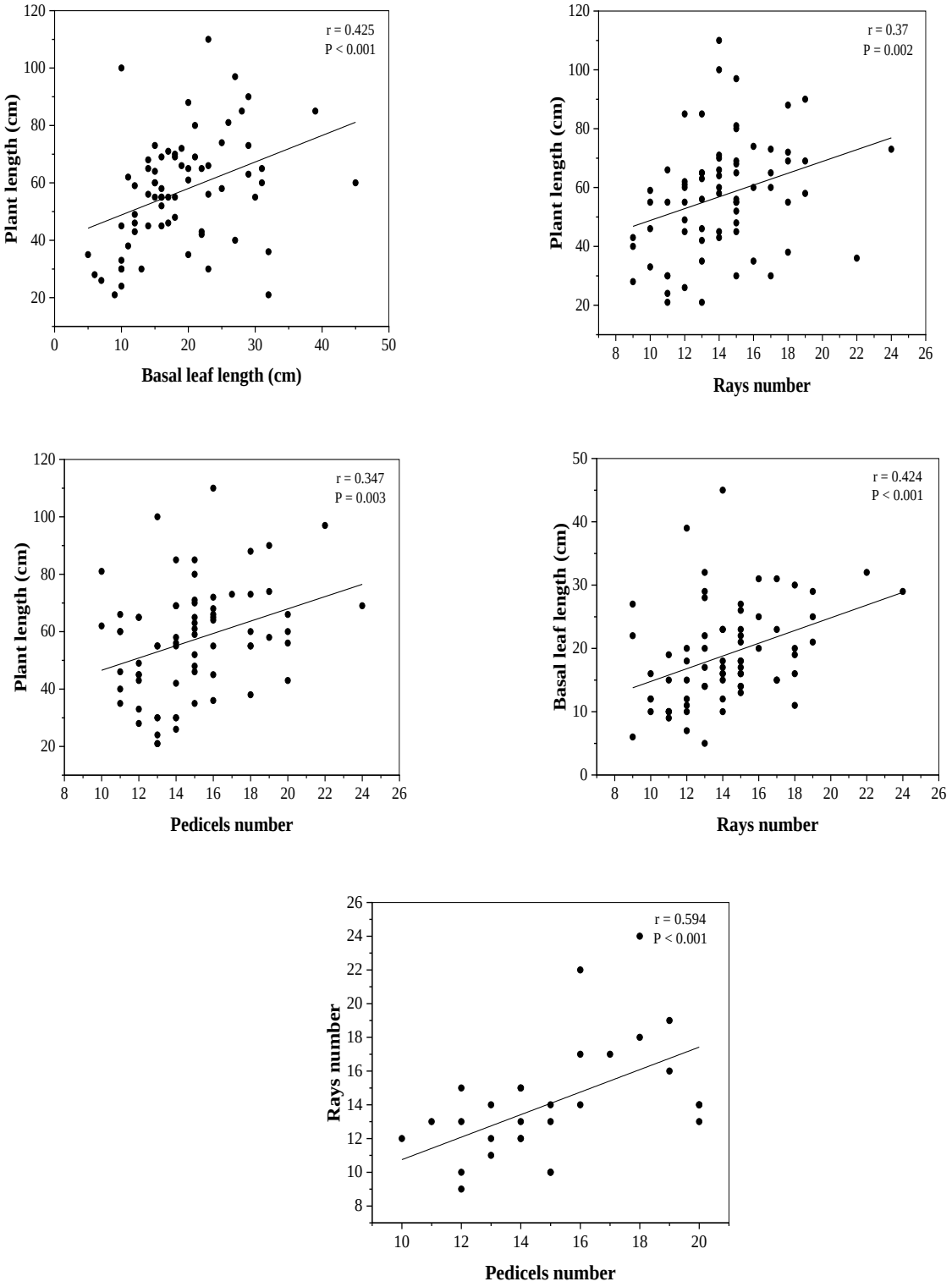


Figure 3.28: Relationship between various quantitative morphological traits of *P. saxifraga* like: plant length with basal leaf length, rays number, and pedicels number, basal leaf length with rays number, and rays number with pedicels number. Correlations were estimated by Spearman's rank correlation test.

Also, the results by Table 3.10, Figure 3.29 presents that, there is a medium positive correlation between basal leaf length with leaflets number in basal leaves (r = 0.264, *P* =0.05) and pedicels number (r = 0.235, *P* =0.05).

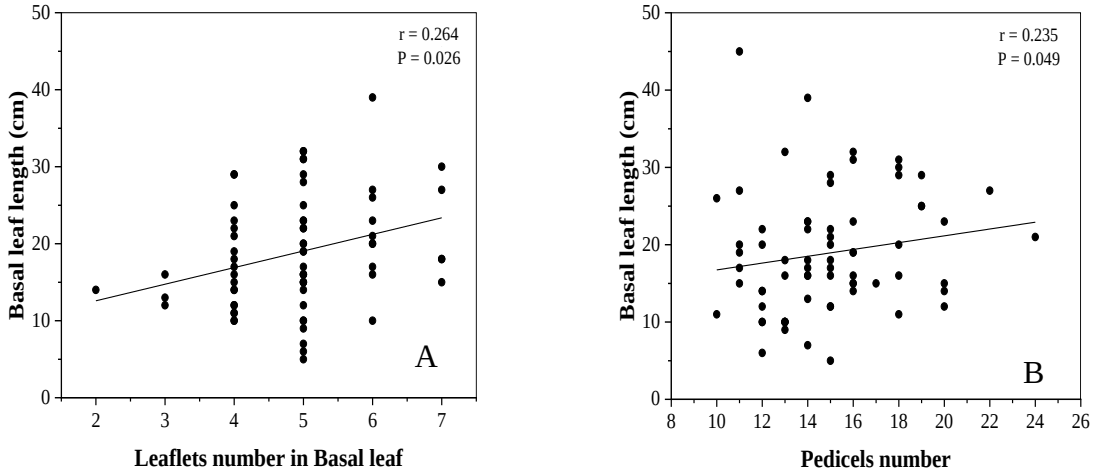


Figure 3.29: Relationship between three quantitative morphological traits of *P. saxifraga.* A) Basal leaf length with leaflets number in basal leaf and B) Basal leaf length with pedicles number. Correlation were estimated by Spearman's rank correlation test.

Meanwhile, a negative linear correlation was found between the leaflets number in basal leaves with pedicles number (r = -0.270, *P* =0.05) (Table 15, Figure 3.30).

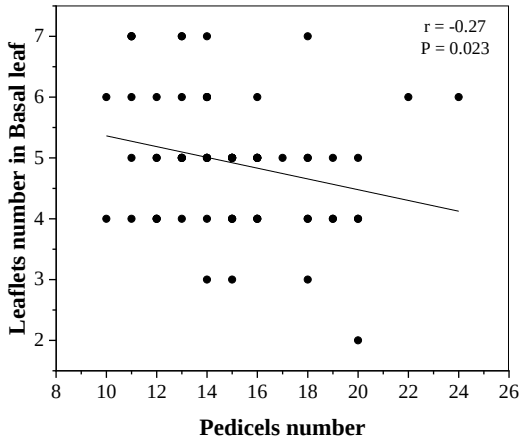


Figure 3.30: Relationship between quantitative morphological traits of *Pimpinella saxifraga*. Leaflets number in basal leaf with pedicles number. Correlation were estimated by Spearman's rank correlation test.

3.3.1.3. Correlation analysis between morphological traits in four geographical regions.

In this part, the relationship between morphological traits according to the distribution of individuals into four geographical regions was studied. Significant differences were observed in plant length with basal leaf length in the central upland region (r = 0.499, *P* = 0.004), as presented in Figure 3.31.

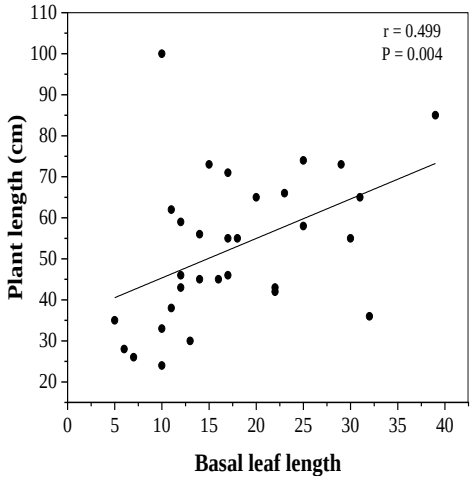


Figure 3.31: Relationship between two quantitative morphological traits of *Pimpinella saxifraga*. Plant length with basal leaf length in the Central upland (Germany). Correlation were estimated by Spearman's rank correlation test.

Also, highly positive correlation between basal leaf length and rays number was found in south (r = 0.635, *P* = 0.026) and central (r = 0.487, *P* = 0.005) geographical regions, (Figure 3.32).

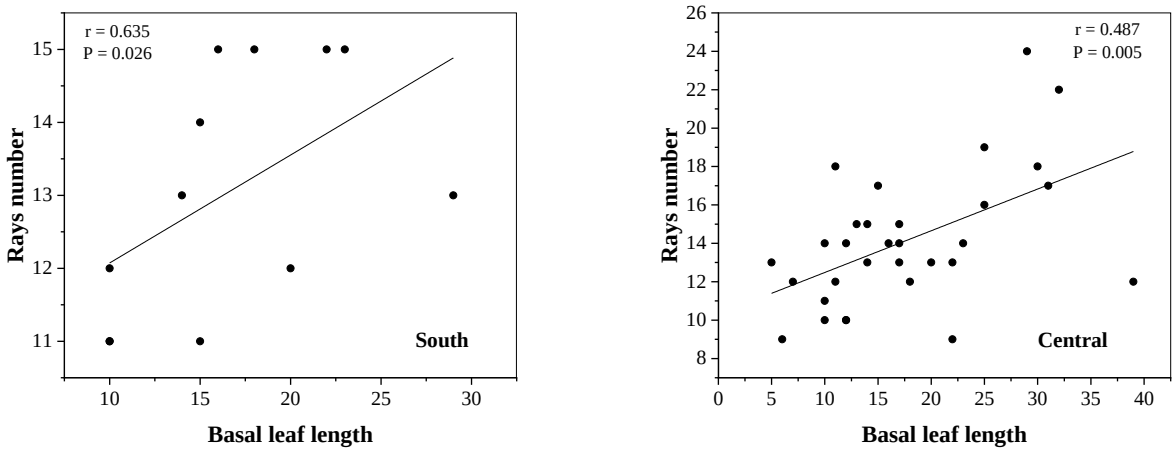


Figure 3.32: Relationship between two quantitative morphological traits of *Pimpinella saxifraga* in two German regions. Basal leaf length with rays number in south and central region. Correlation were estimated by Spearman's rank correlation test.

Strong positive significant differences were detected in alpine, central and north regions ((r = 0.903, *P* = 0.004), (r = 0.594, *P* = 0.001), and (r = 0.511, *P* = 0.026)) between rays numbers and pedicels number (Figure 3.33).

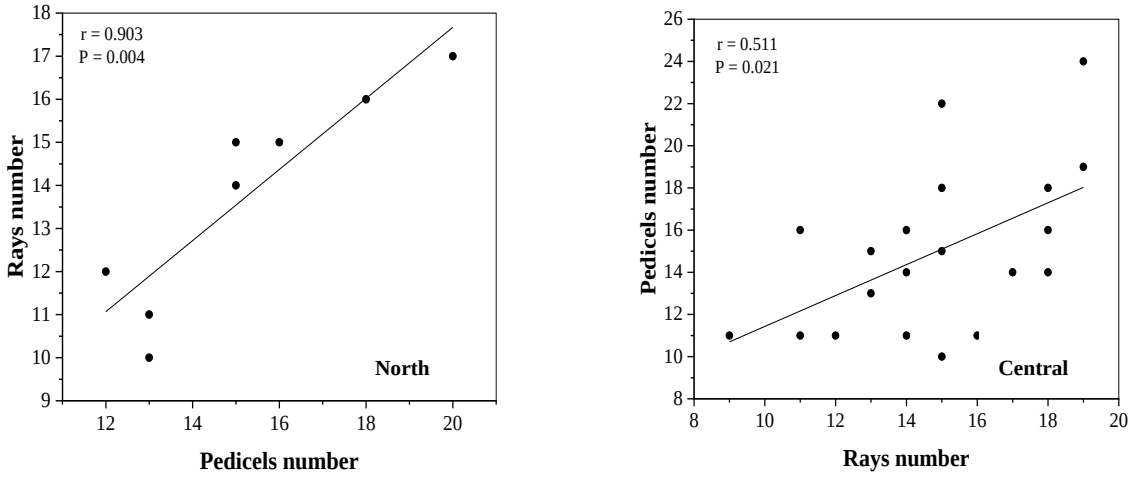


Figure 3.33: Relationship between two quantitative morphological traits of *Pimpinella saxifraga* in different geographic regions of Germany. Like rays number and pedicels number. Correlation were estimated by Spearman's rank correlation test.

As illustrated in Figure 3.34, pedicels number was negative significantly correlated with leaflet number in basal leaf (r = - 0.497, *P* <0.026) in north region.

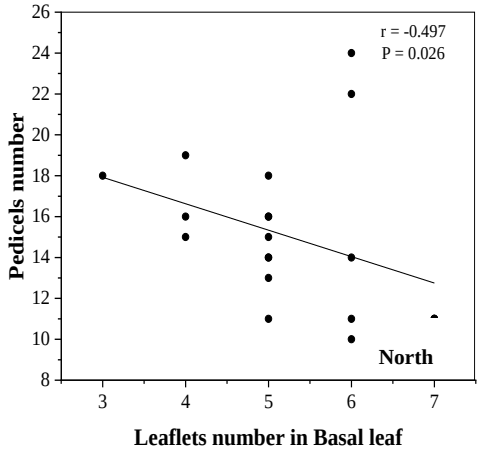


Figure 3.34: Relationship between quantitative morphological traits of *Pimpinella saxifraga*. Pedicels number with leaflets number in basal leaf .Correlations were estimated by Spearman's rank correlation test.

3.3.1.4. Correlation between morphological traits and altitude

Among the *Pimpinella saxifraga* individuals of the four geographical regions, negative significant correlation was found for plant length, basal leaf length, leaflets number in basal leaf, and rays number (Table 3.11, Figure 3.35).

Table 3.11: Spearman's rank correlation coefficients of the five quantitative morphological traits among 7 individuals with altitude in the Germany

Morphological traits	Altitude (m)	
	Correlation (R)	*P*- values
Plant length (cm)	-0.31*	**0.009**
Basal leaf length (cm)	-0.353**	**0.003**
leaflets number in basal leaf	-0.241*	**0.043**
Rays number	-0.272*	**0.021**
Pedicels number	-0.057^{ns}	0.636

*,**Correlation significant $P < 0.05$, $P < 0.01$ respectively ns: No correlation Significant.

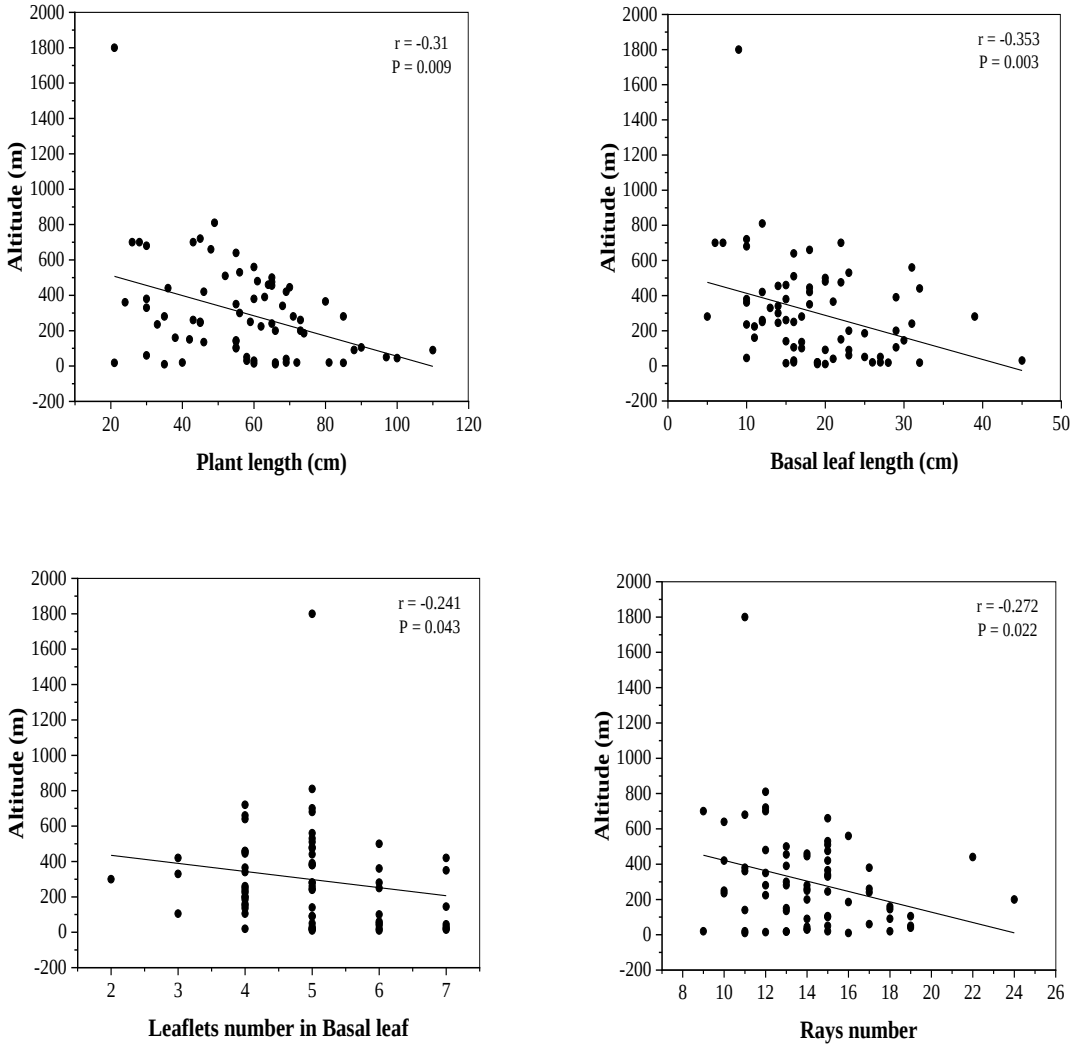


Figure 3.35: Significant relationships of morphological traits versus altitude among 71 individuals at geographical regions in the Germany.

3.3.1.5. Relationship of geographical elevation to morphological traits.

According to the geographical elevation in Germany, we divided three groups according to this site world atlas web 2014 (https://sites.google.com/site/germanyfactsandhistory/geography). These groups were: under 201 m, between 201 and 500 m, and above 500 m. Quantitative morphological traits were tested by Kruskal - Wallis test across these groups based on the median (Table.3.12). Three quantitative morphological traits (plant length, basal leaf length, and rays number) have been significant ($P< 0.05$) through grouping by elevation in Germany, while two quantitative morphological traits (Pedicels number and leaflets number in the basal leaf) were not significant through difference according elevation.

Table 3.12: Kruskal Wallis test for quantitative morphological traits in *P. saxifraga* across three groups of elevation (under 201 m, 201-500 m and above 501 m) in Germany. *P –values* that are significant with $P < 0.01$ are in bold.

Median ± SD	Under 201 m	201-500m	Above 500m	*P*-value Kruskal-Wallis
Plant length (cm)	63 ± 21.27	61 ± 16.06	46.5 ± 13.19	**0.004657**
Basal leaf length	21.5 ± 7.12	15 ± 7.51	14 ± 7.51	**0.00215**
Leaflets number in Basal leaf	5 ± 1.13	5 ± 1.13	5 ± 0.45	0.273
Rays number	15 ± 3.18	14 ± 2.55	12 ± 2.45	**0.0136**
Pedicels number	15.5 ± 3.62	15 ± 2.39	13 ± 1.78	0.1663

As shown in Figure 3.36, plant length (cm) above the height group (500 m) showed significant differences ($P < 0.01$ and $P = 0.002$) to the other groups, compared to plant length with the inverse relationship to elevation.

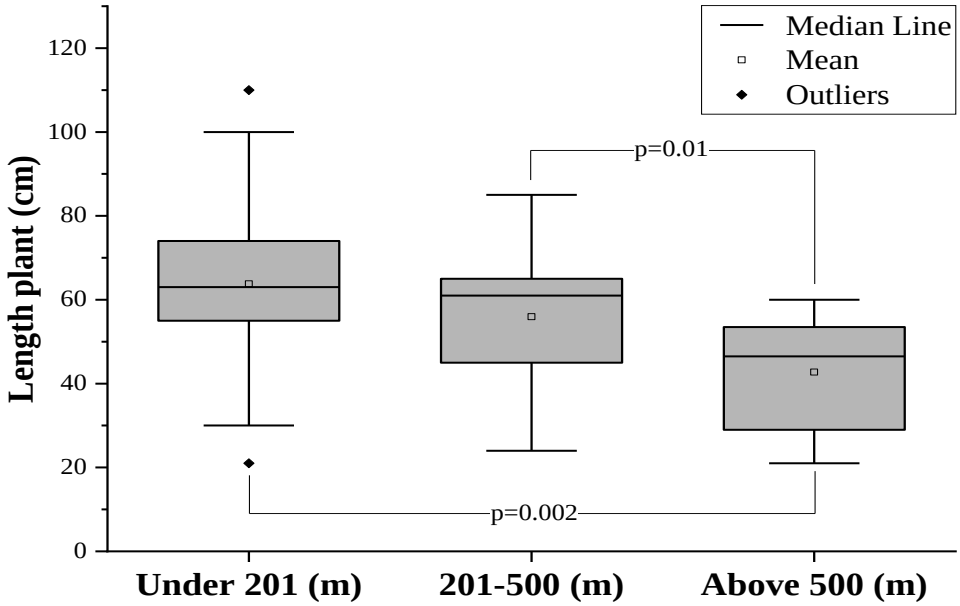


Figure 3.36: Plant length (cm) in *Pimpinella saxifraga* across three groups of elevation. Inside each box represent median the lower and upper borders of the box. Kruskal – Wallis *P*- value for the differences in the median was $P < 0.01$.

Figure 3.37 showed the basal leaf length among three groups of elevation, it has been significant ($P < 0.001$), in under 201 m elevations, compared with other groups. Therefore, the highest basal leaf length is under 201 m elevation.

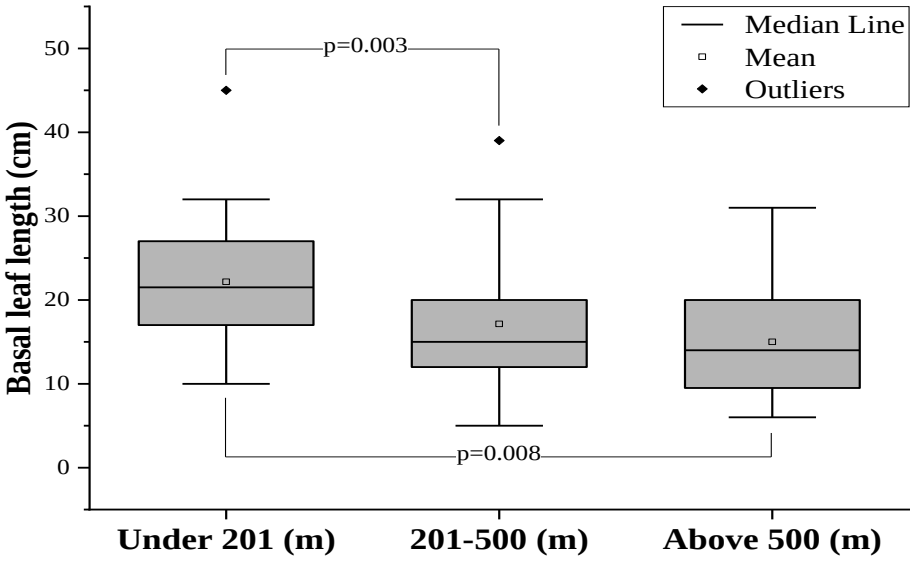


Figure 3.37: Basal leaf length differences (cm) in *Pimpinella saxifraga* across three groups elevation. Inside each box represent median the lower and upper borders of the box. Kruskal – Wallis *P*- value for the differences in the median was < 0.01.

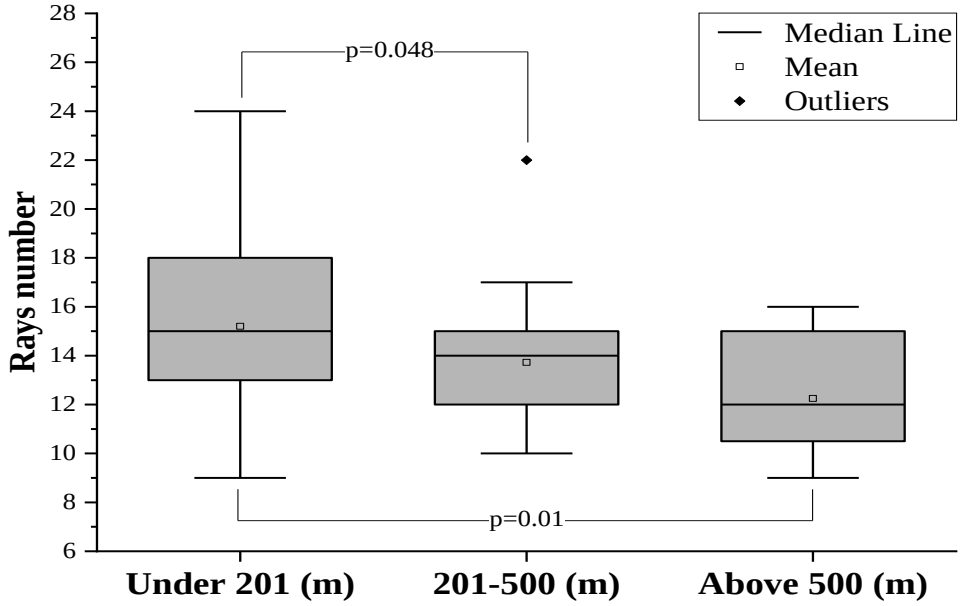


Figure 3.38: Rays number (cm) in *Pimpinella saxifraga* across three groups elevation. Inside each box represent median the lower and upper borders of the box. Kruskal – Wallis *P*- value for the differences in the median was $P < 0.05$.

As illustrated in Figure 3.38 rays number in the under 201 m elevation group showed significant ($P < 0.05$) differences compared to the other two groups. Therefore, the maximum number of rays has appeared under 200 m elevation.

3.3.1.6. Morphological traits across different leaflet shapes of *P. saxifraga*

If you divide the individuals according to the clearly different leaf shapes, you get two groups one with 48 samples with ovate serrate leaflets and the other 23 samples with pinnately lobed leaflets. Using the Mann-Whitney test for this grouping one trait (leaflet number in the basel leaf) of five traits, shows significant differences ($P < 0.01$) (Table 3.13, Figure 3.39).

Table 3.13: Mann-Whitney test investigating significant values of five morphological traits according to grouping after leaflet shape for 71 investigated. *P. sxifraga* samples across Germany

Median ± SD	Ovate serrate teeth	Pinnatifid lobed teeth	*P*-value
Plant length (cm)	59.5 ± 18.26	58 ± 20.62	0.9212
Rays number	14 ± 2.77	14 ± 3.44	0.9874
Pedicels number	15 ± 2.81	14 ± 3.15	0.1185
Basal leaf length	16.5 ± 6.75	20 ± 9.6	0.3229
Leaflets number in basal leaf	5 ± 0.99	5 ± 1.04	**0.0064***

**P*<0.01 significant

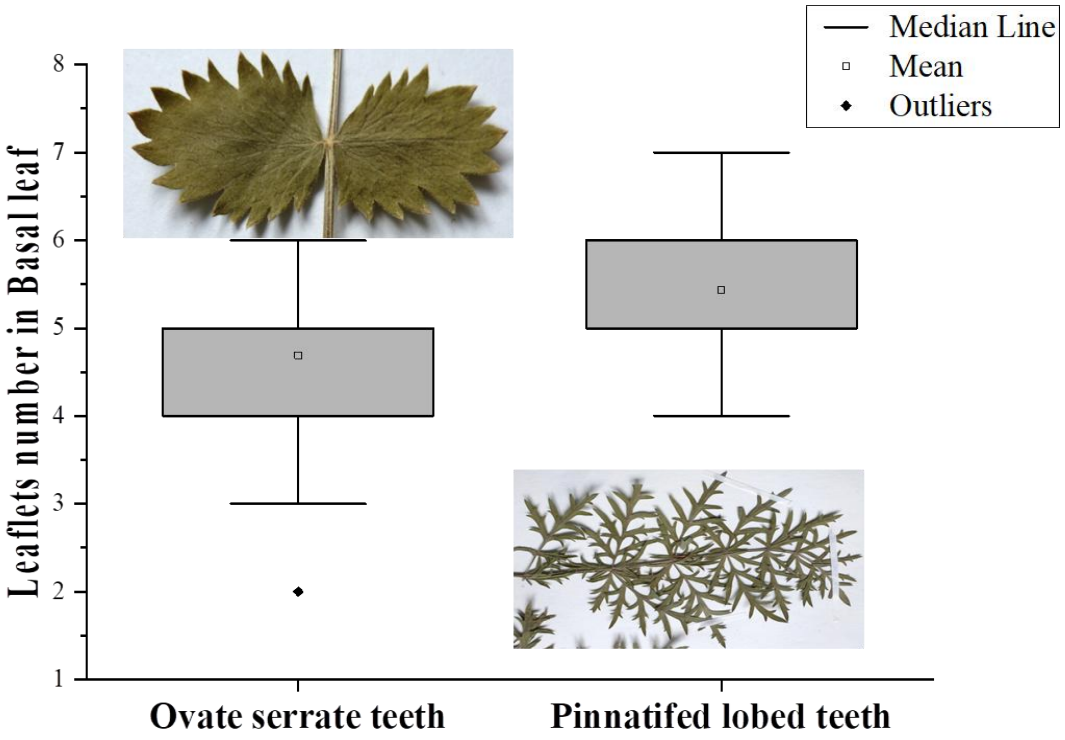


Figure 3.39: Mann Whitney test applied on morphological traits showed that there is a significant difference in leaflet number in basal leaf trait in *Pimpinella saxifraga* across two leaflet morphological trait shape in Germany. Level of significance $P < 0.05$.

According to the significant differences between the two morphological leaflet groups, a factorial analysis (Figure 3.40) was calculated. Factorial analysis was given to the two groups with a clear differentiation, the first group included the ovate serrate teeth leaflet and the second group included the pinnatifid lobed teeth leaflet. Seven individuals showed combined traits between the two different leaflet shapes.

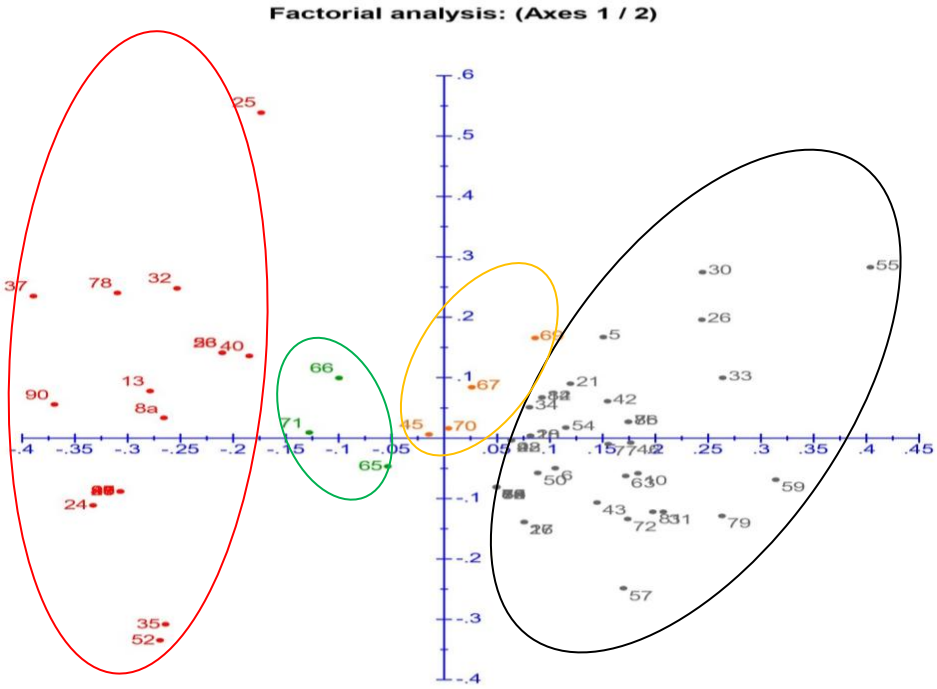


Figure 3.40: Factorial analysis of axis 1 and 2 based on dissimilarity of morphological traits for 71 individuals of *Pimpinella saxifraga* using DARwin software. Red color (Ovate serrate teeth) black color (Pinnate lobed teeth) green and orange (common traits between two main groups.

3.3.1.7. Principle Coordination's Analysis (PCoA) and Cluster Analysis

The Principle coordination's analysis (PCoA) was used to demonstrate the variability in different quantitative and qualitative morphological traits on *P. saxifraga* according to their geographical location (Figure 3.41). The first and second axis explained 57.97% of the total variation (first axis 31.86%, second axis 14.87% and third axis 11.25%). With four geographical regions the overall pattern revealed no clear groups of the samples, in general individuals did not assemble according to their regions.

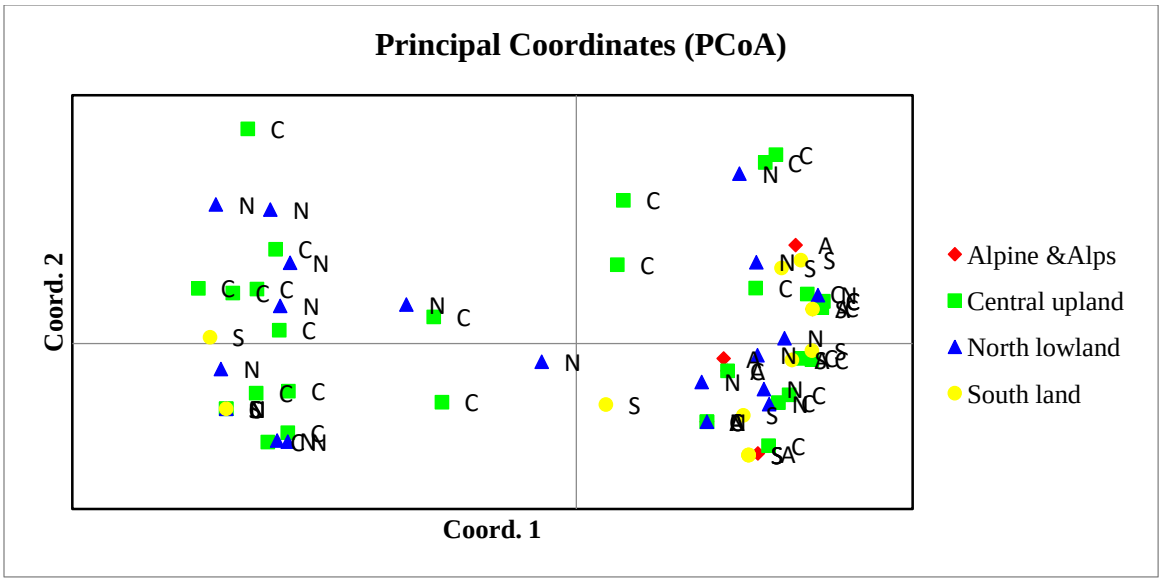


Figure 3.41: Principal Coordinate Analysis (PCoA) of different individuals from the four geographical regions of selected quantitative and qualitative morphological traits of *Pimpinella saxifraga* distribution in Germany.

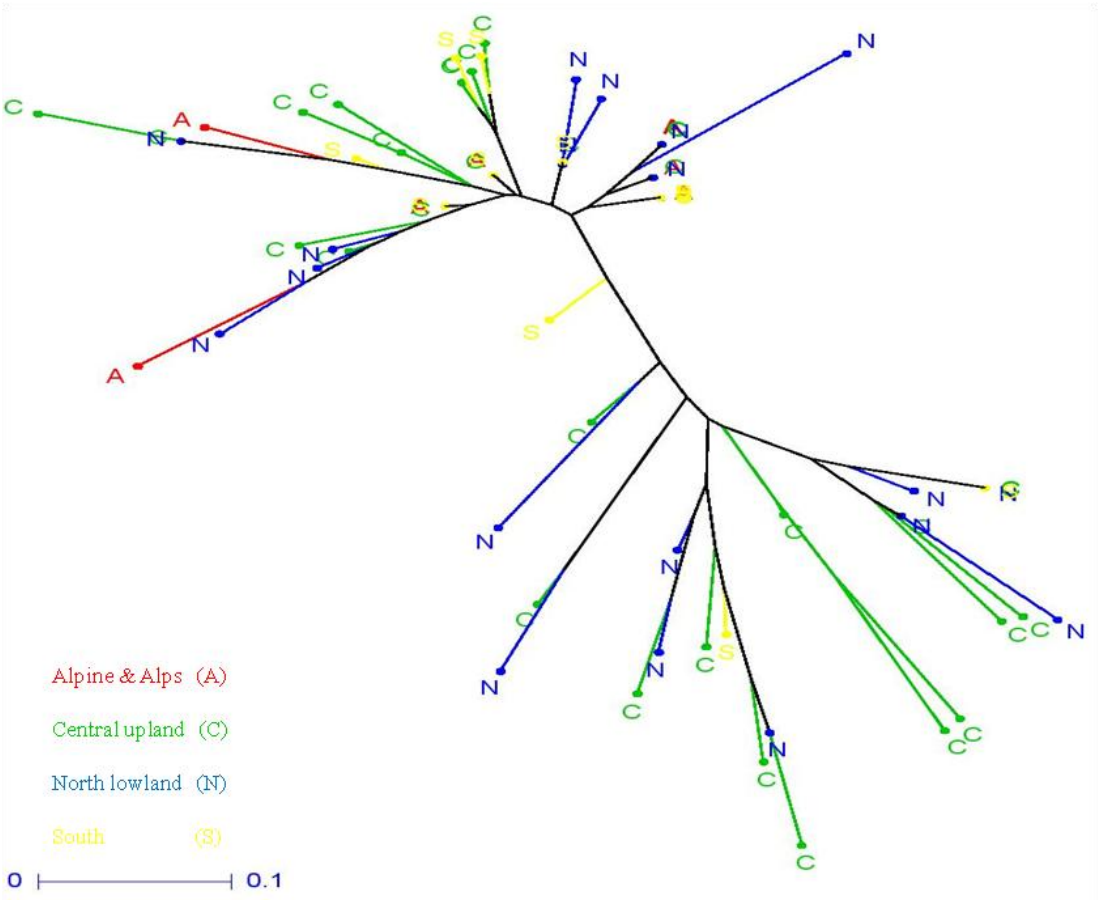


Figure 3.42: Relationships of the 71 *Pimpinella saxifraga* individuals. Unweighted NJ (neighbor-joining) tree based on the dissimilarity matrix of 11 morphological traits, depend on the four geographical regions in Germany.

The unweighted pair group method (UPGMA) based on the 11 morphological traits indicated that the trees for the 71 *Pimpinella saxifraga* individuals were clustered into two major branches (Figure 3.42). The individuals from north and central regions were widely distributed across the two major branches.

Overall, the two multivariate analysis (PCoA, NJ) suggested the presence of two main clusters in examined *Pimpinellla saxifraga* individuals. However, the associations with their geographic regions were very low, except for the individuals from alpine & alps and some individuals in southern region.

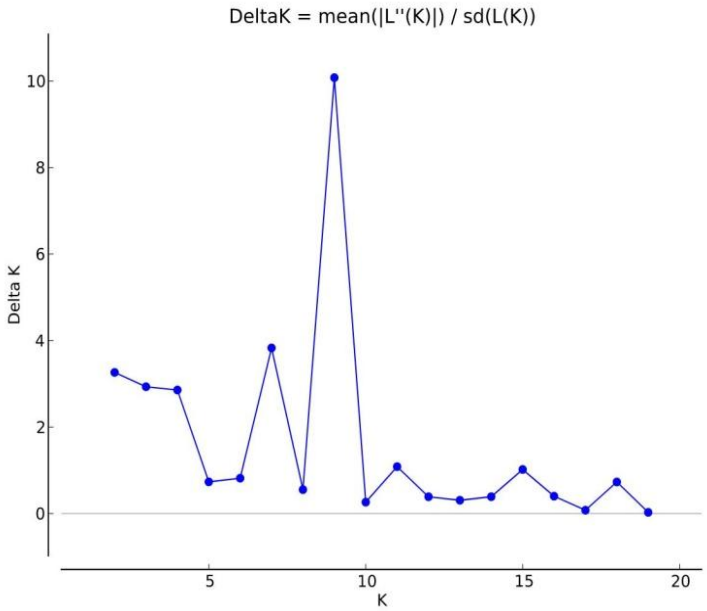


Figure 3.43: The relationship between the number of clusters K and the identical ΔK plot calculated from *K* = 2 to *K* = 20. Best K = 7, 9 based on the structure analysis.

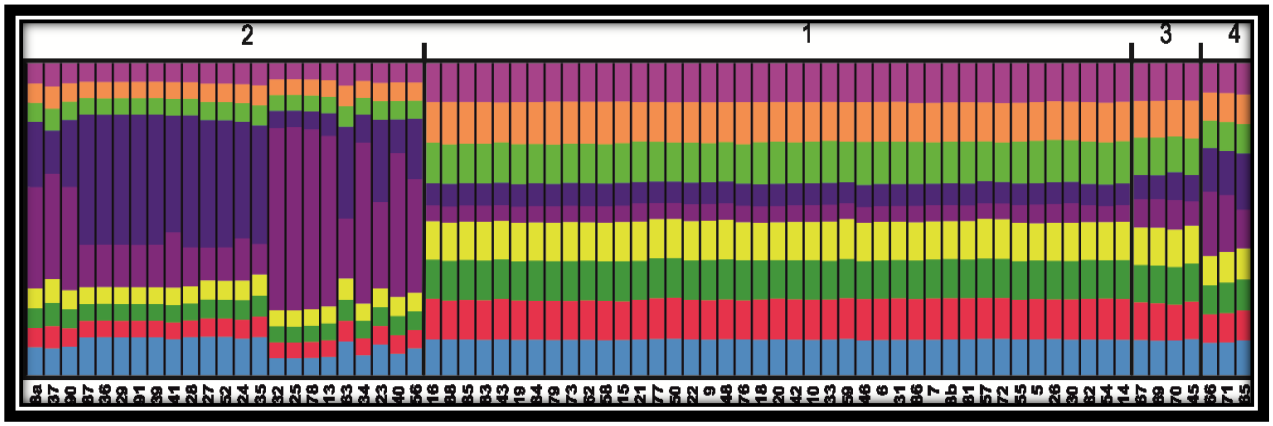


Figure 3.44: Morphological traits evaluated a STRUCTURE analysis for the individuals of *Pimpinella saxifraga* main groups group 1 (Ovate serrate teeth) group 2 (Pinnate lobed teeth).

As shown in Figures 3.43 and 3.44 the Bayesian analysis with the full data set of morphological traits identified values of K = 7 and 9. The analysis revealed morphological cluster visibly assured the factorial analysis results in Figure 3.41 have divided two major groups originating from different morphological traits first group ovate serrate teeth second group Pinnate lobed teeth in all geographical regions in Germany. Group 3 and 4 showed the intermediate samples.

The morphological trait's relatedness between *Pimpinella saxifraga* individuals was analyzed by unweighted Neighbor-Joining (NJ). The rooted method based on a dissimilarity matrix (Figure 3.45). The 71 individuals were grouped into two major clusters (named as I red colored, II grey colored) with 20, 44 individuals, respectively. The other 7 individuals common traits between cluster I and cluster II. Cluster I with higher pinnate lobed teeth. Cluster II consisted of ovate serrate teeth, the grouping of individuals was supported the result by STRUCTURE. And factorial analysis of these cluster groupings depends on significant morphological traits.

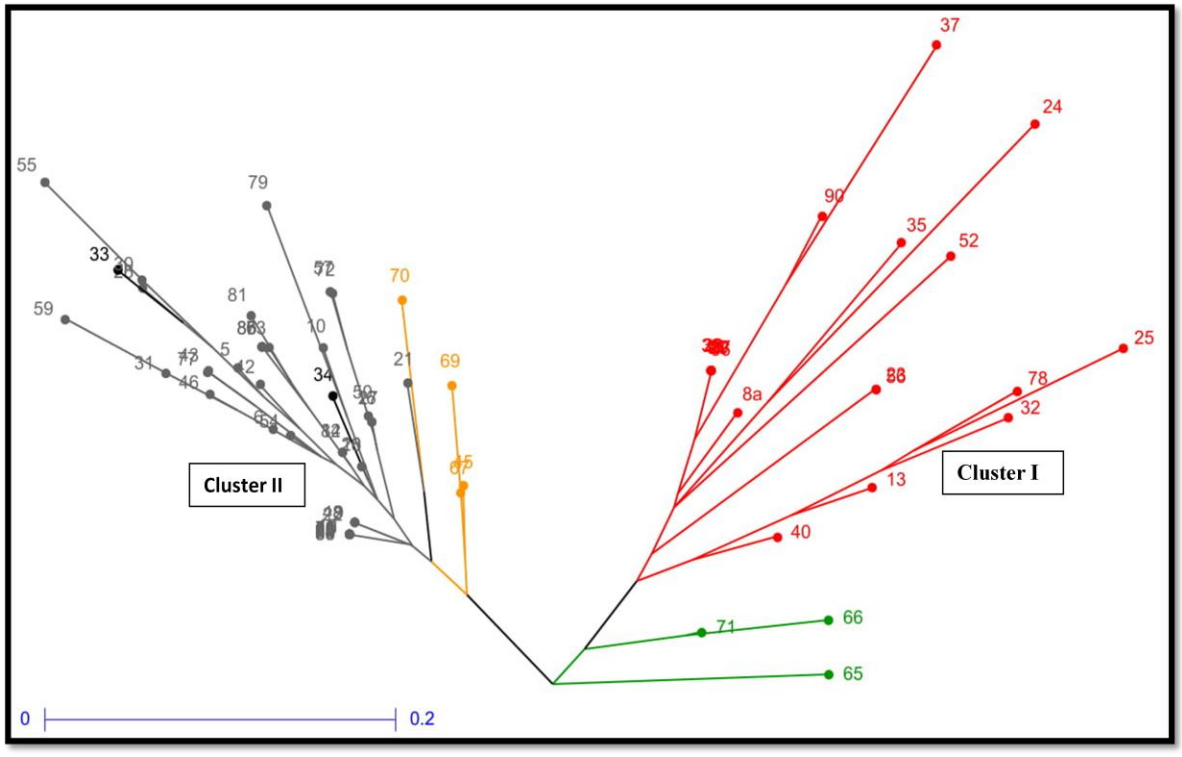


Figure 3.45: Neighbor-joining (NJ) graph was computed by using DARwin 6.0.21 software obtained with the dissimilarity matrix of the 11 morphological traits and unweighted pair group method Cluster analysis clearly divides two groups based on morphological traits. Two major clusters (named as Cluster I, red colored and Cluster II grey colored).

Another network based on Median-joining (MJ) network was used to analyze the morphological traits of 71 individuals. By this network performed in the Popart software (Figure 3.46), identical groupings to the clusters in the Neighbor-joining method and STRUCTURE analysis become visible.

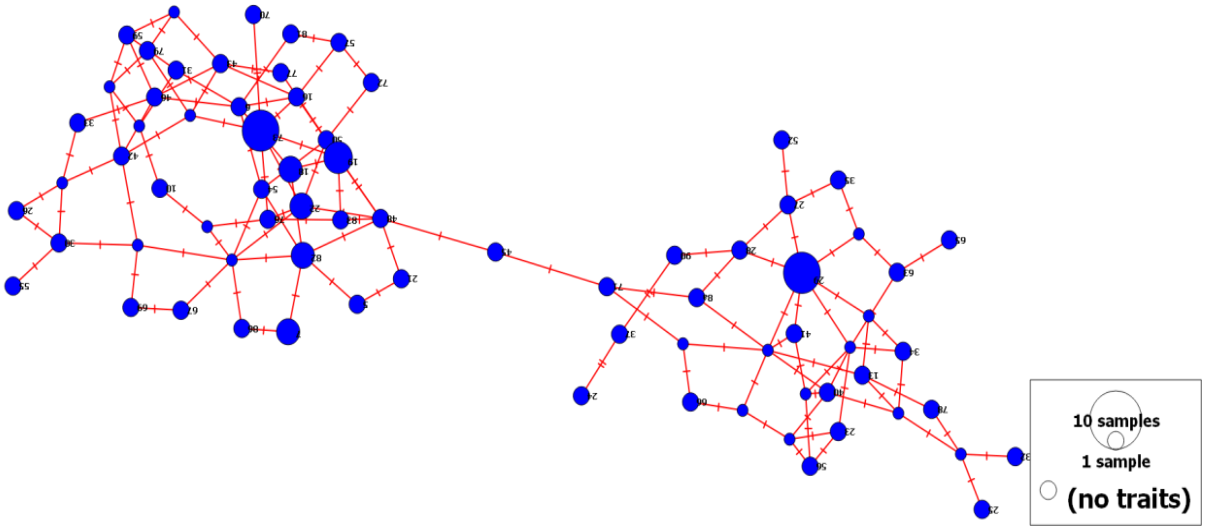


Figure 3.46: Median-joining (MJ) network constructed in PopART inferred from phenotypes traits of *Pimpinella saxifraga* from Germany is represented by circles whose sizes are proportional to the number of individuals.

With the morphological traits the factorial analysis, STRUCTURE analysis, Neighbor-joining (NJ) and Median-Joining network (Figures 3.43-3.45) placed the majority individuals in the same two clusters. The clustering of the *Pimpinella saxifraga* individuals using STRUCTURE analysis, Neighbor-joining (NJ) and Median-Joining the network was very consistent. However, this grouping was not in concordance to their geographical regions.

CHAPTER IV
DISCUSSION

4. Discussion

In recent years, an increasing number of researchers have understood that it is essential to preserve the genetic diversity of natural populations to include their continued survival and supply a chance for the evolution of species (Frankham et al., 2002Traditionally, analyses of differences in plant morphology and phytochemical traits have been used to estimate diversity (Kubeczka et al., 1989 and Latowski et al., 2009). However, when using these methods, only limited information is available for *Pimpinella saxifraga*, because such traits are not constant in different environmental conditions. Recently, DNA molecular marker techniques have been used to analyze *P. saxifraga*; this has become possible through the use of ISSR (Gemeinholzer *et al*., 2020). However, these studies have been very much focused on the study of genetic relationships within and between the regions of *P. saxifraga*. It is generally recognized that a greater number of molecular markers is required for genetic phylogenetics, thereby ensuring evolutionary and diversification of the species.

The Apiaceae family includes several species that are economically important and used by mankind. One of these species is *P. saxifraga* in the genus *Pimpinella.* However, there are only limited numbers of SSR markers in the literature for the Apiaceae family (Tew *et al.,* 2012; Rijal *et al*., 2015 and Michalczyk *et al.,* 2016), and no study on the development of SSR markers in *Pimpinella* currently exists in the literature.

SSR is an extremely practical molecular marker in population genetics studies because it is able to measure co-dominant alleles and it also offers high levels of polymorphism. The current study is actually the first to research the genetic diversity and population structure of *P. saxifraga* using SSR markers.

In the present study, we used data from morphological and molecular (SSR) markers to evaluate the diversity within the *P. saxifraga*-group. Morphological analyses of *P. saxifraga* taxa showed that quantitative indicators (from the Kruskal-Wallis test results) and qualitative characteristics are clearly differentiated from each other. PCoA analysis suggests that morphological characters, such as leaflet shape, leaflet teeth, leaflet margin, and indument on the surface of the leaflets, have the potential to identify and delimitate *P. saxifraga* taxa. The results from the principal

component analysis suggest that the utilization of morphological characters that play a key role in plant systematics and taxonomy are able to identify and delimitate *P. saxifraga* taxa.

Our work also highlighted the significance of morphological characters and molecular data in identifying and studying the species' genetic diversity. In general, the genetic relationships obtained from SSR data show a low agreement with morphometric results. This is in accordance with the parameters of AMOVA and with genetic diversity results. SSR molecular markers detected low-level genetic differences among taxa. However, these results indicate that SSR markers do not have enough potential to enable plant systematics and taxonomy in *P. saxfraga* taxa to be fully studied.

The aim of this study is to present an analysis of the genetic diversity and genetic differentiation of *P. saxifraga* individuals within the entire distribution of the biogeographical region in Germany and to correlate this with morphological groups based on literature delimitation. The following four main results, derived from an analysis of 78 individuals with nine SSR markers, were: (1) the transferability the SSR markers originally developed for *D. carrota* in *P. saxifraga* taxa, (2) the molecular data separated individuals from their biogeographic regions and the analyzed markers showed a low level of genetic variation among biogeographical regions in Germany, (3) the morphological character states discriminated taxa from Germany and, (4) molecular and morphological data were congruent in their information content concerning diversity.

4.1.Hypothesis I

We tested the inter-generic transferability of 44 microsatellite markers that were originally developed from *Daucus carrota* in *Pimpinella saxifraga* and both of which belonged to the Apiaceae family. This study used nine microsatellite markers, namely, specific and reliable amplified products that would allow a detailed study of the genetic diversity and structure of this particular plant.

4.1.1. Transferability of SSR markers

Simple sequence repeats (SSRs) have both a co-dominant nature and a high polymorphism. For this reason, they are widely considered to be very robust and informative concerning plant and

animal species (Ellegren, 2004 ; Han *et al.,* 2015). The highly variable nature of SSRs produces very high allelic differences, even among very close-linked varieties (Litt and Luty, 1989). SSR markers are credible materials for genetic diversity and population structure analysis as well as for genetic mapping, fingerprinting, and identification in plants However, sequence information is needed for the primer development for each species; the development process is, therefore, both expensive and time-consuming, with the result that SSR markers have been hardly used extensively for plant diversity or genotype identification (Parmer *et al*., 2015). As the development of SSR is labor-intensive, time-consuming, and costly, it is indeed both highly valuable and extremely useful to investigate the transferability of SSR markers through linked species/genera (Kumar *et al.,* 2014).

SSR markers are therefore considered to be the markers of preference for the characterization of core collections in addition to for the management of germplasm collections. Furthermore, one of the characteristics that produce these markers particularly interesting in studies of genetic diversity is their rise average of transferability to closely related species (Zhou *et al*., 2014). However, significantly lower values of cross-transferability have been observed for genomic SSRs, which are known to be more polymorphic but are located in less conserved regions of the genome (Sourdille *et al.,* 2004 ; Aiello *et al.,* 2020).

SSRs have been developed and extensively used in other Apiaceae species (cumin (Kumar *et al*. are, 2014), celery (Li *et al*., 2014), coriander (Choudhary *et al.,* 2017), dill (Jethra *et al*., 2018), and Fennel (Aiello *et al*., 2020), etc.). The transferability of molecular markers across species or genera has been used in several families, such as, for example, Sapindaceae (Ekue *et al*., 2009), Apocynaceae (Mishra *et al*., 2011) Rosaceace (Fan *et al*., 2013), Myrtaceae (Rai *et al*., 2013), and Solanaceae (Parmar *et al*., 2015).

Overall, the primer's transferability results showed a similar success, compared to what had been found in other studies The use of microsatellite markers, however, was particularly useful in these earlier studies. In the present study, we used groups of SSR microsatellite markers from *D. carota* to assess the transferability and phylogenetic relationship across the *P. saxifraga* in the Apiaceae family.

The probability of microsatellite marker transferability increases with any enlarging phylogenetic relationship (Varshney *et al.,* 2005). Molecular ecologists are increasingly demanding genetic markers that can be easily transferred between species. The distribution of the nuclear microsatellite's cross-species transferability is highly variable in flowering plants. There is considerable evidence of microsatellite marker transferability within genera but scant evidence for a higher taxonomic level than the genus. For example, the success rate of microsatellite transferability within genera in eudicots is about 60%, compared to 10% across genera (Barbara *et al.,* 2007).

Therefore, it is always useful to identify evolutionarily close relatives before checking the SSR markers for cross-species transferability when microsatellites markers are not available for sister species within the genus. In our own phylogenetic analysis, we found *D. carota*, *Apium graveolens*, *Heracleum mantegazzianum* and *Eryngium alpinum* were the only taxa with microsatellite markers developed within the clade and they were only distantly related to *P. saxifraga* (Piya and Nepal, 2013). Previous studies demonstrated the transferability of microsatellite markers *Daucus* and *Heracleum* to other species (Henry *et al*., 2008; Cavagnaro *et al.,* 2011). We therefore deliberately chose microsatellite markers that had been developed for these two species to test their transferability to *P. saxifraga* We chosen and tested 44 genomic SSRs of *D. carota* that had already been evaluated by Henry *et al*. 2008 and Cavagnaro *et al*. 2011 for its cross-transferability in other Apiaceae species.

The *Daucus* primers examined for the amplification of *P. saxifraga* were categorized as follows: nine primers were amplified and provided cross species transferability by producing a clean band with a nearly expected amplicon size (20%), and 11 primers produced multiple bands (25%) but 24 primers did not produce any band (56%). These results indicated that they were transferable to *P. saxifraga* (Table 3.1, Figure 3.3). The identified nine nuclear microsatellites were then used for genotyping *P. saxifraga* samples that had been collected from different geographical regions in Germany. Our results were in concordance with many former studies that had shown the intergeneric transferability of SSR, such as, for example, in *Setaria italica* to six grass species (74 %; Gupta *et al.,* 2012), *Psidium guajava* to *Syzygium aromaticum* (73.9 %; Rai *et al*., 2013), *Litchi chinensis* to *Blighia sapida* (58% Ekue *et al.,* 2009) and *Daucus carrota* to *Cuminum cyminum* (38 %; Kumar *et al*., 2014). When transferring SSRs from *Eucalyptus* spp. to

Escherichia dysentery, the percentage of polymorphic loci decreased to 70% (Zucchi *et al*., 2002), and the high level of genomic transferability of maize SSRs (74.5%) has also been reported to affect sugarcane too (Hernandez *et al.*, 2001), indicating that the potential of SSR markers for transferability across races belongs to the same families.

A review analysis of cross transferability by Rosetto (2001) brief the data from 19 studies and found that 58% of microsatellites were polymorphic within the same family and 78% within the same subgenus. Thus, the results acquired in this study were within the previously referred to range.

Of all the nine primer pairs tested, 3 were considered to be compound markers and six were considered to be simple markers (Table 2.2). Among these six simple pairs, four were perfect and two were imperfect markers. Four of the six perfect markers and just one of the imperfect SSRs were transferable to *P. saxifraga*. Interestingly, this was also a finding that had been observed in a previous study by Ekue *et al.* (2009) on transferable markers from Lychee to Ackee .This clear association between the transferability and perfection of repeat sequences had also been suggested previously by Cottle & Williams (2001).

Di and tri-nucleotide repeats were the major types in many plants, but the predominant motifs were different in different species. such as, for example, the most abundant repeat motif di-nucleotides in *Scaligeria lazica* (Baldemir *et al*., 2017) and also in *Centella asiatica* (Sahu *et al.*, 2015). In our study, however, the di- and compound nucleotide repeat motifs were at the same frequency and proved to be the most abundant motifs (33.33%), followed by the tetra-nucleotide repeats that accounted for 22%, and the tri-nucleotides repeats motifs that were only 11%.

The polymorphism information content (PIC) is an important parameter of a DNA marker to calculate the discrimination power and informativeness of SSR markers; it is also the most important feature to determine the quality and efficiency of primers. In general, markers with PIC> 0.5 are defined as being highly polymorphic, PIC < 0.25 as being low, and 0.25< PIC<0.50 as being medium (Botstein *et al*., 1980). In our case, the average PIC value of the 9 SSR markers used was 0.57, which is higher than many of the SSR markers developed from other species , such as *Foeniculum vulgare* (0.267) (Aiello *et al*., 2020), *Trigonella foenum-groecum* (0.43) (Jethra *et al*., 2018), and *Cuminum cyminum* (0.38) (Bharti *et al*., 2017) Three markers

(GSSR154, BSSR53, and BSSR76) had a moderate informative value of the used nine microsatellite primers. The high PIC values observed in our work gratifyingly pointed towards a high efficiency of this particular set of SSR markers in the genetic diversity in *P. saxifraga* and towards an invaluable usefulness in detecting the polymorphism rate at a particular marker. Our data provided indisputable evidence for the transferability of SSRs in plants more closely related to carrots such as *Pimpinella*, for it has already been previously reported, that closely related species share similar SSR priming sites that tends to result in the easy transferability of SSRs (Rai *et al.,* 2013). The effect of the evolutionary relationship between source and target species on SSR transfer success has been widely observed in many plant families (Rossetto *et al*., 2001; Arnold *et al.,* 2002).

The transferability of carrot SSR markers to *P. saxifraga* is demonstrated and proven in this study, thereby showing that screening of SSR markers from different species can lead to the development of SSR markers in *P. saxifraga* (Choudhary *et al*., 2013). It may even be potential to raise the success rate of cross-species SSR amplification by a database of SSRs and searching for expressed sequence tags (ESTs) (Choudhary *et al*., 2013).

4.1.2. Characterization of transferable carrot molecular markers

The genetic diversity of a wide range of plants was estimated using SSR markers to select and recommend particular populations for conservation (Garcia *et al*., 2004; Acquadro *et al*., 2005; Cavagnaro *et al*., 2011; Matin *et al*., 2012; Wangari *et al*., 2013; Piya *et al*., 2014; Olango *et al*., 2015; Krupa, 2017 and Frese *et al.,* 2018).

To characterize the genetic diversity and population structure of 78 *P. saxifraga* individuals, collected from Germany, we evaluated 9 microsatellite markers. *P. saxifraga* is distributed in Europe and Western Asia but has also been introduced to Northern America (Tutin *et al*., 1968 and Kisiel *et al*., 1998) and even used in folk medicine (Jodral, 2004) However, there are as yet in Germany no reports available on genetic diversity analysis using SSR markers with a significant degree of polymorphism. This is in actually the first study to date to detail the genotypes of *P. saxifraga* and to analyze and compare a large number of individual plants collected from herbaria in Germany. This original study was designed to characterize diversity in *P. saxifraga* genotypes at the molecular level.

The molecular weight amplicons ranged from 151 to 324bp, which suggested a remarkable variation in the number of repeats between the different alleles. The amplicon size was in agreement with the results of Cavagnaro *et al* (2011), where the SSR amplicon ranged from 190 to 355bp with the same set of markers.

There are several reports on SSR development in Apiaceae. Tew *et al.* (2012) reported eight SSR markers from *Lilaeopsis schaffneriana* subsp. *recurva* by fingerprinting 48 individuals from two natural populations and thereby acquiring 4.4 different alleles per locus. Rijal *et al*. (2014) created 25 SSR markers in *Heracleum*. *persicum* by characterizing eight geographically distant samples of *H. persicum* and acquired different allele (Na) of 2.3 alleles per locus. The mean values were *Ho* = 0.57 and *He = 0.42* for the 25 SSR markers. Baldermir *et al*. (2017) used 40 SSR markers in their genetic diversity analysis of *Scaligeria Lazica*, with a mean of 6.6 different alleles per markers by characterizing 40 individuals. The mean values were *Ho* =0.47 and *He* = 0.66. Maksylewicz and Baranski (2013) developed 27 SSR markers from carrot (*Daucus carota* L. ssp. *sativus*) and tested them in two F1 hybrids, six landraces and ten open pollinated cultivars, obtaining an average of 9.4 alleles. *Ho* = 0.56 and *He* = 0.39 values were also reported. Michalczyk *et al*. (2011) generated 12 SSR loci from *Cnidium dubium* and acquired a mean of 8.3 different alleles per markers by characterizing 40 individuals with the mean values of *Ho* =0.62 and *He* = 0.63. Ipek *et al*. (2016) tested 20 SSR markers from *Daucus carota* in order to study genetic variation within the purple carrot population And thereby obtained a mean of 5.78 different alleles per markers with a mean value of He = 0.68.

In the present study, nine SSR markers were used in the genetic diversity analysis of *P. saxifraga*. These markers produced 112 alleles, the generated and characterized SSR marker being higher than those in the above studies for Apiaceae (Tew *et al.*, 2012; Rijal *et al*., 2015 and Michalczyk *et al*., 2016). The mean *Ho* value for the nine markers in this study was 0.74, which was higher than reported in the previous studies in Apiaceae. The mean *He* for nine SSR markers in this study was 0.61 which was higher than that for the *L. schaffneriana* subsp. *recurva* and *H. persicum* (Tew *et al.*, 2012 and Rijal *et al.*, 2015), but lower than that for *C. dubium* and *S. lazica* (Baldemir *et al*., 2017 and Michalczyk *et al*., 2011).

The difference in the mean number of alleles per marker (6.30) and the effective number of alleles (3.2) indicates the presence of some allele species specific to *P. saxifraga*. The effective number of alleles indicates in turn the minimum number of alleles required to maintain genetic diversity in that marker. The *P. saxifraga* specific alleles, observed in this present study, indicate the discriminative power of these alleles to achieve such diversity.

The mean fixation index equal (-0.258) indicates heterozygosity for the respective markers in *P. saxifraga* in this study. However, the average gene diversity (0.63) and Shannon's information index (1.26) indicated that the carrot markers in terms of their genetic and allelic diversity were of particularly effective use in *P. saxifraga,* and this is also supported by the higher PIC value measured in most of the markers.

These findings provide further evidence for the potential transferability of SSRs across genera. Previous studies involving many plant families have revealed that generally only a low percentage of markers amplify fragments from species belonging to different genera (Weising *et al.,* 2005). An analysis of *P. saxifraga* from regions outside the native range using nuclear markers can supply important insight into the genetic structure of populations. Because nuclear DNA markers are bi-parental, they differ in their facilities to detect the real-time operating population processes. Therefore, analyses of *P. saxifraga* regions from a native range but using these markers can indeed provide insights into the whole *P. saxifraga* evolution (Sunnucks, 2000).

Overall, the present investigation is the first inclusive report on the development and applicability of SSR markers. It also provides additional evidence for the potential transferability of SSRs across genera and supplies supportive proof for the potential transferability of SSRs' markers across the *P. saxifraga* taxa of Apiaceae. This group of developed SSR markers was found to be cross-transferable, thereby serving as a precise resource for genetic research in *P. saxifraga* on a variety of aspects, such as genetic diversity and marker-assisted selection studies and also in the genera/species of the Apiaceae family for which at the moment no report yet exists on phylogenetic relatedness as well as on the characterization and population structure of *P. saxifraga*.

4.2. Hypothesis II

Using microsatellite SSR, we characterized the selected biogeographical regions of *P. saxifraga* individuals in Germany. To understand the contribution of each region to the overall diversity of the entire group, we analyzed their genetic diversity and genetic differentiation The analyzed markers revealed a low level of genetic variation among the various biogeographical regions in Germany.

Species with a wide geographical distribution face several environmental conditions and may thus exhibit a broad genetic and morphological diversity to deal with the environmental challenges (Freeland *et al*., 2011). This work highlighted the significance of morphological characters and molecular data to identify and study species' genetic diversity and genetic differentiation. SSR molecular markers detected genetic diversity among individuals But also a low-level differentiation among biogeographical regions.

Studying population genetics provided valuable information about the genetic structure of plants, the genetic variation of the populations, etc. (Sheidai *et al.,* 2014). Genetic changes can switch actively, thereby decreasing the genetic diversity within a population (Setsuko *et al*., 2007; Sheidai *et al.,* 2016). This information, however, has different applications, ranging from a pure understanding of species biology for conservation to a selection of suitable parents for crossbreeding, and also to phylogeography (Freeland *et al.,* 2011).

In recent years, an increasing number of researchers have realized that it is important to maintain the genetic diversity of natural populations to ensure their continued survival, fitness and potential for the evolution of species (Frankham *et al*., 2002). Traditionally, differences in plant physiology and morphological traits have been used to assess diversity. However, using these methods, only somewhat limited information was available about this species, because these traits are not stable in different environmental conditions

Recently, a range of DNA molecular marker techniques have been used to analyze *P. saxifraga,* including the use of ISSR (Gemeinholzer *et al*., 2020).

P. saxifraga is widespread in Germany, but we did not have information on its genetic structure or any detailed taxonomic information. The current study, however, revealed interesting data

about *P. saxifraga* genetic diversity, genetic differentiation and morphological divergence in Germany. However, these studies were focused on investigating the interspecific relationships and it was generally recognized that a greater number of molecular markers are now required for genetic studies within *P. saxifraga*. SSR is the most practical molecular marker in population genetics studies because it is able to measure co-dominant alleles and the present high levels of polymorphism. SSR markers are credible tools for studying genetic diversity and carrying out a population structure analysis as well as for genetic mapping, fingerprinting, and parental identification in plants. The current study is in fact the first to investigate the genetic diversity and population structure of *P. saxifraga* using microsatellite markers, which contribute significantly to the conservation, management, and further understanding of genetic relationships. However, we did not have sufficient information on its genetic diversity and structure and also lacked detailed taxonomic data.

4.2.1. Genetic diversity of *P. saxifraga* among various regions

It is crucial to assess genetic diversity to ensure that the most diverse populations are selected, thereby expanding the base of genetic resources. The genetic variance was higher than in previous genetic studies of *Pimpinella*. However, these studies cannot be directly compared because different marker systems can lead to slight differences in the results obtained.

Generally, species with wind dispersal, wide distribution, and outcrossing show a high genetic diversity, in which population size mutation can also affect genetic diversity (Amos and Harwood, 1998; Nybom, 2004). Genetic diversity is based on different specific factors, such as the mating system, the reproductive mode, biological traits, life history, geographical range and evolutionary history: species with small geographic ranges tend to have a lower genetic diversity than geographically dispersed species (Hamrick and Godt, 1989), and others originating in human activity factors, such as overgrazing, habitat degradation, overutilization of natural resources, and ground rehabilitation, are all regarded as important elements that affect the genetic diversity level (Hamrick and Godt, 1996; Leimu *et al.,* 2006; Dong *et al*., 2007; Qiao *et al*., 2010 and Wu *et al*., 2016).

The studied regions had a high level of genetic diversity. Genetic diversity is of fundamental importance in the continuity of a species as it is used to bring about the necessary adaptation to

cope with changes in the environment (Shedai *et al.,* 2013). The degree of genetic variability within a species is highly correlated with its reproductive mode, with the higher degree of open pollination bringing about a higher level of genetic variability in the studied taxon (Freeland *et al*., 2011).

In this study using SSR makers, nine microsatellites were developed and used to screen 78 *P. saxifraga* individuals . The results from our study showed that the genetic diversity level of *P. saxifraga* in biogeographical regions in Germany was of a moderate to high level (Ho = 0.74, He = 0.61) among the *P. saxifraga* species, even though it is a widespread species in Germany. Compared with former research into. *saxifraga*, the genetic diversity parameters observed in this study were slightly higher than those of *Apium graveolens* (Ho = 0.14, He = 0.36) (Fu *et al.,* 2013), *Scaligeria Lazica* (Ho=0.47, He= 0.66) (Baldemir *et al*., 2017), but lower than *D. carrota* (He= 0.83, Ho=0.76) (Reiker *et al*., 2015) and *Daucus carota* (He = 0.68) (Ipek *et al*., 2016) which was like the same primer, equal to the *Ferula tadshikorum* (He = 0.72 , Ho = 0.605) (Yang *et al*., 2021). The genetic diversity compared with the indigenous *P. saxifraga* but, on using ISSR markers, a level that was not so high (He = 0.356) was also noted (Gemeinholzer *et al*., 2020). This also occurred when using SNP alleles in the *Pimpinella* species (Ho=0.67, He=0.49) (Mehravi *et al*., 2021) The relatively higher frequency of the observed heterozygosity (Ho) than the one expected (He) for this study suggests that in this species it may be due to the presence of more heterozygous individuals but comparing results from different studies is particularly difficult because of the type of markers used (Powell *et al.,* 1996). However, our results are in line with those supporting a moderate to high genetic diversity in species with small populations (Luan *et al*., 2006; Yang *et al*., 2021)

Using genotype identification and similarity analysis in *P. saxifraga*, a means of 6.3 observed alleles and of 3.20 effective alleles per locus were revealed in this study. The difference between the average number of observed alleles and the effective number of alleles was due to the unequal number of individuals per region.

PIC supplied an assessment of the discriminatory power of a marker to differentiate genotypes that depend on both the number of alleles being expressed and on their relative frequencies (Nagl

et al. 2011). The average PIC value was 0.57, which indicates the isolation of highly polymorphic microsatellites.

The overall genetic variability for the individuals studied, according to Shannon's indexes, was especially high with an average of 1.17. The high value of Shannon's information index supports the effectiveness of microsatellite markers to detect a variation. The results indicated that all markers deviated significantly from HWE.

Mean negative inbreeding coefficient (Fis) values (-0.25) indicated an excess of heterozygotes, but, although *P. saxifraga* had an exogamous system, all four biogeographic regions presented highly negative (Fis) values. We hypothesized that this result might have been due to different causes, such as the destruction of critical habitats, which can lead to non-random mating between individuals within regions (Tong *et al*., 2019).

Of all the regions, the central ones had the highest genetic diversity values, He = 0.65, while the northern region had the lowest levels of genetic diversity (Table 3.3). The lowest level of private alleles was in the Alpine region, and eventually the number of samples in this region were not enough to show any greater diversity. This marginal distribution would decrease the opportunity to connect with other regions and might thus lead to a low level of genetic diversity.

P. saxifraga presents a moderate to high level of genetic diversity in all geographical regions, and this may be attributed to the biological characteristics of *P. saxifraga*. The geographical boundary of *P. saxifraga* ranges from the northern part to the southern part of Germany with gradual altitude in the Alps. The widespread distribution and varied habitats may lead to high levels of genetic diversity. On the other hand, the mating system with *P. saxifraga* is predominantly outcrossing (Willemstein, 1987), as shown in previous studies in which insect pollination occurs in the genus *Ferula* (Yaqoob and Nawchoo, 2016), which can also lead to a high level of genetic diversity. However, according to Knuth (1908), East (1940), and Berger *et al*. (1975), self-pollination can also be used. The main reason for this could be the different collection sampling sites overall 70 years ago, but another reason could be the various types of climatic environments and soil conditions in these sampling sites

4.2.2. Genetic differentiation of *P. saxifraga*

Genetic differentiation coefficient important parameters are used to assess the genetic structure of a population (Hamrick and Godt, 1989). The present study provided information about the genetic diversity of *P. saxfraga* taxa in a variety of geographical regions of Germany. The result of the AMOVA analyses showed that there was significant genetic variability (98% of total genetic variation) within regions, whereas merely 2% of genetic differentiation existed within the evaluated regions. The breeding system was one of the key factors determining the distribution of genetic differentiation in plant regions (Dumini *et al*., 2007; Nybom and Bartish, 2000) and in the studied regions, and this could be related to the outcrossing substance of the species. In reality, gene migration increases the genetic variability of the regions (Sheidai *et al*., 2014).

According to Wright (1965), the level of genetic differentiation among regions is low when the coefficient of genetic differentiation (*Fst*= 0.02) is less than 0.25 of the four biogeographical regions. Our results showed that the genetic differentiation level of *P. saxifraga* individuals was very low among the regions of *P. saxifraga* (*Fst* less than 0.05), and the AMOVA results showed that only 2.0% of the total genetic variation occurred among regions due to their insect pollination, high out-crossing rates, and transfer of pollen by humans and animals. Gene flow and the genetic differentiation coefficient are negatively correlated (Perdereau *et al*., 2014); this can be attributed to the massive gene flow (Nm = 12.725) that counteracted the effect of genetic drift and reduced genetic variation between regions.

4.2.3. Genetic structure of *P. saxifraga*

The genetic structure depends on the Bayesian model, which presented 78 genotypes of *P. saxifraga* and which itself is coordinated with the results of a cluster analysis, based on Nei's genetic distance. These were then divided into two genetic clusters with a maximum ΔK value at K = 2. However, the Bayesian model failed to assign populations into groups depending on their geographic origins. Furthermore, the Mantel test showed a positive but not significant correlation between geographic and genetic distance patterns (r = 0.096, *P* = 0.094), indicating no clear isolation by distance among the investigated *P. saxifraga* biogeographical regions. Therefore, it was estimated that the genetic structure of the *P. saxifraga* population in Germany could not be affected by geographic distance. Notably, each cluster displayed a high admixture of alleles, and no refined lines were observed. These results were further promoted by the PCoA and factorial

analysis, also supported by the hierarchical neighbor-joining tree (NJ) Thus, the average of gene flow, Mobility is perhaps the most important factor affecting the rate of gene flow between different populations (Mayr,1997). Slatkin (1985) considered that if Nm>1, the exchange of genes between populations can prevent the effect of genetic drift and thus reduce the genetic variation between regions. *P. saxifraga* is pollinated by wind- and insects; pollen spreads easily over medium and long distances. In addition, the seed shape of *P. saxifraga* allows the animals to move easily over long distances. All of these factors could contribute to the high gene flow observed between different regions.

4.3. Hypothesis III

We analyzed morphological traits, both quantitative and qualitative characteristics in the different herbaria in Germany. Among morphological traits five diagnostic delimiting characteristics, namely, leaflet shape, leaflet margin, hair on the surface of the leaflets (indument), plant length and basal leaf length, exist to clearly separate the species of *P. saxifraga*

Problem sources. The identification of species in a taxonomic context methodically implies the recognition of morphological features, which usually present a high intraspecific variation within the *Pimpinella saxifraga* taxa, resulting in species delimitation problems. Among the main causes of such variation are plasticity, convergence, hybridization and introgression, as well as incomplete divergence.

Identifying lineages and delimiting species within German *P. saxifraga* is not clear due to the recent radiation of the species (Tutin *et al.*,1968) and to morphological data from herbarium specimens of the *P. saxifraga* group that were analyzed as a first step to identify sub-specific taxa within it. In general, the taxon *P. saxifraga* can be defined depending on morphology, but not all currently recognized taxa of labeled name entities are morphologically characterized. These morphological clusters will be further tested by SSR microsatellite and then incorporated within the *P.saxifraga* canon.

Among the morphological characteristics that we measured in our collection of entries were quantitative and qualitative characters (Table 2.4) Here, we have the first comparative study on the morphological and genetic parameters of *P. saxifraga* taxa, For all of these traits, we found

significant variations between each other and with different altitudes indicating morphological differentiation between taxa.

In the present study, we found morphological characteristics to be important for the differentiation of species or subspecies varies significantly among entries for *P. saxifraga*. Morphological analyses of the *P. saxifrafga* species clearly showed that quantitative indicators (Kruskal-Wallis test results) and qualitative characteristics are well differentiated from each other.

Among the morphological characteristics of the quantitative traits studied in the *P. saxifraga* species taxa, it could be seen that they are well differentiated between the various biogeographical regions in significant quantitative measures, such as plant length and basal leaf length (the Kruskal -Wallis test result), and the main reason for this may be because it is based on different temperatures, the average precipitation and different altitudes.

The correlation between quantitative morphological traits, such as basal leaf length and plant length, with graduated altitude was a significantly negative correlation coefficient. Basal leaf length and plant length, which are important botanical features of plants adapting to the environment. inclusively reflect an important taxonomic and function reference; when comparing plant length, basal leaf length and the number of leaflets in a basal leaf, they decrease with elevation, indicating that *P. saxifraga* growing at higher altitudes have smaller plant lengths and basal leaf lengths than those at lower altitudes. This has been observed in previous studies on leaf size (Körner, 2003; Liu *et al*., 2018; and Liu *et al*., 2020),the main reasons for which being given as mean annual temperatures and altitude wind exposure. These results were in fact further supported when the altitude was divided into three groups, with the first group being under 200m, the second group between 201 to 500m and third group above 501m,. This was then compared with morphological traits: the plant highest and most significant under 200m (This mean the lower elevation), the plant length increase, also the basal leaf length most significant under a 200m altitude with other groups of altitude, the plant length's high significance in lower altitude, and how it plant length is decreased by increasing the elevation and also the basal leaf length.

Despite their difficult morphological traits for differentiation, the results of the PCoA analyses of the morphological trait data set clearly showed that *P. saxifraga* cannot be differentiated in accordance with geographical regions but it can be morphologically distinguished, although a few specimens do show a somewhat intermediate morphology.

The qualitative morphological traits, such as the leaflet shape, the leaflet margin, the leaflet teeth, and the hairs on the surface of the leaflets, were tested with PCoA and a factorial analysis (Figures 3.33; 3.34).

Our morphological traits investigation revealed that using a neighbor-joining tree (NJ) divided two main clusters: cluster I which had pinnatifed lobed liner teeth irregular deeply margin contained 23 individuals, cluster II ovate serrate teeth regular shallowly margins contained 48 individuals, and 7 individuals intermediate between two clusters. This was particularly supported by the STRUCTURE plot (Figure 37) and by the Median-joining (MJ) network (Figure 39) that identified the two main groups.

The PCoA results suggest that morphological characters are used to identify and delimitate *P. saxifraga* taxa. Morphological characters play a key role in plant systematics and taxonomy and may be used in species group delimitation. This morphological difference is due to qualitative traits, namely, the ovate serrate margin in *P. saxifraga* var. *eu- saxifraga* (Figure 4.1) while there were pinnatified linear lobed teeth in *P. saxifraga* var. *dissecta* among the studied species (Figure 4.2)

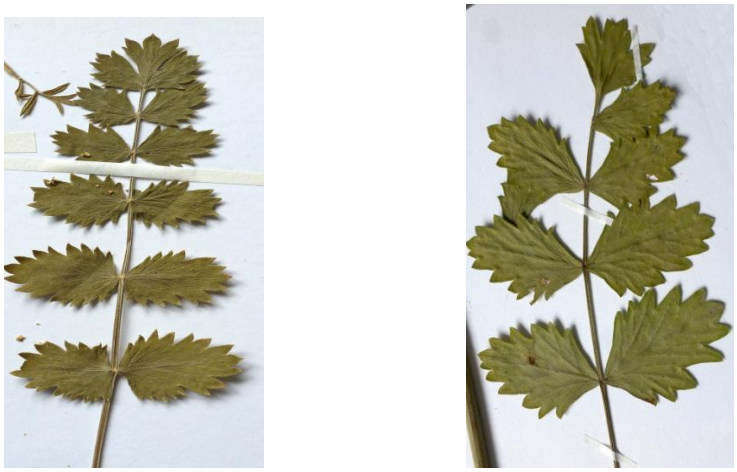


Figure 4.1: Leaf shape of *Pimpinella saxifraga* ovate serrate margin leaflet.

Figure 4.2: Leaf shape of *Pimpinella saxifraga* with a innatified lobed linear teeth margin leaflet (Maybe *var dissecta*).

These characters and several others resulting from further examination of the herbarium specimens, such as the leaflet shape, the leaflet margin, the leaflet teeth, the hair on the surface leaflet, the plant length, and the basal leaf length can be used to reliably distinguish *P. saxifraga* taxa from each other. All of these characteristics can be easily observed in a field setting with the use of a hand lens and a dissecting microscope. Some previous studies that depended on morphological characteristics support what we have obtained about the morphological characteristics of the samples that were studied. For example, Hegi (1926, 1975) showed how *P. saxifraga* taxa differentiated in the shape of its leaf and leaflet among different taxa (Figure 4.3). Sell and Murrell (2009) in Flora of Great Britain described basal leaves in various dissecta with segments dissected into linear and hairy lobes. Tutin (1968**)** focused on the sub-species *Pimpinella dissecta*, the main distinguishing trait of which was pinneatisect with linear lobes.

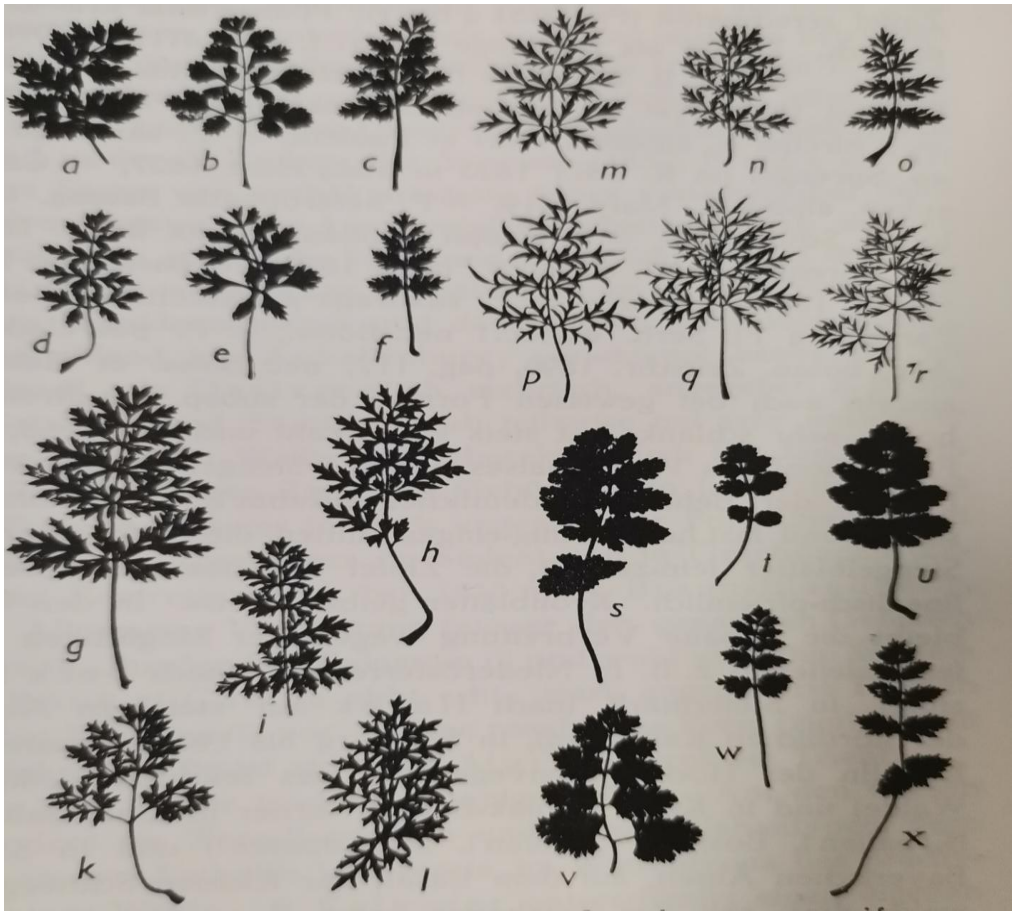


Figure 4.3: *Pimpinella saxifraga* L. subsp. eu-saxifragaThell.- *s* bis *x* : var.minor Sprengl und var. *ovate* Sprengl, *a* bis *f* var. intercedensThell. *g* bis *r* var. *dissecta* (Ratz.) Sprengl (c bis l *intermedia* – Dissecta Petersen; *m* bis *r* Type *dissecta*. (Hegi, 1965).

In Latowski *et al*.'s (2009) study the morphological traits of *Pimpinella saxifraga* seven quantitative traits were detached (plant height, the number of petioles in the main umbel and in the side umbels, number of leaflets in basal leaves, length of schizocarp measured with each other with the nectar-disk, pistil neck length in the flowers and on ripe fruits), in addition two qualitative traits (surface colour) the outer surface of the root with its blue aspect after the root is broken and degree of appearance of the stem. As a result of these morphological traits studies, there are two separate forms (*Pimpinella nigra* and *Pimpinella saxifraga*), in which the *Pimpinella nigra* form is significantly more refined than the *Pimpinella saxifraga* one. Biometric ranges overlap for some features indicating plants of the middle (hybrids).

The great morphological variability resulted in being were no characteristic taxa within each group in *P. saxifraga*. The geographic distribution of the genera is synchronized with the

complex style of climatic and soil conditions throughout the whole *P. saxifraga* range but this creates problems for its taxonomic treatment. Because environmental conditions can alternate sharply or gradually change across a region, it is hard to separate taxa or regions with unique complexes of characters or to draw boundaries between them. Some local regions possess morphological traits no less important than those of large geographical taxa. However, when it comes to taxonomic segregation, the tendency is to hesitate rather than to explain the patterns of variation within the species.

Different mechanisms may cause differentiation among regions that may, as a result, differ in phenotypic characters and allelic composition (Schmid and Guillaume, 2017). We must mention that the studied individuals differed in quantitative and qualitative morphological characteristics and we still do not know the number of morphological differences between the genetically studied individuals; they may also be influenced by environmental conditions and altitudes (Guo *et al*., 2018 ; Leal-Saenz *et al*.,2020) and for this reason, we have not attempted to suggest any new taxonomic forms below the species level for this taxon but considered as different morphotypes only.

4.4. Hypothesis IV

Congruence between morphological characters in the study with SSR microsatellite markers

Determining species delimitation in complex groups and in those in which there are different degrees of morphological overlap of species is indeed a daunting and challenging task. In these cases, we decided to use different and aggregated approaches, such as cytological, molecular, morphological, etc., to define the species' boundaries (Carstens *et al*., 2013). In the past few decades, the use of molecular markers as tools for identifying species and subspecies delimitation has increased dramatically (Esfandani- Bozchaloyi *et al*., 2017).

Population genetic studies provide valuable information about the genetic structure of plants, as well as about the stratification and genetic divergence of the plant populations, etc. (Sheidai *et al*., 2016). AMOVA and STRUCTURE analysis have revealed that the species are genetically differentiated as being low between biogeographical regions. The present study revealed that the use of SSR molecular markers was not enough along with morphological characters in *P.*

saxifraga species delimitation. A previous study (Gemeinholzer and Bachmann 2005) of species delimitation between *Cichorium intybus* and *Cichorium spinosum* in Asteraceae supports the contention that such a study can distinguish between species by focusing on morphological traits but that molecular markers maynot be enough or that we need other molecular markers to support differentiation between subspecies of *P. saxifraga*.

Incongruence across the results from various methods is proof of a difference in the ability to reveal cryptic lineages across one or more methods applied to delimit species and could signalize that assumptions of one or more of the methods have been violated. anyway, the conclusion drawn from species delimitation studies has to be conservative, because in most contexts it is best to fail to delimit species than to fail to delimit entities that do not perform actual evolutionary lineages (Carstens *et al*., 2013).

5. Conclusions and Future research

Pimpinella saxifraga L. (Apiaceae) is native in Germany,. Due to large morphological variation and the wide range of geographical distribution, it is taxonomically complex. For these reasons, in this study, molecular data and morphological traits were used for separating individuals from biogeographic regions and discriminate taxa from Germany.

The results of the present study showed the successful transferability of nine SSR microsatellite markers from *Daucus carota* to *Pimpinella saxifraga*. Since these two species belong to two distantly related genera, the transferability of SSR microsatellite markers between these species suggests that these microsatellite markers may work with other genera within the family Apiaceae. These microsatellite markers are polymorphic and are useful for testing the population structure and genetic diversity of *Pimpinella saxifraga*.

This study provided analysis and quantification of genetic diversity, population structure, and genetic relationship in *P. saxifraga* from Germany. SSR analysis showed low genetic differentiation and closed genetic relationships of *P. saxifraga* individuals. This can be assigned to outcrossing by insects pollination and maybe the exchange of the seed by animals through different regions. Therefore, the correlation of the individuals with their geographical regions was very low. Depending on molecular genetics analyses, there may be value in an additional and more in depth study of the morphological traits.

Morphological traits did not reveal any connection with the geographic region but separate taxa as groups. Thus, morphological characters of *P. saxifraga* provide a key for taxon identification but do not explain their taxonomic status as species. The applicability of our results to the formerly published morphological records is limited, mainly due to ambiguous terminology.

As we only focused on the taxa occurring in Germany with only 78 individuals, we need a broader and more comprehensive study of the species in Germany. Further studies involving all taxa from all over the world distribution, would be needed to gain a comprehensive understanding of the variety and subspecies delimitation in *P. saxifraga*.

The present study is by no means complete, but it does give a preliminary idea of the types of data needed for morphological and molecular phylogenies. More work is needed to define the

effect of cultivation on qualitative vegetative and reproductive traits of *P. saxifraga* taxa, in addition to the application of further specific molecular markers.

5.1. Future research

This thesis tells researchers what to do with morphological and genetic information. SSR markers have generated ideas about some of the relevant questions that need to be addressed in the future, questions which will most likely provide additional background information on how species delimitation in *P. saxifraga* is investigated, while conducting this research. Among these ideas that can be done in the future to reach a real investigation that helps scientists distinguish between *P. saxifraga* taxa. Five of these do indeed provide potential future directions for research and are therefore briefly identified below.

I. Chromosome count.

There are in fact only a few published chromosome counts that have been taken regarding *P. saxifraga* but these potentially show different chromosome races within *P. saxifraga*. The advantage of conducting a census is to then be aware the presence of any hybridization of species with other species in the same genus. Based on the pollination mechanism, I would suggest that the chromosome ploidy should be studied.

II. Mendelian analysis

These results show that *P. saxifraga* to be a particularly is an interesting example of the theory and practice of species delimitation and identification. Indeed if the *P. saxifraga* species can be crossed with other species in the same genus and produce fertile offspring, a direct Mendelian analysis of the genetic basis of their morphological differences is actually possible. The results of such an analysis could then form the basis for an experimental examination of the factors that maintain these differences in nature.

III. Sequencing microsatellite marker

The development of 9 microsatellite markers was determined; the number of alleles present at each locus varied between 6 and 26 (when only the *P. saxifraga* data set was considered [Table 3.5]). In a number of cases, those alleles diverged by a single base pair in size, despite the

microsatellite repeat being a double, a triple, or even a complex nucleotide. When microsatellite alleles differ by numbers other than the size of a nucleotide repeat, it can be due to mutations other than those that change the number of microsatellite repeats that have occurred, Microsatellite sequencing allows the revelation of these mutations. Also, transcriptome sequencing analysis of *Pimpinella saxifraga* will supply beneficial molecular information for functional and genetic characterizations.

IV. Different molecular markers

More genetic data (from other sources, or clarifying what to look for) are invaluable, perhaps as a perspective to also research the reproductive system. Molecular analyses have shown that there is no differentiation according to the biogeographical region, which is currently possible. But, as far as genetic distance is concerned there are some significant differences between individuals who emerge. As a result of this finding, we propose to use not only more samples and but also to use, in addition to SSR markers, other molecular markers, such as SNP and EST, or to design a specific primer by making use of Primer3 software. We will then compare the results derived from the various markers.

V. Morphological traits

A study was undertaken of the variation in leaf characteristics of *P. saxifraga* at different heights and of the adaptation strategy of plants to environmental changes are desirable research topics for the next steps, also the comparison with other *P. saxifraga* in Europe at the same sea level above 0, as we only focused on the species occurring in Germany. Further studies involving all taxa from all over the distribution of *P. saxifraga* would be needed to gain a comprehensive understanding of species delimitation. To complete the investigation of morphological data necessary to add characters common garden experiments in greenhouses are necessary, to check such as reproductive parts and other specific morphological traits; this could be done by using a scanning electron microscope or a leaf area meter instrument.

6. References

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Declaration

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" in carrying out the investigations described in the dissertation."

Giessen, 05.01.2022

Mohammad Saleh Jawarenh **Ort, Datum**