DETERMINATION OF GENETIC DIVERSITY OF RHODODENDRON SPECIES IN TURKEY

by

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B.S., Molecular Biology and Genetics, Bilkent University, 2013

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics
Boğaziçi University

2017

ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my thesis supervisor Prof. Müge Türet for her guidance, supervision and evaluations throughout my studies and for her endless efforts and contributions to the beginning of my academic life. I would like to thank my thesis committee members Prof. Aydın Türkeç and Assoc. Prof. İbrahim Yaman for their support, criticisms and valuable time.

I would like to thank my dearest interns Aybeg Nafiz Günenç, Burcu Özcan, Özge Osmanoğlu and Umut Batman for their dedication and hard work. Their motivation and positive attitude towards science motivated me further towards my thesis studies.

I would also like to thank my dearest colleagues Harun Niron and Mahmut Can Hız and all former and present members of PLANT, CMT and RETINA groups also for their endless support and friendship inside and outside of the lab. Emirhan Taşöz, Hayri Oğuz Arı, Burcu Ersoy, Nazmiye Özkan and Betül Demirkaya deserve special thanks. I would also thank to other members of MBG from the head to the technicians and janitors.

The last but not least, I have special thanks to my great family for their support and encouragement throughout my life.

This thesis was financially supported by The Scientific and Technological Research Council of Turkey (TUBITAK 112O500) and Bogazici University Research Funds (BAP 11140).

I gratefully acknowledge financial support from the Scientific and Technological Research Council of Turkey (TUBITAK 2210-E).

ABSTRACT

DETERMINATION OF GENETIC DIVERSITY OF RHODODENDRON SPECIES IN TURKEY

The Rhododendrons of Turkey are presented by 10 taxa and they grow naturally along the coast of the Black Sea region from Artvin province at the east to Istranca Mountains at the west. Although in many countries, cultivated natural forms or breeding varieties of Rhododendron genus are highly used in landscape planning and as ornamentals, in Turkey there are no cultivated local forms available and all commercial varieties are imported as potted plants. Recently, a national project has started with an objective to generate well documented ex-situ conservation of all naturally growing Rhododendron species of Turkey following a detailed morphological, molecular studies and tissue culture propagations. As partners of this project our aim is to analyze the genetic diversity by incorporating possible impacts of location and altitude to their diversity. The plant material has been collected from nature at different GPS locations and diverse altitudes along the cost of Black Sea and Marmara region. Total of 22 primers consisting of RAPD, ISSR and ITS markers were selected to study the genetic diversity and phylogenetic relationship among the samples. Genetic distance calculations of the data matrix to generate cluster analysis were performed by UPGMA using Pearson and Jaccard coefficient. The dendograms reflecting the genetic distance were drawn using MATLAB. All primers have shown 100% polymorphism, producing amplicons ranging between 200-3000bp. The average number of bands obtained for ISSR and RAPD primers were 19.1 and 20.9 respectively. The impact of geographic range on the species genetic diversity were clearly observed in cluster analysis when highly wide spread wild species were clustered as separate groups from the other wild species which has very narrow range of natural growth locations.

ÖZET

TÜRKİYE ORMAN GÜLLERİNİN GENETİK ÇEŞİTLİLİĞİNİN BELİRLENMESİ

Türkiye'nin Ormangülleri 10 takson ile temsil edilmekte ve doğudaki Artvin ilinden batıdaki Istranca dağlarına kadar Karadeniz ve Marmara kıyılarında doğal olarak büyümektedirler. Günümüzde Rhododendron cinsi içerisinde yer alan bitkiler, karakteristik özelliklerinden dolayı dış mekân düzenlemelerinde ve saksılı süs bitkisi olarak kullanım açısından son derece popüler bitkilerdir. Kentsel peyzaj planlamalarında kültüre alınmış doğal orman gülü türlerinin yanı sıra ıslah çalışmaları ile geliştirilmiş ve günümüzde sayıları binlerle ifade edilen orman gülü çeşitleri de sıkça kullanılmaktadır. Ülkemizde doğal olarak yayılış gösteren orman gülleri gösterişli süs bitkileri olmalarına rağmen yerli türler henüz kültüre alınmadığı için kentsel peyzaj uygulamalarında süs bitkisi olarak kullanılmamaktadır. Peyzaj planlamalarda kullanılan orman gülleri ise ithal edilerek, yüksek fiyatlarla tüketiciye sunulmaktadır. Türkiye'nin doğal olarak yetişen Rhododendron türlerinin ex-situ korunması için iyi belgelendirilmiş bir kaynak oluşturmak amacıyla ulusal bir proje başlatılmıştır. Bu projenin ortakları olarak, yer ve yüksekliğin çeşitliliğe olası etkilerini de dahil ederek genetik çeşitliliği analiz etmeyi amaçlanmıştır. Örnekler arasındaki genetik çeşitlilik ve filogenetik ilişkiyi incelemek için RAPD, ISSR ve ITS işaretçilerinden oluşan toplam 22 primer seçilmiştir. Küme analizi oluşturmak için veri matrisinin genetik uzaklık hesaplamaları, Pearson ve Jaccard katsayısı kullanılarak UPGMA metodu ile gerçekleştirilmiştir. Genetik benzerliği yansıtan dendogramlar MATLAB kullanılarak çizilmiştir. Tüm primerler, 200-3000 bp arasında değişen amplikon üretmiş ve % 100 polimorfizm göstermiştir. Coğrafi aralığın tür genetik çeşitliliği üzerindeki etkisi, oldukça geniş yayılmış yabani türlerin çok dar doğal gelişim yerlerine sahip diğer yabani türlerden ayrı gruplar halinde kümelenmesyle açıkça görülmüştür.

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LIST OF SYMBOLS

bp	Base Pairs
gr	Gram
hr	Hour
m	Meter
min	Minute
ml	Milli Liter
mM	Milli Molar
nM	Nano Molar
nt	Nucleotide
rpm	Revolutions per Minute
s	seconds
μg	Micro Gram
μM	Micro Molar
μmol	Micro Mol
°C	Centigrade Degree

LIST OF ACRONYMS/ABBREVIATIONS

dH2O	Distilled Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxynucleotide tri phosphate
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
KCl	Potassium Chloride
MgCl2	Magnesium Chloride
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
TAE	Tris Acetate EDTA
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA

1. INTRODUCTION

1.1. Rhododendron Species

The name of the Rhododendron comes from Ancient Greek "*rhódon*" (rose) and "*déndron*" (tree). The genus Rhododendron is one of the largest among angiosperms, comprised of more than 1000 species of woody plants in Ericaceae family, and still growing, as new species are continuously being described [1, 2]. Rhododendron species are mostly grown in Northern Hemisphere (Figure 1.1), China and Papua New Guinea are the centers of overwhelming diversity for the distribution globally. Apart from these countries, they are naturally found in eastern Russia, northern Japan, Himalayas, eastern Afghanistan and Pakistan through Caucasians. Spain, Northeastern America, and Turkey are also listed as natural habitats of the genus [3].

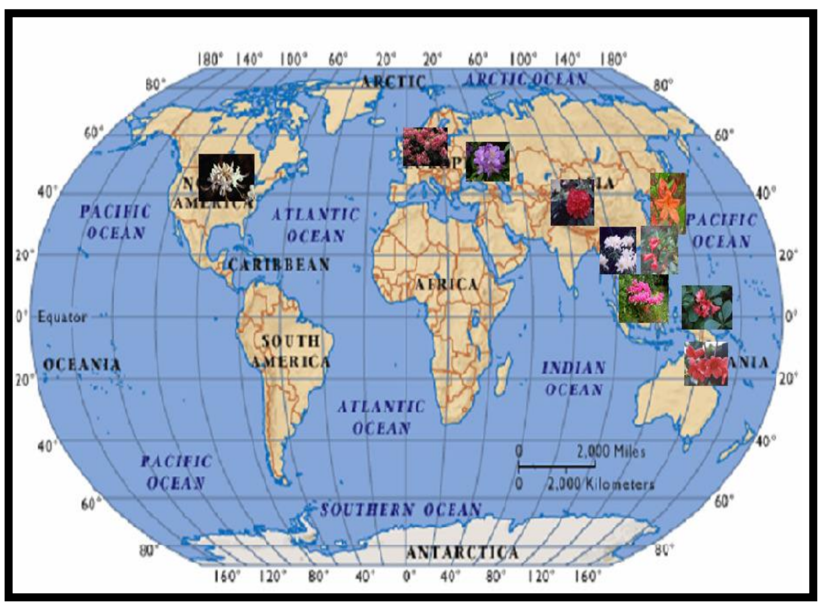

Figure 1.1. Global distribution of Rhododendron species.

Rhododendron species vary in shape and size, ranging from shrubs to trees. Varieties include low creeping plants with 10 cm long bodies to 30 m long trees (i.e. *Rhododendron protistum var. giganteum)*. They are mostly evergreen and they can grow in diverse climatic conditions having few deciduous species. Rhododendrons are mostly grown at

altitudes between 90 to 5500 m above sea level. There is also variation in the size and shape of the leaves having linear to orbicular type with varying sizes between one to over fifty centimeters long. Some species have their leaves covered with hairs and scales. Fruits are found as capsule-shaped while seeds are found flattened or spindle-shaped to ellipsoid (Figure 1.2) [4, 5].

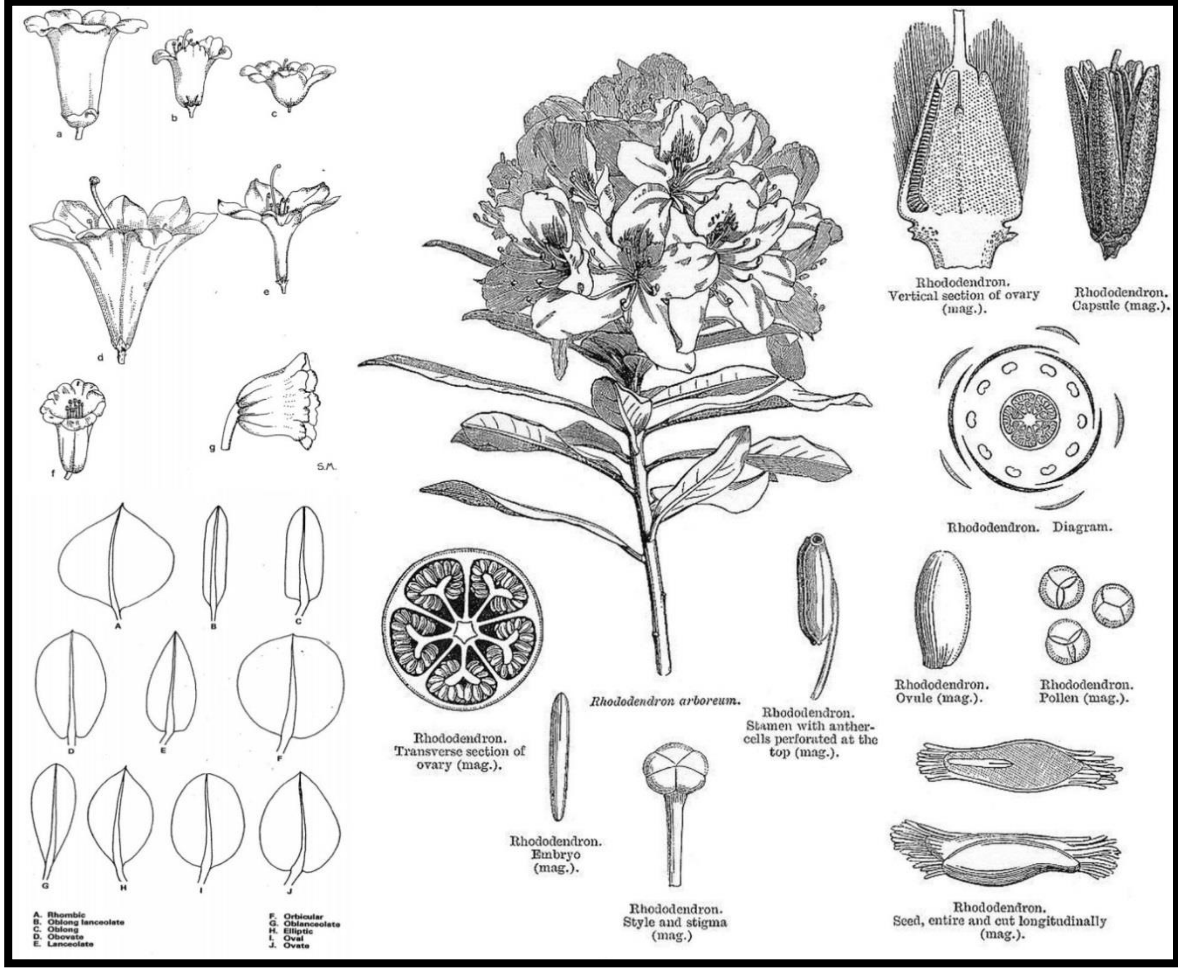

Figure 1.2. Flower, leaf and seed structure of Rhododendron species (Modified from [6-8]).

Rhododendron species has been under ongoing taxonomic debate due to hybridizations between species of different subsections in the wild. Also, taxonomic classifications do not clearly represent the morphological differences which itself challenges the taxonomic significance of the species [6]. Additionally, speciation of the

Rhododendrons take place in areas where many different types of species overlap and merge leading to variations within natural populations [7, 9].

The first documented description of Rhododendron which had been referred as Chamaerhododendron (low growing rose tree) occurred by Charles de l'Écluse (Clusius) in sixteenth century with the *Rhododendron hirsutum*. However, later on classification on six types of Rhododendrons as species of Azalea was published by Carl Linnaeus in the book of Species *Plantarum* in 1753 [10]. Although the distinction between Azalea and Rhododendron has created questions among other botanists, finally in 1836, *Azalea* was incorporated into Rhododendron. Classification of the Rhododendron genus interests not only the botanists and taxonomists but also horticulturists and hobbyists. This has created changes in the taxonomy of Rhododendrons difficult to implement to the literature because the majority of the individuals in the field had stuck to the “Series” system introduced by Sir Isaac Bayley Balfour and the system itself was reluctant to accept changes [7]. Balfour’s system was intended to deal with the massive influx of the species coming from Asia due to the early botanical explorations in that region. His system consists of 43 series; each represented a single, well-known typical species which was adopted by the British Rhododendron Society [11]. A complete revision of the genus was undertaken after years of modification by several different authors up to 21st century with the incorporation of molecular studies [12]. Although today the genus Rhododendron is divided into 9 subgenera, 16 sections and 56 subsections [2], taxonomic challenges and debate are still an ongoing process.

Rhododendrons are aggressive colonizers that limit the biodiversity of the invaded site and obstruct the regeneration of new woodlands. Once they are established at a site, it is costly to eradicate them. Invaded sites have limited physical access due to the occupation of high density bushes. Rhododendrons blocks 98% of total daylight, as compared to oak trees which blocks 91% of light [13]. They also inhibit the growth of nearby plants by poisoning them with aliphatic acids or simple phenols [14]. Furthermore, Rhododendron species have mycorrhizae roots which increase their ability to compete for soil nutrients. Aliphatic acids, phenols and mycorrhizae residues remain in the soil even after the removal of Rhododendron species enabling them to give growth advantage to their seedlings [15]. First direct effect of Rhododendrons to other species has been

observed in southwestern Ireland where they replace oak trees in the areas they grow together, to the point of total destruction which is referred as sudden oak death [16].

In order to battle against the uncontrolled invasion of Rhododendron species in the wild, natural solutions have been taken into consideration such as natural rival insects that target them as a food source and cause variety of diseases. For example, Jassid bug (*Graphocephala coccinea)* which carries the disease Bud Blast (*Pycnosteanus azalea)* and it has effects on controlling invasion of Rhododendron species naturally [17].

Rhododendron species generally grow in acidic organic soil with ideal pH values of 5.0 – 6.0 at partial shade and open conditions. Their high tolerance to temperatures as high as +25 °C and as low as -10 °C makes them quite hardy plants [18]. Rhododendron species has been internationally introduced as ornamental species for planting in gardens and parks. Different varieties and colors make them popularly available as potted plants in markets, shops and gardening centers [19]. Their aesthetic beauty made them chosen as national symbols such as *Rhododendron arboretum* is the national flower of Nepal, *Rhododendron ponticum* is the state flower of Kashmir and *Rhododendron niveum* is the state tree of Sikkim, India. Apart from being ornamentals, they can also be used in landscaping applications as walls, hedges and roadside plantations. In nature they also serve as nesting site for birds and animals because of their bushy body and wide leaf area [20]. Rhododendron species symbolizes the warning and danger since the flowers of the species contain toxic compounds to animals and humans.

Rhododendron species have been also traditionally used for the treatment of different disorders. They have been used for treating asthma, bronchitis and arthritis in China where they are overwhelmingly diverse [21-25]. *Rhododendron brachycarpum* has been used in Korea in order to treat headache, arthritis, diabetes and hypertension [4, 26]. The leaves and flowers of the *Rhododendron anthopogon* and *Rhododendron campanulatum* are used in Tibet to treat inflammation and skin, lung disorders [4, 27, 28]. In Nepal, *Rhododendron lepidotum*, *Rhododendron campanulatum* and *Rhododendron anthopogon* are used for the treatment of several different disorders [4, 29, 30]. Ayurvedic medical system in India uses 12 different Rhododendron species for treating diarrhea, dyspepsia, altitude sickness, nose

bleeding and headaches [4, 31, 32]. In Austria, *Rhododendron hirsutum* and *Rhododendron ferrugineum* flowers have been used for the treatment of gastro-intestinal, cardio-vascular, urinary and respiratory tract infections [4]. *Rhododendron ferrugineum* leaves have been employed in Germany for the treatment of muscle pain, hypertension and metabolic disease [4, 33]. Native Americans have used *Rhododendron calendulaceum*, *Rhododendron maximum* and *Rhododendron albiflorum* to treat the common cold, gastro-intestinal swelling, and rheumatism [34]. Rhododendron species have also been used as psychoactive incense in Europe and Asia, narcotic in North America, diaphoretic in Caucasus, fruit preservative by Native Americans and as an insect repellant in Russia [4].

Rhododendron species have been tested in a variety of studies to find out the pharmacological effects on human health. Studies on different parts of the species revealed the anti-inflammatory, analgesic, anti-microbial, cardio-protective, tyrosinase inhibitory, immune-modulatory, anti-oxidant, anti-spasmodic and anti-diabetic activities of the Rhododendron species which has been linked to their flavonoid-rich chemical composition [35-45].

1.1.1. Rhododendron in Turkey

Rhododendrons of Turkey are represented by 12 taxa which consists five wild species, three subspecies and four hybrid species. Two of the subspecies which are *R. ponticum* L. subsp. Ponticum var. heterophyllum Ansin and *R. ponticum* L. subsp. Baeticum has been extinct (Dr. Altun pers. comm.).

Remaining ten taxa consists of five wild species which are *Rhododendron ponticum* L., *Rhododendron caucasicum* Pallas, *Rhododendron ungernii* Trautv., *Rhododendron smirnovii* Trautv. and *Rhododendron luteum* Sweet [46, 47]. *Rhododendron ponticum* L. subsp. *ponticum* forma album is the only natural form of *Rhododendron ponticum* in Turkey. Remaining four species are the hybrids of wild species and they are mainly located at Eastern Black Sea region (Table 1.1).

Table 1.1. Rhododendron hybrid species in Turkey.

Hybrid Name	**Hybrid Between**	**Altitude Range (Meter)**
Rhododendron x *sochadzeae* Charadze and Davlianidze	*Rhododendron caucasicum* L. X *Rhododendron ponticum* L.	1800 – 2300 m
Rhododendron x *rosifaciens* R. Milne	*Rhododendron smirnovii* Trautv. X *Rhododendron ungernii* Trautv.	1900 – 2100 m
Rhododendron x *davisianum* R. Milne	*Rhododendron smirnovii* Trautv. X *Rhododendron caucasicum* L.	1900 – 2300 m
Rhododendron x *filidactylis* R. Milne	*Rhododendron ponticum* L. X *Rhododendron ungernii* Trautv.	1600 – 1750 m

In Turkey, Rhododendrons are acclimatized from eastern black sea region (Artvin) through central and western black sea regions to the slopes of Istranca Mountains (Figure 1.3) [46]. They grow in wet, and mild climatic conditions within the altitudes between 100 to 3100 m above sea level, containing acidic soils within the pH range of 3.5 – 5.0 [13]. They can establish in moorlands, bogs, mires, grasslands, riverbanks and woodlands. Deciduous woodlands provide the most suitable conditions for their spread [48].

Rhododendrons in our country are mainly imported from other countries and extensively sold as potted plants in nurseries. Native species are used as timber wood in constructions and highly destroyed by villagers since considered as toxic and invasive species danger the fields.

Every year, millions of dollars being spent on imported Rhododendrons for their sole use as potted plants. Ornamental values of the species are not known to local people due to the absence of domesticated cultivars, which disables their use in landscaping as hedges or roadside plantations.

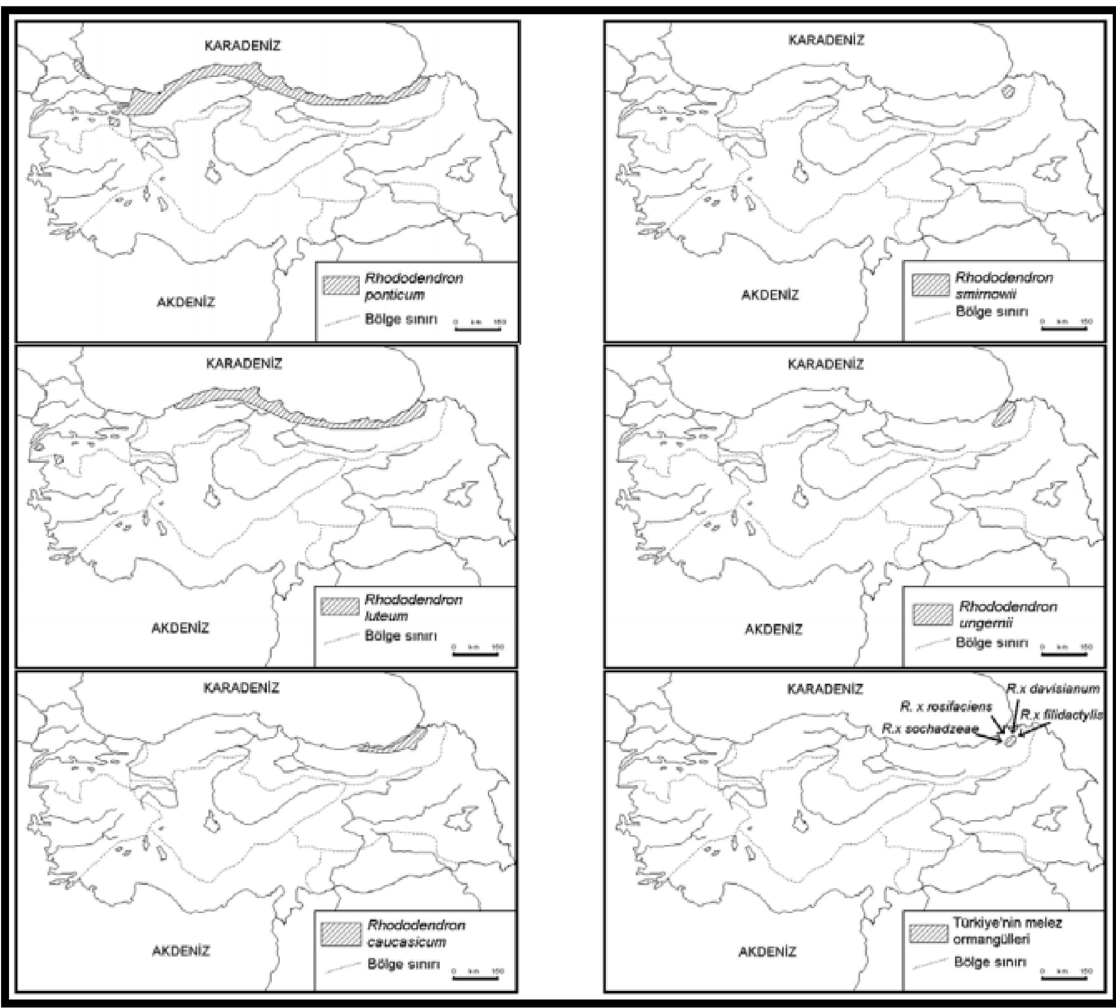

Figure 1.3. Regional distribution of Rhododendron species in Turkey (Adapted from [3]).

Two species, *Rhododendron ponticum* L. and *Rhododendron luteum* Sweet are used in honey making process and produced honey is referred as “mad honey” or “toxic honey” [49]. Xenophon of Athens describes the strange behavior of Greek soldiers after ingesting the honey made in a village covered with *Rhododendron ponticum* bushes during the march of Greek mercenaries called the “Ten Thousand” in 401 BC [50]. In 67 BC, during the Third Mithridatic War, Pontic soldiers used mad honey in order to stop the invasion of Pompey soldiers to their villages. Pompey soldiers suffered casualties because of the mad honey which deliberately left behind by the enemy Pontic soldiers [51]. Mad honey is mainly produced and consumed in Black Sea region of Turkey [49]. It is sold at local markets in Trabzon where commonly used against gastritis, flu and as sexual stimulant (Anonymous). Besides Turkey, the mad honey is also produced in Brazil, Nepal, Japan and

few regions of North America and Europe [52]. Ingestion of the mad honey at lower doses can cause dizziness, bradycardia, hypotension and gastro-intestinal problems. When ingested at higher doses, it can cause seizures, unconsciousness and atrial-ventricular blockages [49].

1.2. Molecular Characterization of Rhododendron Species

Molecular markers are powerful tools for estimating genetic diversity and distinguishing between different individuals that have different origins or morphologies [53]. Depending on the dominant or codominant nature of the markers, they provide vast amount of genetic information about studied organisms. Additionally, they present several advantages over other identification techniques as no prior knowledge of genome size or sequence identity is required. The fast development and widespread use of PCR-based molecular markers in plants during the last decades contributed to the accumulation of information such as genetic diversity of species, stability or dynamic nature of population structure, introgression in cultivation process of breeding programs, evolutionary history of the species and adaptive changes to the habitats [54-58]. Development of appropriate conservation programs especially for endangered wild species cannot be envisaged without the availability of such information of a species.

Over the years several studies accumulated in the literature from different countries about the Rhododendron genus mainly concentrating on genetic diversity analysis of species and populations as well as genetic lineage analysis of hybrid / introgressed varieties and phylogenetic relationships using mostly SSR (simple-sequence repeat), ISSR (inter-simple sequence repeat), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) and ITS (Internal Transcribed Spacer) marker systems [59-65]. Such marker systems have been preferred for the reasons of being easy to apply, fast, less labor intensive and efficient in detection of high level of polymorphisms [65, 66].

2. PURPOSE

Global changes and human factors threaten the genus Rhododendron for years. Economical and ornamental values of these species are highly underestimated in our country. As the research partner of the Tubitak 112O500 project which aims to characterize the Turkish Rhododendron genus, our purpose is,

(i) to determine the genetic diversity of the Rhododendron genus using molecular marker systems.

(ii) to understand the population structure relating altitudinal and locational information of the plants.

3. MATERIALS

3.1. General Enzymes, Kits and Reagents

Enzymes, kits and reagents in Table 3.1 were purchased from Thermo Fisher Scientific, (Wilmington, DE) unless stated otherwise.

Table 3.1. List of enzymes, kits and reagents.

Name	Brand
Total DNA Extraction Kit	69104, DNeasy Plant Mini Kit, Qiagen, Hilden, Germany
Hot Start DNA polymerase	203603, HotStarTaq Plus DNA Polymerase, Qiagen, Hilden, Germany
DNA ladder	SM0311, GeneRuler 1 kb DNA Ladder SM0321, GeneRuler 100 bp Plus DNA Ladder SM0241, GeneRuler 100 bp DNA Ladder
DNA loading Dye	R0611, DNA Gel Loading Dye (6X)
DNA Polymerase	EP0402, *Taq* DNA Polymerase, recombinant (5U/µL)
Magnesium Chloride	R0971, $MgCl_2$ (magnesium chloride) (25 mM)
dNTPs	R0191, dNTP Mix (10 mM each)

3.2. Chemicals, Plastics and Glassware

All chemicals were purchased from *Sigma-Aldrich* (USA), *Merck* (Germany), *Applichem* (Germany) or *Duchefa* (Netherlands) unless stated otherwise. Tips and tubes, sterile plates and falcon tubes were ordered from *Axygen* (USA), *Interlab*, *and BD Biosciences* (USA), respectively. All glassware, tips and tubes were sterilized by autoclaving at 121°C for 20 minutes.

3.3. Buffers and Solutions

Table 3.2. List of common buffers and solutions.

Solution Name	**Concentration**
10X TBE buffer	1M Tris base, 1M Boric Acid, 20mM EDTA
50X TAE buffer	2M Tris base, 5.71% (v/v) Glacial acetic acid, 50 mM EDTA, pH:8.0
1X TE buffer	10mM Tris HCl, 1mM EDTA, pH:8
CTAB extraction buffer	3% (v/v) Cetyltrimethyl ammonium bromide (CTAB), 28% (v/v) 5 M NaCl, 4% (v/v) 0.5 M EDTA, 10% (v/v) 1 M Tris-Cl, 3% (w/v) Polyvinylpyrrolidone (PVP) (MW 40 kDa), 0.2% (v/v) β-Mercaptoethanol, dH_2O pH: 8.0

3.4. Equipments

Table 3.3. List of equipments.

Name	**Model and Brand**
Thermal Cycler	C1000 Thermal Cycler, Bio Rad, USA Runik Thermal Cycler, Sacem Life Technologies, Turkey
Agarose Gel Electrophoresis	Minicell Primo EC320, Thermo Fisher Scientific, USA
Power Supply	164-5050 PowerPac Basic, Bio Rad, USA EC250-90, Thermo Fisher Scientific, USA
Gel Documentation System	Gel Doc EQ Bio Rad, USA
Spectrophotometer	NanoDrop 1000, Thermo Fisher Scientific, USA
Micro-centrifuge	miniSpin Plus, Eppendorf, Germany Centrifuge 5414R, Eppendorf, Germany

Table 3.4. List of secondary equipments.

Name	Model and Brand
Microwave Oven	MD551, Arcelik, Turkey
Autoclaves	Mac-601, Eyela, Japan ASB260T, Astell, UK
Deep Freezer (-20°C)	A2021-D, Arcelik, Turkey
Deep Freezer (-80°C)	Forma 860-ULT, Thermo Fisher Scientific, USA
Ice machine	AF20, Scotsman, Italy
Scanner	GT15000, Epson, Japan
Pipettes	Pipetman Classic, Gilson, USA
Multi-Channel Pipettes	Transferpette, TreffLab, Switzerland
Vortex	NM110, Nuve, Turkey
Magnetic Stirrer	RCT basic, IKA, Germany
Balances	AY123, Sartorius, Germany
Incubator	EN500, Nuve, Turkey

3.5. Biological Material

3.5.1. Plant Material

As a research partner of the Tubitak project (112O500), the plant material used in this thesis were collected from their native locations and provided by Asst. Prof. Bahadır Altun (Ahi Evran University Department of Horticulture, Kırsehir, Turkey). Total of 147 plant leaf samples belonging to Rhododendron genus were categorized as; five wild species, four wild hybrid species, one subspecies, one outlier species (Table 3.5), 13 variants (Table 3.6), 22 different *Rhododendron ponticum* L. populations (total of 110 individuals) and 3 different *Rhododendron luteum* Sweet populations (total of 13 individuals) (Table 3.7).

Out of 147 plants, 55 of them were sampled from Marmara region, 46 of them were sampled from eastern black sea region, nine of them were sampled from central black sea region and 37 of them were sampled from western black sea region (Figure 3.1). The plant materials which are referred as variants cover the samples that were classified by taxonomists under certain Rhododendron species however individually diverges from the nominated species with a unique morphological difference as specified in Table 3.6. They are included in our sample groups to study whether these morphological differences are also reflected in molecular level as they were analyzed by marker system.

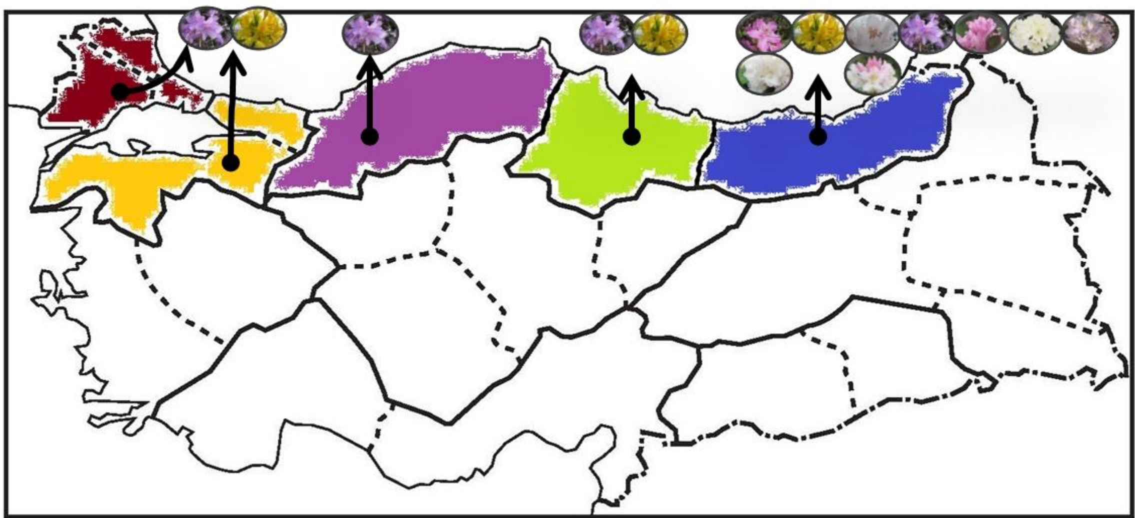

Figure 3.1. Regional distribution of Rhododendron species and populations used in this study.

Table 3.5. List of plant material; wild and hybrid species of Rhododendron genus and outlier species. Bold marked samples have been selected for molecular marker analysis.

No	Sample code	Wild and Hybrid Species	Coordinates	Altitude (meter)	Location	Image
1	**08 AR 03**	*Rhododendron ungernii* Trautv.	41°18’870 K 41° 53’495 D	1249	Borcka / Artvin	
2	**08 ART 01**	*Rhododendron caucasicum* Pallas	41°13’450 K 42° 28’376 D	2289	Savsat / Artvin	

Table 3.5. List of plant material; wild and hybrid species of Rhododendron genus and outlier species. Bold marked samples have been selected for molecular marker analysis (cont.).

No	Sample code	Wild and Hybrid Species	Coordinates	Altitude (meter)	Location	Image
3	**08 MU 01**	*Rhododendron luteum* Sweet	41° 10'424 K 42° 18'943 D	900	Murgul / Artvin	
4	**08 MU 02**	*Rhododendron ponticum* L.	41° 16'174 K 41° 32'823 D	834	Murgul / Artvin	
5	**08 MU 05**	*Rhododendron smirnovii* Trautv.	40° 14'743 K 41° 35'699 D	1332	Murgul / Artvin	
6	**08 BO 05**	*Rhododendron x filidactylis R. Milne* (hybrid)	41° 22'902 K 41° 51'680 D	1561	Borcka / Artvin	
7	**08 MU 08**	*Rhododendron x rosifaciens* R. Milne (hybrid)	41° 13'832 K 41° 35'959 D	1663	Murgul / Artvin	
8	**08 MU 09**	*Rhododendron x davisianum* R. Milne (hybrid)	41° 13'170 K 41° 37'038 D	2231	Murgul / Artvin	
9	**08 MU 10**	*Rhododendron x sochadzeae* Charadze & Davlianidze (hybrid)	41° 13'076 K 41° 37'161 D	2248	Murgul / Artvin	
10	**08 MU 07**	*Rhododendron ponticum forma album* (subspecies)	41° 14'311 K 41° 34'233 D	1154	Murgul / Artvin	
No	**Sample code**	**Outlier Species**	**Coordinates**	**Altitude (meter)**	**Location**	**Image**
1	**A. unedo**	*Arbutus unedo*	41° 07'120 K 31° 03'348 D	810	Akcakoca / Duzce	

Table 3.6. List of plant material; morphologically divergent variants of Rhododendron genus. Bold marked samples have been selected for molecular marker analysis.

No	Sample Code	Variants	Coordinates	Altitude (meter)	Location	Morphological Difference
1	**08 MU 11**	*Rhododendron smirnovii* Trautv.	41° 13'133 K 41° 37'155 D	2256	Murgul / Artvin	Wavy petal edges
2	**08 MU 03**	*Rhododendron ponticum* L.	41° 16'172 K 41° 32'820 D	840	Murgul / Artvin	Petal color tone
3	**08 SA 02**	*Rhododendron luteum* Sweet	41° 10'424 K 42° 18'943 D	1671	Savsat / Artvin	Silvery color leaves on both side
4	**81 DU 01**	*Rhododendron ponticum* L.	40° 75'847 K 31° 29'046 D	707	Kaynasli / Duzce	Hairy ovarium
5	**08 BO 01**	*Rhododendron ponticum* L.	41° 18'880 K 41° 53'487 D	1260	Borcka / Artvin	Lack of spots in petals
6	**28 GO 01**	*Rhododendron ponticum* L.	40° 99'169 K 39° 05'415 D	734	Gorele / Giresun	Petal color tone
7	**39 DE 01**	*Rhododendron ponticum* L.	41° 87'155 K 27° 83'313 D	363	Igneada / Kirklareli	Longer Calyx leaf
8	**52 UL 01**	*Rhododendron ponticum* L.	40° 45'951 K 37° 49'272 D	1205	Ulubey / ORDU	Longer Calyx leaf
9	**52 KU 01**	*Rhododendron luteum* Sweet	40° 41'325 K 37° 08'822 D	1578	Kumru / Ordu	Totally different color petals
10	**08 AR 01**	*Rhododenron ponticum* L.	41° 13'349 K 43° 31'696 D	1688	Arhavi / Artvin	Petal color tone
11	**08 AR 02**	*Rhododendron x sochadzeae* Charadze & Davlianidze	41° 16'618 K 41° 29'444 D	1688	Arhavi / Artvin	Petal color tone
12	**17 CA 01**	*Rhododendron luteum* Sweet	39° 99'092 K 26° 71'053 D	400	Bayramic / Canakkale	Silvery color leave on lower side
13	**67 AL 01**	*Rhododendron ponticum* L.	41° 03'349 K 31° 37'923 D	1095	Alapli / Zonguldak	Petal color tone

Table 3.7. List of plant material; populations of *Rhododendron ponticum* L. and *Rhododendron luteum* Sweet species. Bold marked populations with diverse altitudes have been selected for molecular marker analysis.

No	*Rhododendron ponticum* L. Population code	Population number /individual number	Altitude (meter)	Location
1	08 ART	1p/5	511	Hopa / Artvin
		2p/5	1326	Murgul / Artvin
		3p/5	896	Murgul / Artvin
		4p/5	1631	Kafkasor / Artvin
2	81 DU	**1p/5**	**719**	Kaynasli / Duzce
3	41 YUVA	**1p/5**	**1088**	Golcuk / Kocaeli
		2p/5	1241	Golcuk / Kocaeli
	41 SUA	**1p/5**	**669**	Suadiye / Kocaeli
	41 KAR	1p/5	1262	Kartepe / Kocaeli
4	54 HEN	**1p/5**	**565**	Hendek / Sakarya
		2p/5	**821**	Hendek / Sakarya
	54 KARA	1p/5	835	Karapurcek / Sakarya
		2p/5	**407**	Karapurcek / Sakarya
		3p/5	765	Karapurcek / Sakarya
		4p/5	**786**	Karapurcek / Sakarya
	54 SA	1p/5	1122	Sapanca / Sakarya
		2p/5	**1023**	Sapanca / Sakarya
		3p/5	571	Sapanca / Sakarya
5	28 GO	1p/5	675	Gorele / Giresun
6	77 YA	1p/5	704	Ciftlikkoy / Yalova
7	39 DE	1p/5	363	Igneada / Kirklareli
8	34 CAT	**1p/5**	**183**	Catalca / Istanbul

Table 3.7. List of plant material; populations of *Rhododendron ponticum* L. and *Rhododendron luteum* Sweet species. Bold marked populations with diverse altitudes have been selected for molecular marker analysis (cont.).

No	*Rhododendron luteum* Sweet Population Code	Population number /individual number	Altitude (meter)	Location
1	08 AR	**1p/5**	**1633**	Sallet / Artvin
		2p/5	**1182**	Kafkasor / Artvin
2	17 CA	**1p/3**	**400**	Bayramic / Canakkale

3.5.2. PCR Analysis Primers

Total of 10 RAPD, 10 ISSR dominant marker primers (Macrogen Ltd) and a pair of ITS primers (Macrogen Ltd) were used to perform molecular marker analysis on the plant samples (Table 3.8).

ITS primers were selected to amplify the internally transcribed spacer regions between 18S and 26S ribosomal DNA genes (Figure 3.2). All primers were optimized via gradient PCR analysis and only the determined optimum annealing temperatures were used as indicated in Table 3.8.

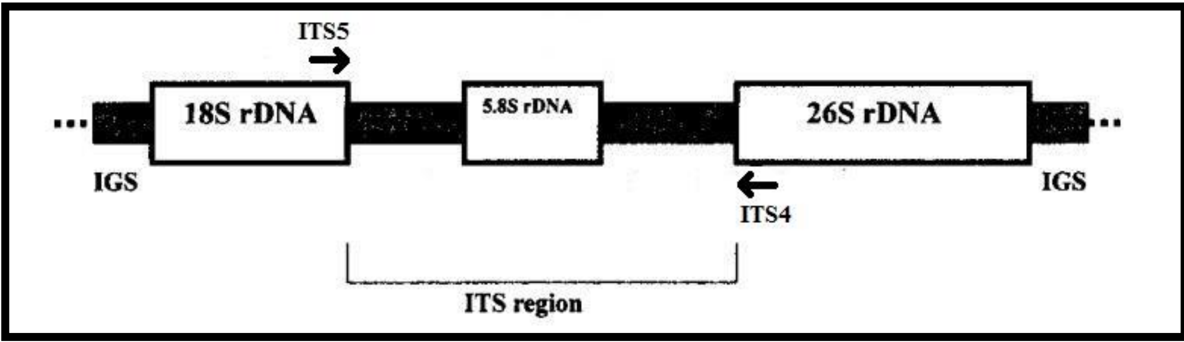

Figure 3.2. ITS primers binding sites relative to 26S and 18S rDNA genes (Modified from [67]).

Table 3.8. List of primers used in this study.

Primer Name	Sequence (5' – 3')	Annealing Temp. (°C)	References
RAPD_F3	CCTGATCACC	36	[68]
RAPD_F7	CCGATATCCC	36	[68]
RAPD_F8	GGGATATCGG	36	[68]
RAPD_F10	GGAAGCTTGG	36	[68]
RAPD_F12	ACGGTACCAG	36	[68]
RAPD_F13	GGCTGCAGAA	36	[68]
RAPD_F14	TGCTGCAGGT	36	[68]
RAPD_G14	GGATGAGACC	36	[69]
RAPD_K11	AATGCCCCAG	36	[69]
RAPD_M5	GGGAACGTGT	36	[69]
ISSR_UBC817	CACACACACACACACAA	48	[68]
ISSR_UBC826	ACACACACACACACACC	52	[68]
ISSR_UBC827	ACACACACACACACACG	52	[68]
ISSR_UBC835	AGAGAGAGAGAGAGAGYC	48	[68]
ISSR_UBC836	AGAGAGAGAGAGAGAGYA	48	[68]
ISSR_UBC841	GAGAGAGAGAGAGAGAYC	52	[68]
ISSR_UBC842	GAGAGAGAGAGAGAGAYG	52	[68]
ISSR_UBC855	ACACACACACACACACYT	52	[68]
ISSR_UBC856	ACACACACACACACACYA	52	[68]
ISSR_UBC857	ACACACACACACACACYG	48	[68]
ITS4	TCCTCCGCTATATGATATGC	52	[67]
ITS5	GGAAGTAAAAGTCGTAACAAGG	52	[67]

4. METHODS

4.1. Sampling and Maintenance of Plant Materials

For all plant materials used in this study, freshly sampled leaves were collected in ziplock bags containing 1:10 (gr/gr) silica gels to eliminate DNA degradation risk by complete desiccation of leaf tissues. Ziplock bags were labeled with the name, species code, location and altitude information for each of the plant material individually. Only fresh baby leaves were sampled. To eliminate repetitive sampling from the same vegetative clones of the same population, at least three-meter distance was considered. For different population sampling, at least 100-meter separation distance was considered. To obtain diverse sampling materials, all natural habitats of Rhododendrons in Turkey at various altitudes (183 m - 2256 m) were covered starting from Eastern Black sea (Artvin) to west of Marmara region (Canakkale). Collected materials were delivered to Bogazici University, Department of Molecular Biology and Genetics, Istanbul, Turkey. They were stored in -80°C deep freezers until used for genomic DNA isolation.

4.2. DNA Extraction from Plant Materials

Genomic DNA was extracted from silica gel dried Rhododendron leaves using the modified version of CTAB protocol [70]. Twentyfive milligrams of leaves were homogenized in liquid nitrogen with autoclaved aquarium sand using mortar and pestle. Homogenized leaf samples were suspended in 600 µl CTAB extraction buffer. Homogenates were incubated at 55°C for two hours in shaker incubator at 450 rpm. Equal volume of 24:1 (v/v) Chloroform:Isoamyl alcohol were added before centrifugation at 13,200 rpm for 10 minutes. Approximately 400 µl of supernatant was transferred to a fresh Eppendorf tube. Half volume of ice cold isopropanol was added to the supernatant and samples were incubated for one hour at -20°C for the precipitation of genomic DNA. Samples were centrifuged for 10 min at 13,200 rpm and pellets were washed three times with 700 µl of 70 % ice cold ethanol and air dried. The pellets were dissolved in 50 µl sterile dH_2O.

4.3. Quantitative and Qualitative Analysis of DNA

4.3.1. Spectrophotometric Determination of the DNA Concentration

Concentrations of the isolated plant genomic DNA were measured by Nanodrop 1000, Thermo Fisher Scientific, USA. OD260/OD280 ratios were used to determine the RNA contamination and samples within the range of 1.8 were considered RNA free. OD260/OD230 ratios were calculated for other contaminants and samples within the range of 2.0 – 2.2 were considered contaminant free.

4.3.2. Agarose Gel Electrophoresis for the Analysis of DNA Integrity

Isolated DNA integrity and concentrations were verified by 1% (w/v) agarose gel electrophoresis. Five microliters of extracted genomic DNA mixed with 1µl of 6X DNA loading dye and run at 80 V for two hours.

4.4. PCR Analysis

4.4.1. PCR Analysis Using RAPD Primers

Total of 10 RAPD primers were used for fingerprinting genomic DNA samples (Table 3.8). PCR amplifications were performed in a total of 25 µl reaction mixture containing 40 ng of genomic DNA, 2.5 µl 10X HotStart Taq Polymerase buffer, 0.2 mM each dNTPs, 0.4 µM primer, 1.5 mM $MgCl_2$ and, 1.25 U HotStart Taq Polymerase. PCR reactions were prepared in 0.2 ml Eppendorf tubes and run using following reaction cycle: Initial denaturation at 94°C for 5 min, 45 cycles of denaturation at 94°C for 45 s, annealing at 36°C for 30 s and extension at 72°C for 90 s. The final cycle was completed by 10 min extension at 72°C. Products of PCR amplification were separated by 2% (w/v) agarose gel electrophoresis at 80 V for 90 min and visualized under Gel Doc EQ, Bio Rad, USA.

4.4.2. PCR Analysis Using ISSR Primers

Total of 10 ISSR primers were used for fingerprinting genomic DNA samples (Table 3.8). PCR amplifications were performed in a total of 25 µl reaction mixture containing 40 ng of genomic DNA, 2.5 µl 10X HotStart Taq Polymerase buffer, 0.2 mM each dNTPs, 0.4 µM primer, 1.5 mM $MgCl_2$, 2 mg/ml BSA and, 1.25 U HotStart Taq Polymerase. PCR reactions were prepared in 0.2 ml Eppendorf tubes and run using following reaction cycle: Initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C or 52°C for 45 s and extension at 72°C for 90 s. The final cycle was completed by 7 min extension at 72°C. Products of PCR amplification were separated by 2% (w/v) agarose gel electrophoresis at 80 V for 90 min and visualized under Gel Doc EQ, Bio Rad, USA.

4.4.3. PCR Analysis Using ITS Primers

A pair of ITS primers (ITS4-ITS5) was used for fingerprinting parental genomic DNA samples (Table 3.8). PCR amplifications were performed in a total of 50 µl reaction mixture containing 40 ng of genomic DNA, 5 µl 10X HotStart Taq Polymerase buffer, 0.2 mM each dNTPs, 0.5 µM primer, 1.5 mM $MgCl_2$, 2 mg/ml BSA and, 2.5 U HotStart Taq Polymerase. PCR reactions were prepared in 0.2 ml Eppendorf tubes and run using following reaction cycle: Initial denaturation at 94°C for 3 min; 10 cycles of denaturation at 94°C for 45 s, annealing at 58°C or 52°C for 45 s and extension at 72°C for 60 s, 30 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 45 s and extension at 72°C for 60 s. The final cycle was completed by a 10 min extension at 72°C. Products of PCR amplification were separated by 2% (w/v) agarose gel electrophoresis at 80 V for 90 min and visualized under Gel Doc EQ, Bio Rad, USA.

4.5. Data Analysis

The band patterns obtained from each RAPD and ISSR PCR analysis were scored based on their presence (1) or absence (0) and a binary data matrix was generated. The amplicons migrated to the same position across the whole samples for each primer was considered the same alleles. The migration distances of the amplicons were analyzed by a

custom made MATLAB code based on relative position of each band with respect to DNA molecular weight ladder band positions in agarose gels. Resulting positional information used as the input data set for the code to produce the binary data matrix with given threshold values. The data matrix was converted to the distance matrix using Jaccard's Correlation Coefficient. Distance matrix was used to generate UPGMA (Unweighted Pair Group Method with Arithmetic Average) dendrograms and the cophenetic value was calculated by MATLAB algorithm [71].

Polymorphic Information Content (PIC) measure the markers detection limit for the polymorphism within the population [72]. Diversity Index (DI) indicates the allelic variation for each primer [73]. Effective Multiplex Ratio (EMR) reflects the effectiveness of the marker system by comparing the percentage of polymorphic loci to total number of loci. Marker Index (MI) shows the overall utility of the marker system and resolving power (Rp) measures the ability of each primer to distinguish between large numbers of genotypes [74]. Each of these parameters was calculated via specialized MATLAB algorithm using the generated distance matrix [71].

Shannon's Information Index (I), gene diversity (He) and unbiased gene diversity (uHe) were calculated in each population over all loci using GenAlEx 6.5.2 software [75,76]. Gene diversity or expected heterozygosity (He) and unbiased gene diversity (uHe) are the two parameters estimating the genetic diversity within a population.

Analysis of molecular variance (AMOVA) has been performed by the program GenAlEx 6.5.2 in order to partition the total genetic variance into within-population and among-population groups. Degree of freedom (df), sum of squares (SS), mean sum of squares (MS), estimated variance and total variation have been calculated with 9999 random permutations. Principal Coordinate Analysis (PCoA) is a technique allowing the analysis and plot of the multivariate data sets. PCoA reflects the variations between individual members of a data set with different locational and altitudinal parameters. It essentially combines the patterns of genetic differentiation with these parameters in order to plot these combinations over individual axes. PCoA have been completed by GenAlEx 6.5.2 [75, 76].

In order to analyze the genetic structure among populations, STRUCTURE 2.3.3 software has been used to determine the approximate number of clusters in the sample (K) [77]. The cluster model applied to the raw data pool assumes the existence of K clusters which significantly contributes the gene pool and assigns species to clusters via Markov chain Monte Carlo (MCMC) simulations. These simulations assign the members of each population to one cluster depending on the allele frequencies at each locus and two or more clusters if their genotypes and allele frequencies indicate that they are admixed which is the indicative of a hybrid zone. For each cluster, 20 independent MCMC simulation runs have been performed. Each MCMC simulation run comprise of 100,000 iterations with initial burn-in period of 20,000. At each iteration, the algorithm randomly assigns one locus into a cluster and calculates a probability for the new state. Then it generates a random number and accepts the state if the random number is smaller or equal to the calculated probability. Otherwise, the new state is rejected and the process continues from the previous state. As the number of iterations increases, the algorithm converges to the optimal state where each member of population is assigned to a cluster (K). An initial burn-in period is necessary to initialize MCMC algorithm with an unbiased sample pool.

5. RESULTS

5.1. DNA Extraction and Quantitative Analysis of DNA

Quality and quantity of genomic DNAs (gDNA) are determining factors for consistent band patterns in molecular marker systems using RAPD and ISSR primers. Initially, the isolation of the plant leaf gDNA has been performed with commercially available kits. However, it has been observed that the isolates were poor in DNA quality and there were RNA contaminations despite of RNase treatments. When the genomic DNAs were extracted with DNeasy Plant Mini Kit (Qiagen) system, the yield was between 30 – 150 ng/µl (Figure 5.1a) and improvement of the yield did not occur even when the starting leaf material quantity was increased.

In some of the Rhododendron species the tough and hairy nature of the leaves negatively affected the success of homogenization, mostly resulted in sticky textured homogenate instead of completely pulverized powders. Change in the company and the genomic DNA isolation kit system (such as Zymoresearch) also resulted in similar yields and thus did not improve the quantity or quality of the DNA.

Therefore, it has been suspected that the lysis buffer of the kits was not adequate in disruption of cell wall components. The CTAB extraction method [70] has been selected and modified as an alternative since it is open to modifications with respect to plant species and also the effects of a cationic detergent are superior for the lysis of the plant cell wall components. Additionally, during homogenization in liquid nitrogen using autoclaved aquarium sands were also added into the mortar in order to generate more abrasive surface over the leaves. The results of the CTAB method has shown significantly improved yield, generating 300 – 4500 ng/µl gDNA (Figure 5.1b) and also completely cleared the contaminating RNAs.

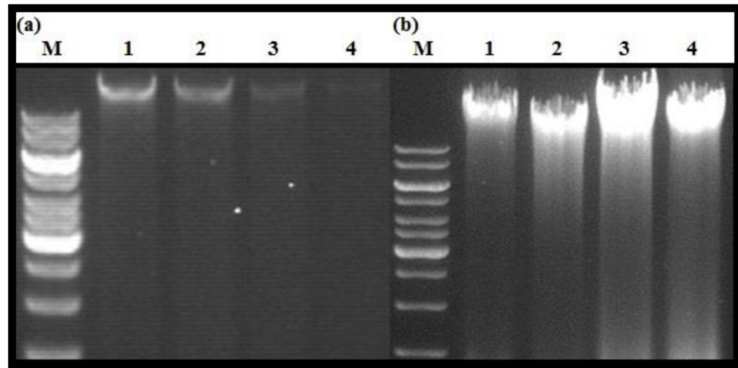

Figure 5.1. Plant genomic DNA samples extracted with kit (a) and CTAB method (b). M: GeneRuler 1kb DNA Ladder. Samples 1 – 4: 08MU07, *A. unedo*, 08BO01 and 08DU01 respectively.

Adverse effect of the low quality genomic DNA extracted by kits was also observed when the band patterns of subsequent PCR amplifications were analyzed. Both RAPD and ISSR primers generated non-reproducible band patterns (Figure 5.2a). Short primer length and degenerate nature of these primers emphasizes the requirement of high quality template DNA for consistent amplification results. When CTAB extracted genomic DNAs of the same samples were amplified using the same primers, reproducible and sharp band patterns were obtained (Figure 5.2b).

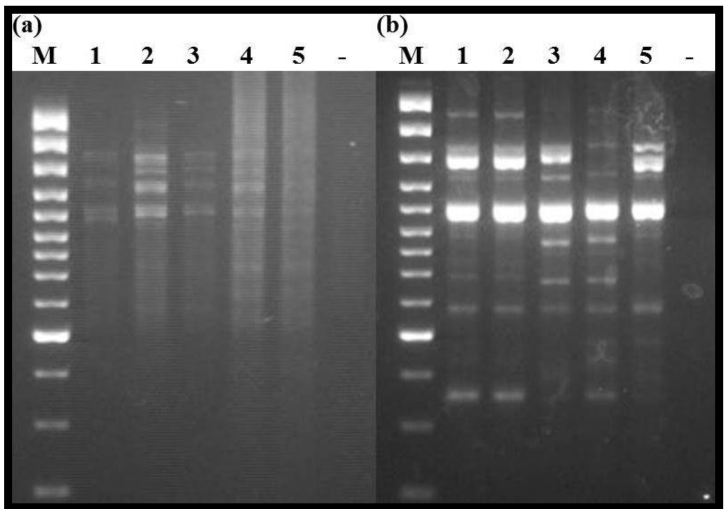

Figure 5.2. PCR analysis results using ISSR ubc817 primers on template DNA extracted with kit (a) and CTAB method (b). M: GeneRuler 100 bp Plus DNA Ladder. Samples 1 – 5: *R. ponticum* L. populations from Artvin.

5.2. Molecular Marker Analysis

Molecular marker analysis have been completed using 10 RAPD, 10 ISSR and 2 ITS primers for 24 Rhododendron species including 5 wild species, 4 wild hybrids, one subspecies and one outlier species (Table 3.5), 13 variants (Table 3.6), 9 different *R. ponticum* L. populations (total of 45 individuals) and 3 different *R. luteum* Sweet populations (total of 13 individuals) (Table 3.7).

Although the plant material listed at Table 3.7 contained the total number of 22 populations for *R. ponticum* L. and 3 populations for *R. luteum* Sweet, due to time limitations in this thesis, total population sizes for molecular marker analysis were cut down to 9 and 3 respectively by preserving the diversity of altitudes and locations. PCR analysis with each primer for all samples was repeated three times to verify the reproducibility of the results.

PCR amplification of 24 species with 10 RAPD and 10 ISSR primers generated a total of 380 bands ranging in size between 200 – 2000 base pairs. Amplified band patterns with each primer across all samples have yielded high polymorphism with 100% PPB (percentage of polymorphic bands) (Table 5.1).

The average number of bands obtained per primer was 20, with the minimum of 16 bands displayed with ISSR 827, ISSR 856 and ISSR 857 primers and the maximum of 24 bands displayed with RAPD G14 primer (Table 5.1). The band patterns of RAPD M5 and ISSR 826 were shown in Figure 5.3 and Figure 5.4 respectively. The gel electrophoresis results for the rest of complete primer sets were given in Appendix A.

Observation of high polymorphic nature of primers was encouraging for further data analysis to generate reliable and informative genetic distance analysis.

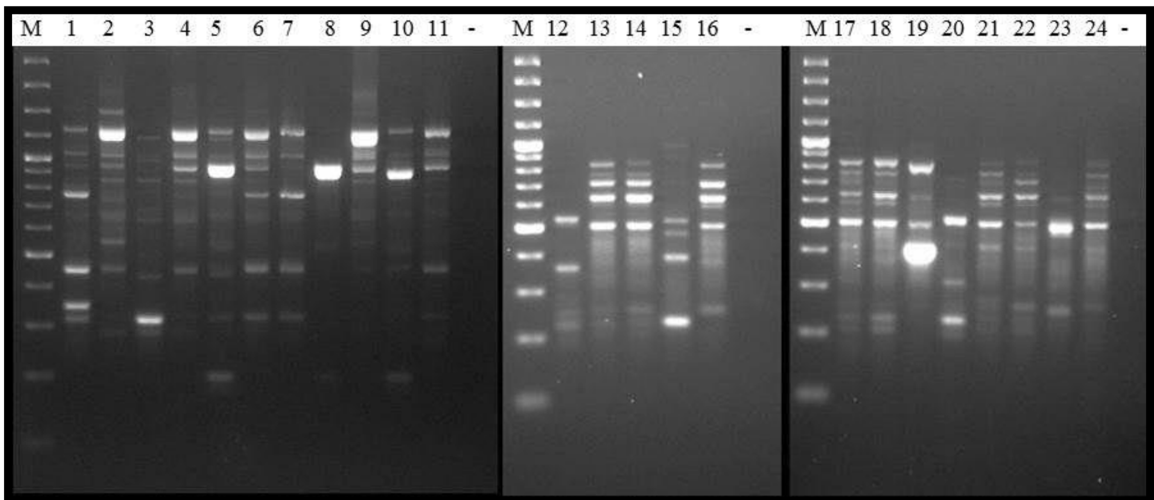

Figure 5.3. PCR amplification of 24 Rhododendron species with RAPD M5 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5, samples 12-24 Rhododendron variants (Table 3.6).

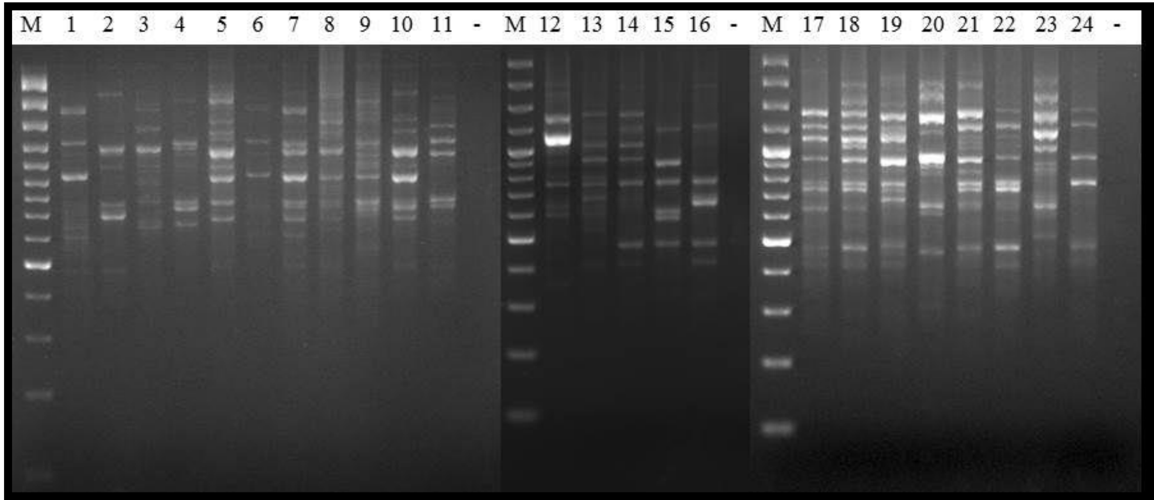

Figure 5.4. PCR amplification of 24 Rhododendron species with ISSR 826 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5, samples 12-24 Rhododendron variants (Table 3.6).

PCR amplification of *R. ponticum* L. and *R. luteum* Sweet populations with 10 RAPD and 10 ISSR primers displayed much lower polymorphisms within populations as expected. For example, ISSR 827 primer displayed average level of polymorphism within five members of the 41 YUVA and 81 DU populations of *R. ponticum* L. (Figure 5.5). On

the other hand, the RAPD F13 primer was highly monomorphic for the same populations (Figure 5.6). For total of 45 individuals of *R. ponticum* L. and 14 individuals of *R. luteum* Sweet populations, the polymorphisms level were ranging from 19.05% PPB to 40.95% PPB for *R. ponticum* L. populations and 22.66% PPB to 59.38% PPB for *R. luteum* Sweet populations for both primer sets. Both RAPD and ISSR primers generated a total of 210 bands (*R. ponticum*) and 128 bands (*R. luteum*) within the range of 300 – 2000 base pairs with average bands per primer of 10.1 (*R. ponticum*) and 8.7 (*R. luteum*) for RAPD and 10.9 (*R. ponticum*) and 6.3 (*R. luteum*) for ISSR. The minimum of 6 bands were displayed by RAPD F10 primer whereas the maximum of 14 bands were displayed by RAPD F14 primer.

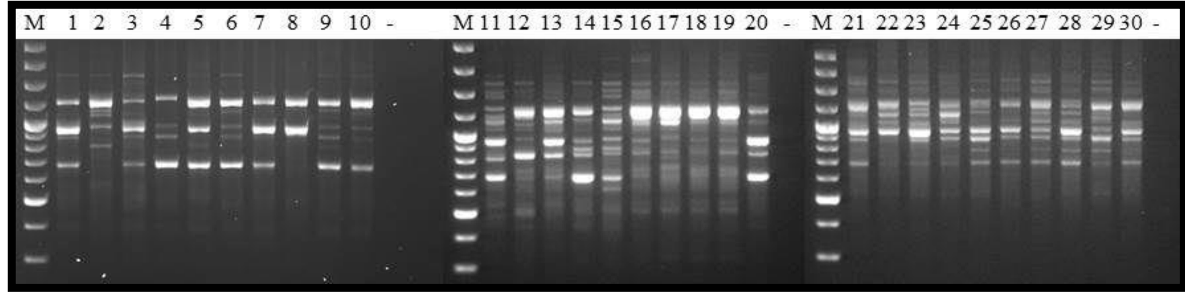

Figure 5.5. PCR amplification of six Rhododendron populations with ISSR 827 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41 YUVA, 6 – 10: 81 DU, 11 – 20: 54 HEN1-2, 21 – 30: 54 KARA1-2.

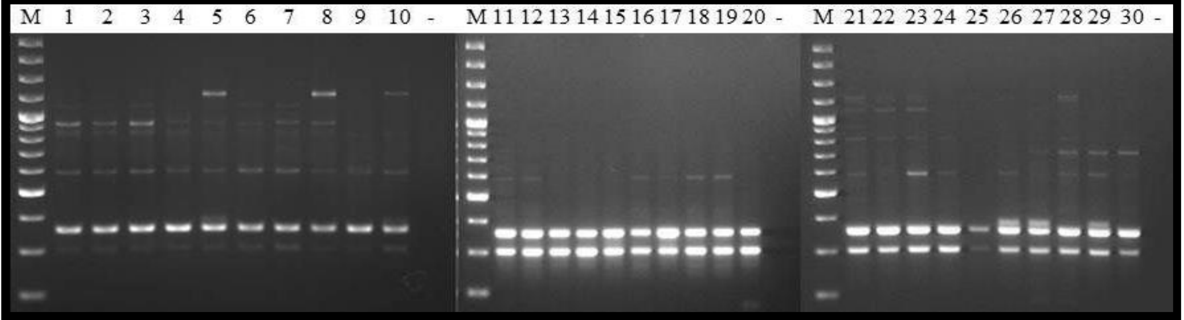

Figure 5.6. PCR amplification of six Rhododendron populations with RAPD F13 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41 YUVA, 6 – 10: 81 DU, 11 – 20: 54 HEN1-2, 21 – 30: 54 KARA1-2.

5.3. Data Analysis

5.3.1. Genetic Relationship Analysis of 23 Rhododendron Genus and *A. unedo*

Band patterns obtained from PCR analysis have been scored as (1) for the presence or (0) for the absence of the bands across the samples for each primer. These scores were used to generate a binary data matrix with 9252 individual loci.

Primer based genetic diversity parameters were also calculated in order to observe the strength of each primer to reflect the overall genetic relationships. The Polymorphism Information Content (PIC) reflects the number of detectable alleles and their frequency with maximum value of 0.5. Therefore, when the PIC value of primer sets is closer to this maximum it is considered to be highly selectable for genetic diversity within the studied sample groups. Average PIC values for RAPD primers and ISSR primers were 0.346 and 0.411 which implicated their high polymorphism in selection of genetic diversity within our samples (Table 5.1). Diversity Index (DI) is considered as another parameter which measures molecular marker strength by reflecting allelic variation with a maximum value to 1 [72-74]. When DI value was calculated for the both RAPD and ISSR set of primers, it was observed to be 0.99, implicating the strong genetic diversity among individuals of the studied plant samples (Table 5.1). Effective Multiplex Ratio (EMR) has been calculated in order to see the fraction of polymorphic loci compared with total number of loci. All RAPD and ISSR primers were observed to have high EMR value which ranges between 16 – 23 due to their highly polymorphic nature. Marker Index (MI) has been calculated in order to understand the overall utility of the system. We have observed high MI values ranging from 15.824 (ISSR 857) to 23.984 (RAPD G14) which indicated the high power of our marker system to evaluate the genetic relationships among samples. Lastly, Resolving Power (Rp) of the marker system was calculated to understand the ability of our system to distinguish between large numbers of genotypes. Our marker system had Rp values ranging from 5.75 (RAPD F10) to 12.667 (ISSR 835) which was a strong indicator of high information content obtained from individual bands (Table 5.1). Overall, primer based genetic diversity analysis results yielded high values for each parameters calculated for our marker system which strongly implicated the representation of genetic relationships with high genetic diversity among studied sample groups.

Table 5.1. Primer based genetic diversity analysis results for 24 Rhododendron species. NB:Number of bands, NPB: Number of polymorphic bands PPB: Percentage of polymorphic bands.

Primer Name	NB	NPB	PPB	PIC	DI	EMR	Rp	MI
RAPD F3	20	20	100	0.400	0.993	20.0	8.083	19.866
RAPD F7	23	23	100	0.324	0.998	23.0	8.583	22.944
RAPD F8	20	20	100	0.369	0.996	20.0	9.250	19.992
RAPD F10	19	19	100	0.257	0.998	19.0	5.750	18.960
RAPD F12	23	23	100	0.327	0.997	23.0	8.583	22.938
RAPD F13	20	20	100	0.381	0.994	20.0	7.833	19.880
RAPD F14	19	19	100	0.330	0.997	19.0	7.250	18.947
RAPD G14	24	24	100	0.303	0.998	24.0	8.750	23.984
RAPD K11	21	21	100	0.382	0.996	21.0	10.333	20.920
RAPD M5	20	20	100	0.389	0.996	20.0	10.583	19.914
ISSR 817	18	18	100	0.429	0.994	18.0	10.750	17.884
ISSR 826	18	18	100	0.435	0.993	18.0	10.167	17.867
ISSR 827	16	16	100	0.375	0.995	16.0	7.667	15.916
ISSR 835	22	22	100	0.449	0.993	22.0	12.667	21.856
ISSR 836	22	22	100	0.404	0.995	22.0	9.250	21.938
ISSR 841	22	22	100	0.342	0.997	22.0	9.250	21.938
ISSR 842	23	23	100	0.394	0.995	23.0	9.917	22.858
ISSR 855	18	18	100	0.392	0.994	18.0	8.250	17.893
ISSR 856	16	16	100	0.427	0.991	16.0	8.000	15.858
ISSR 857	16	16	100	0.467	0.989	16.0	9.684	15.824

Binary data matrix was translated into a genetic distance matrix which then compiled into an (UPGMA) dendogram using Jaccard's standard correlation coefficient. Individual and combined impacts of RAPD and ISSR primer sets in detection of genetic diversity were observed by generation of separate dendograms for RAPD (Figure 5.7) and ISSR (Figure 5.8) primer sets as well as by generation of a dendogram from compiled binary data set (Figure 5.9). Both individual and combined dendograms were reflected the genetic diversity among the sample groups with high significance as determined by Cophenetic correlation coefficient (*r*) values of 0.843, 0.722 and 0.848 for RAPD, ISSR and RAPD – ISSR data sets respectively.

Combined dendogram of 20 primers generated seven clusters with *A. unedo* being the outlier group (Figure 5.9). Cluster I consisted of two *R. ponticum* L. variants (67AL01 and 08AR01) which were collected at the altitudes of 1095 and 1688 meters from Zonguldak/Alapli and Artvin/Arhavi respectively. These two variants as being divergent from the wild *R. ponticum* L. (08MU02 at 834 m) with petal tone color were also reflected 83.6% and 79.9% genetic distance from the wild species which belonged to Cluster VI. Cluster II contained three *R. ponticum* L. variants (39DE01, 28GO01 and 52UL01) and one *Rhododendron x sochadzeae* Charadze & Davlianidze hybrid variant (08 AR 02) with altitudinal values of 363, 734, 1205 meters from Giresun/Gorele, Kirklareli/Igneada, Ordu/Ulubey and 1688 meters from Artvin/Arhavi respectively. Within this cluster, *R. ponticum* L. variant possessing longer calyx leaves in comparison to the wild species 08 MU 02 (834 m) displayed around 76-78% genetic distance. However, the hybrid variant with different petal color tone, 08 AR 02 of cluster II was 82.4% genetically distant from the wild hybrid 08 MU 10 (2248 m in cluster IV). An interesting grouping was observed in cluster III which contained two *R. ponticum* L. variants; 08BO01 (1260 m) from Borçka/Artvin which had highly distinctive morphology with lack of spots on its white petals and 81 DU 01 (707 m) from Kaynasli/Duzce with uncharacteristic hairy ovarium. Together with these two variants, the only natural form of Turkey; *R. ponticum forma album* Sweet. Zab. (08 MU 07; 1154 m) with characteristic white spotted petal and naked ovarium was also grouped at cluster III. While the natural form 08MU07 was only 50.4% and 58% genetically distant from the other two variant members of Cluster III, these variants (08 BO 01 and 81 DU 01) were much more distant from their wild species 08 MU 02 with 78.6% and 83% respectively. Cluster IV contained one wild Rhododendron species

as *R. caucasium* Pallas (08 ART 01) from Savsat/Artvin and one wild hybrid species *Rhododendron x sochadzeae* Charadze & Davlianidze (08MU10) from Murgul/Artvin with similar altitudes of 2289 and 2248 meters respectively. Since 08MU10 hybrid contains a genetic background from both parental species of 08ART01 and 08MU02, it was not surprising to observe similar genetic distance of 70.4% and 73% between them. The wild species *R. smirnovii* Trautv. (08MU05; 1332 m) and two wild hybrids, *Rhododendron x rosifaciens* R. Milne (08MU08; 1663 m), *Rhododendron x davisianum* R. Milne (08MU09; 2231 m) were observed to be grouped in Cluster V together with wavy petal edge morphology in *R. smirnovii* Trautv. variant (08MU11; 2256 m). Despite of their altitudinal range, all members of the cluster V were from Murgul/Artvin. Both hybrid species in this cluster contained common parent, *R. smirnovii* Trautv genetic background and thus displayed 65.5% (08MU08) and 59.4% (08MU09) variability from the parental wild species 08MU05. Similar genetic distance of 59.7% was also observed between 08MU11 variant and its main wild species. Cluster VI included two Borcka/Artvin and two Murgul/Artvin groups as species *R. ungernii* Trautv. (08AR03; 1249 m), *Rhododendron x filidactylis* R. Milne hybrid (08BO05; 1561 m) and *R. ponticum* L species (08MU02; 834m), *R. ponticum* L variant 08MU03 (840 m) with different petal color tone respectively. While the wild species 08AR03 and 08MU02 were shown 74% genetic distance from each other, the hybrid 08BO05 had lower genetic distance of 62% to both of its wild parents. On the other hand, although the *R. ponticum* L. variant with petal color tone difference was collected from the same location and the same altitude with its main wild species 08MU02, it still displayed 55% genetic distance. *R. luteum* Sweet species (08MU01; 900 m; Murgul/Artvin) and three of its variants (17CA01; 400 m); 52KU01; 1578m) and (08SA02; 1671 m) were formed the cluster VII. The wild species *R. luteum* Sweet is well known with its yellow flowers and Pantone 371C/5763C upper/lower side mature leaf colors (Dr. Altun pers. comm.). However, the genotypes 17CA01 (Bayramiç/Canakkale) and 08SA02 (Savsat/Artvin) exhibited shifts in leaf color to silvery tone whereas, the 52KU01 variant (Kumru/Ordu) presented totally different flower colors with dark orange base and yellow stripes. The genetic distances of these genotypes from the main wild species were 87.3%, 81.8% and 80.3% respectively.

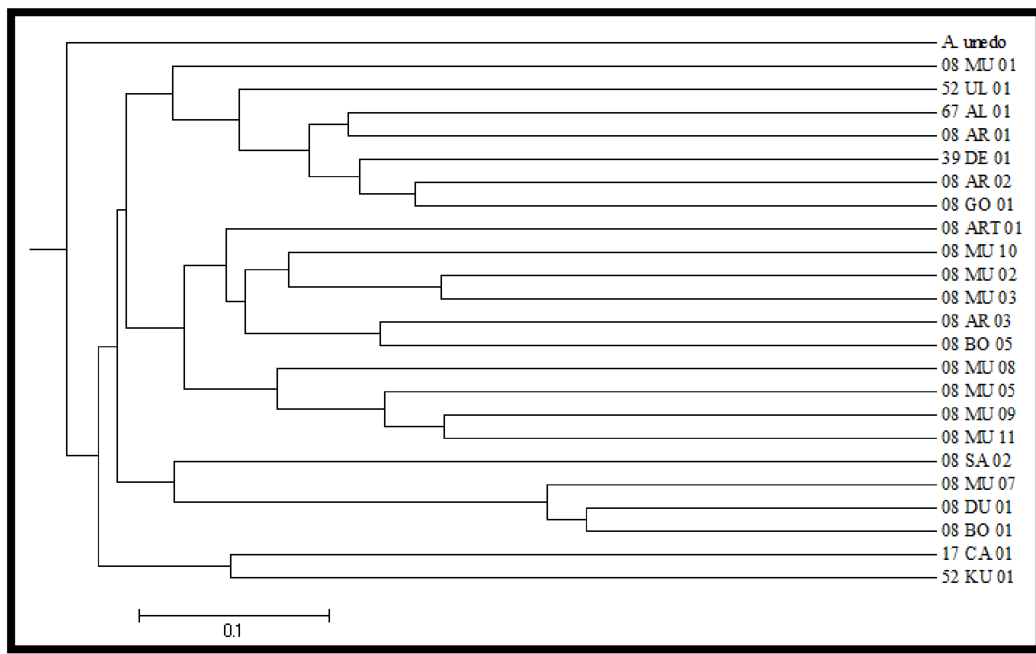

Figure 5.7. UPGMA dendogram of genetic dissimilarity (distance) among 23 Rhododendron genus and *A. unedo* outlier generated by RAPD marker data matrix using Jaccard's standard correlation coefficient.

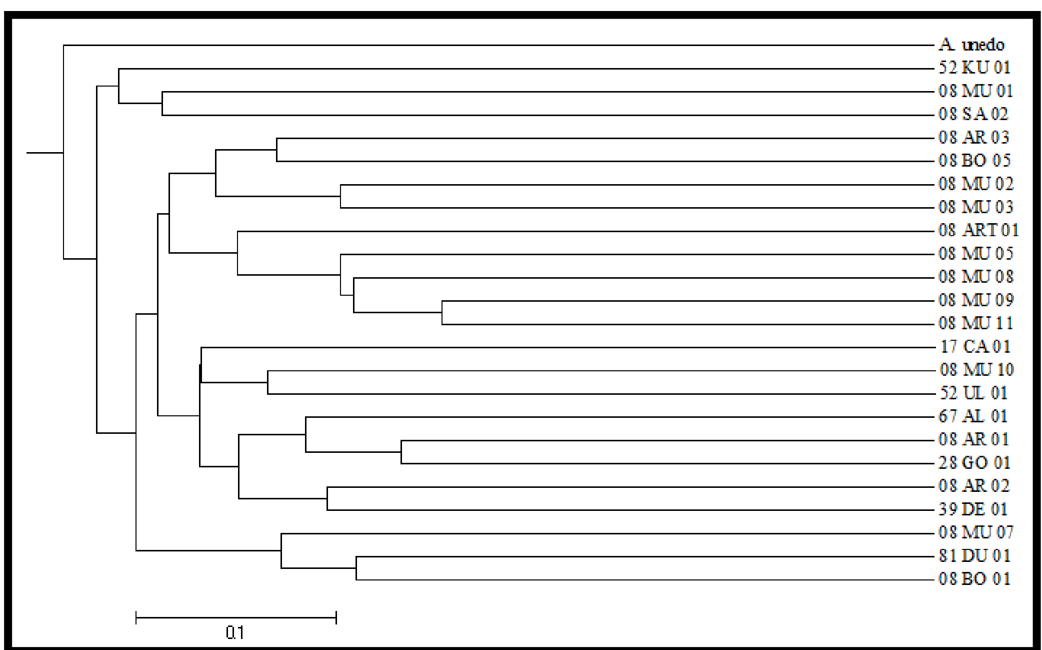

Figure 5.8. UPGMA dendogram of genetic dissimilarity (distance) among 23 Rhododendron genus and *A. unedo* outlier generated by ISSR marker data matrix using Jaccard's standard correlation coefficient.

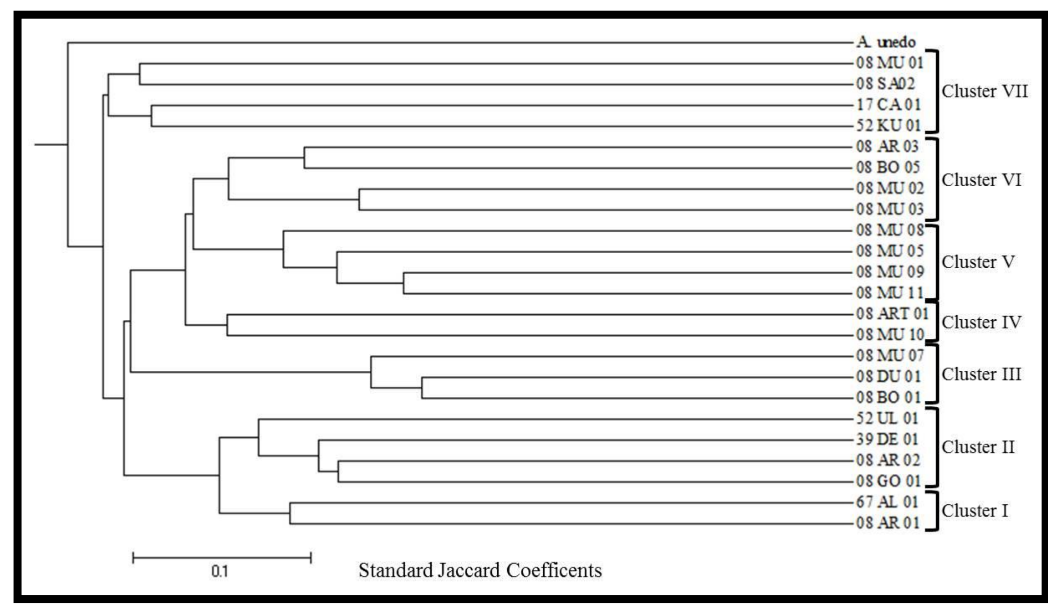

Figure 5.9. UPGMA dendogram of genetic dissimilarity (distance) among 23 Rhododendron genus and *A. unedo* outlier generated by both RAPD - ISSR marker data matrix using Jaccard's standard correlation coefficient.

Genetic differences among 21 Rhododendron genus were also analyzed using a sequencing based approach over the ribosomal DNA (rDNA) ITS region. ITS4 primer has been designed from the conserved region of 18S rDNA at 5' end and ITS5 has been designed from the conserved region of 26S rDNA at 3' end (Figure 3.2). The ITS4/ITS5 primer pair amplified the internal transcribed spacer (ITS) region (Figure 3.2). Resulting single amplicon from each sample ranged between 642 - 644 bp was sequenced with the same PCR primers and genetic differences were determined with MEGA software version 5.2 [78]. Despite of several troubleshooting attempts, the samples 08AR03 (*R. ungernii* Trautv.) and 17CA01 (*R. luteum* Sweet variant) did not produce any PCR products; therefore they were discarded from the phylogenetic analysis. Aligned nucleotide sequence results among 21 Rhododendron genus and *A. unedo* outlier have shown that they contain a partial sequence of ten base pairs from 18S rDNA region, nine base pairs from 26S rDNA region and a total of ITS region majorly 642 base pairs (Figure C.1). Among 21 Rhododendron genus, 36 polymorphic sites with 143 polymorphic characters were found within ITS region (Figure C.1). Aligned sequencing data have been translated into a genetic distance matrix by Kimura's two parameter method [79]. (Table B.2) and has been directly compiled into a dendogram using UPGMA method analysis (Figure 5.10). Genetic

distances among 21 Rhododendron genus ranged between 0 to 0.016, however this distance increased to 0.129 when compared with the outlier *A. unedo* species (Table B.2).

Genetic distance matrix of 21 Rhododendron genus generated six clusters (Figure 5.10). Cluster I contained the wild *R. ponticum* L. species (08MU02) and all of the morphologically different variants of this species (67AL01, 28GO01, 39DE01, 08AR01, 08MU07, 81DU01, 52UL01, 08MU03) except 08BO01. The 08MU10 and 08BO05 wild hybrids with a common parent of *R. caucasium* Pallas were grouped into cluster II. Although the 08BO01 is a variant of *R. ponticum* L. missing yellow spots in its petals, it formed an independent cluster III. 08MU08, 08MU05, 08MU11, 08ART01 and 08AR02 were grouped in cluster IV which contains *R. smirnovii* Trautv. (08MU05) and *R. caucasicum* Pallas (08ART01), as being either the parental species of the hybrid 08MU08 or the main species of the variant 08MU11. Although the 08MU09 is a hybrid of 08AR01 and 08MU05 species, it still formed an independent Cluster V. As being the variants of *R. luteum* Sweet (08MU01), 08SA02 with leaf silvery color tone shift and 52KU01 with unusually dark orange-yellow stripped flower formed the final cluster VI with no genetic difference from each other.

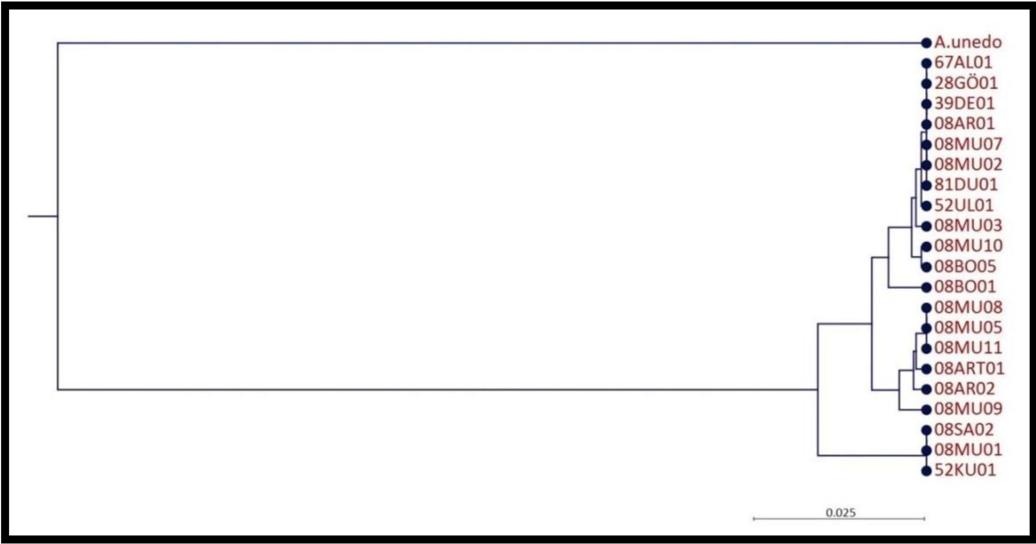

Figure 5.10. A dendogram of 21 Rhododendron genus and an outlier (*A. unedo*) by sequence comparisons of ITS region between 18S rRNA and 26S rRNA genes using Kimura’s two parameter method [79].

5.3.2. Population Structure Analysis of *R. ponticum* L. and *R. luteum* Sweet

5.3.2.1. Genetic Diversity Within Populations of *R. ponticum* L. and *R. luteum* Sweet. Within - population genetic diversities have been evaluated for nine R. ponticum populations (Table 5.2). 41YUVA and 81DU populations displayed the lowest gene diversity with uHe = 0.415 and I = 0.523. However, the highest gene diversities with uHe = 0.460 and I = 0.577 were observed in 34CAT population respectively (Table 5.2). Similarly, when R. luteum Sweet populations were concerned, the 08ARL-1 had the highest gene diversity with uHe = 0.350 and I = 0.268 respectively. Whereas, the lowest values were obtained for 17CA population with uHe = 0.109 and I = 0.132 respectively (Table 5.3). These parameters determine the distinctive representation of overall genetic diversity of population reflected by each individual. Therefore, the results indicated moderate genetic diversity within both R. ponticum L. and R. luteum Sweet populations by RAPD and ISSR primers.

Table 5.2. Genetic diversity estimates over 10 RAPD and 10 ISSR primers for nine populations of *R. ponticum* L.

Population Code	n	I	He	uHe
41 YUVA	5	0.523	0.347	0.415
34 CAT	5	0.577	0.395	0.460
41 SUA	5	0.540	0.361	0.427
54 HEN-1	5	0.548	0.366	0.428
54 HEN-2	5	0.554	0.373	0.438
54 KARA-1	5	0.563	0.380	0.446
54 KARA-2	5	0.543	0.365	0.430
54 SA	5	0.568	0.387	0.450
81 DU	5	0.523	0.347	0.415

Table 5.3. Genetic diversity estimates over 10 RAPD and 10 ISSR primers for three populations of *R. luteum* Sweet.

Population Code	n	I	He	uHe
08 ARL1	5	0.350	0.241	0.268
08 ARL2	5	0.275	0.186	0.207
17 CA	3	0.132	0.090	0.109

The band patterns from PCR analysis have been converted into a binary data matrix containing 210 individual loci for *R. ponticum* L. populations and 138 loci for *R. luteum* Sweet populations. Genetic distance matrix obtained from binary data used to compile UPGMA method to generate a dendogram using Pearson's correlation coefficient (Figure 5.11 and Figure 5.12).

R. ponticum L. populations generated nine clusters in which every population member grouped under unique cluster. Among those nine clusters, the highest genetic distance was reflected between 34CAT and 54SA population with a value of 88% (Figure 5.11). On the other hand, 54HEN-2 and 54KARA-1 were the closest populations with genetic distance of 73%, which implied a significant genetic diversity among those populations (Figure 5.11).

R. luteum Sweet populations grouped into two clusters where 17CA grouped under Cluster I, the two populations collected from Artvin (08ARL-1 and 08ARL-2) grouped under Cluster II (Figure 5.12). Cluster I population has 51% genetic distance within individual members; in contrast, cluster II has higher genetic distance of 71%. It has been observed that high genetic diversity has been maintained within individuals of three populations of *R. luteum* Sweet in spite of low population size.

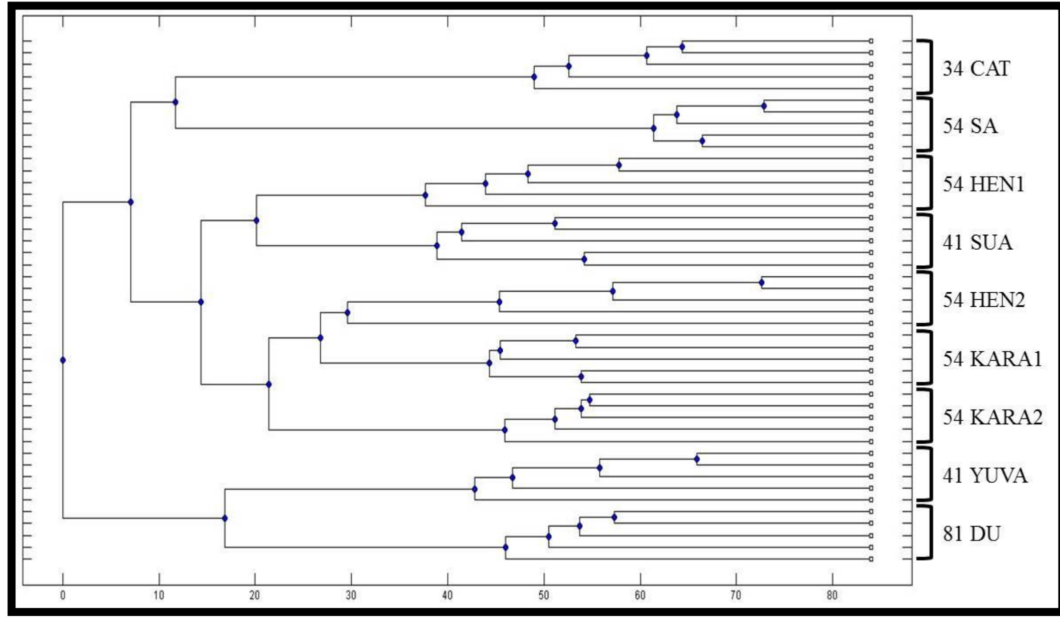

Figure 5.11. UPGMA dendogram of genetic dissimilarity (distance) among nine *R. ponticum* L. populations generated by both RAPD - ISSR data matrix using Pearson's correlation coefficient.

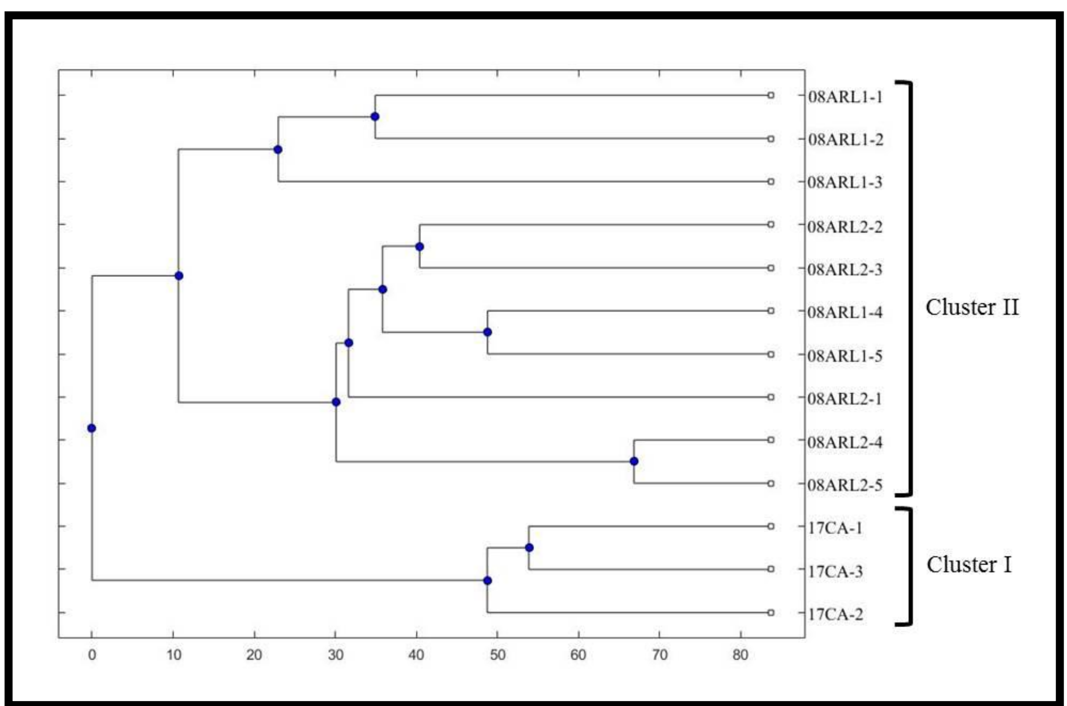

Figure 5.12. UPGMA dendogram of genetic dissimilarity (distance) among three *R. luteum* Sweet populations generated by both RAPD - ISSR data matrix using Pearson's correlation coefficient.

5.3.2.2. Genetic Diversity and Structuration among Populations of *R. ponticum* L. and *R. luteum* Sweet. In order to interpret genetic relationships among populations, the STRUCTURE algorithm was used to estimate the optimal values of hypothetical number of clusters (K). Simulation data were collected for each value of K from one to total number of populations which was nine in our study. The optimal value of K has been estimated using Evanno's deltaK method [80,81] (Figure 5.13a) where values above zero indicates the statistically significant K in which gene pool of populations were successfully clustered.

Comparing all nine populations, deltaK function which is the derivative of K reached the first peak at K = 2 and the second peak at K = 5 (Figure 5.13a). At K = 2, populations from the black sea region which were 54HEN-1, 54HEN-2, 54KARA-1, 54KARA-2, 54SA and 81DU were assigned to the same cluster while populations 41YUVA, 34CAT and 41SUA from Marmara region were grouped in other cluster. Members of 41SUA displayed similar proportion of gene pool belonging to both genetic clusters (Figure 5.13b).

For the value K = 5, although both 41YUVA and 34CAT grouped into the first cluster, 41YUVA has been also observed to admixed with cluster 5 of 81DU thus forming a hybrid zone in cluster 1. 41SUA and 54HEN-1 were grouped in the second cluster where 41SUA admixed with other populations from cluster 1, 4 and 5; whereas 54HEN-1 admixed with cluster 2 and 3 populations, thus forming a hybrid zone in cluster 2. Among the members of cluster 3 (54HEN-2, 54KARA-1 and 54KARA-2), 54HEN-2 admixed with cluster 2 populations thus forming a hybrid zone in cluster 3. However, the cluster 4 and 5 members (54SA and 81DU) formed individual groups without blending with any of the other populations.

The STRUCTURE analysis for *R. luteum* Sweet populations could not be performed due to insufficient population / individual size to detect meaningful gene pool variations from binary data matrix.

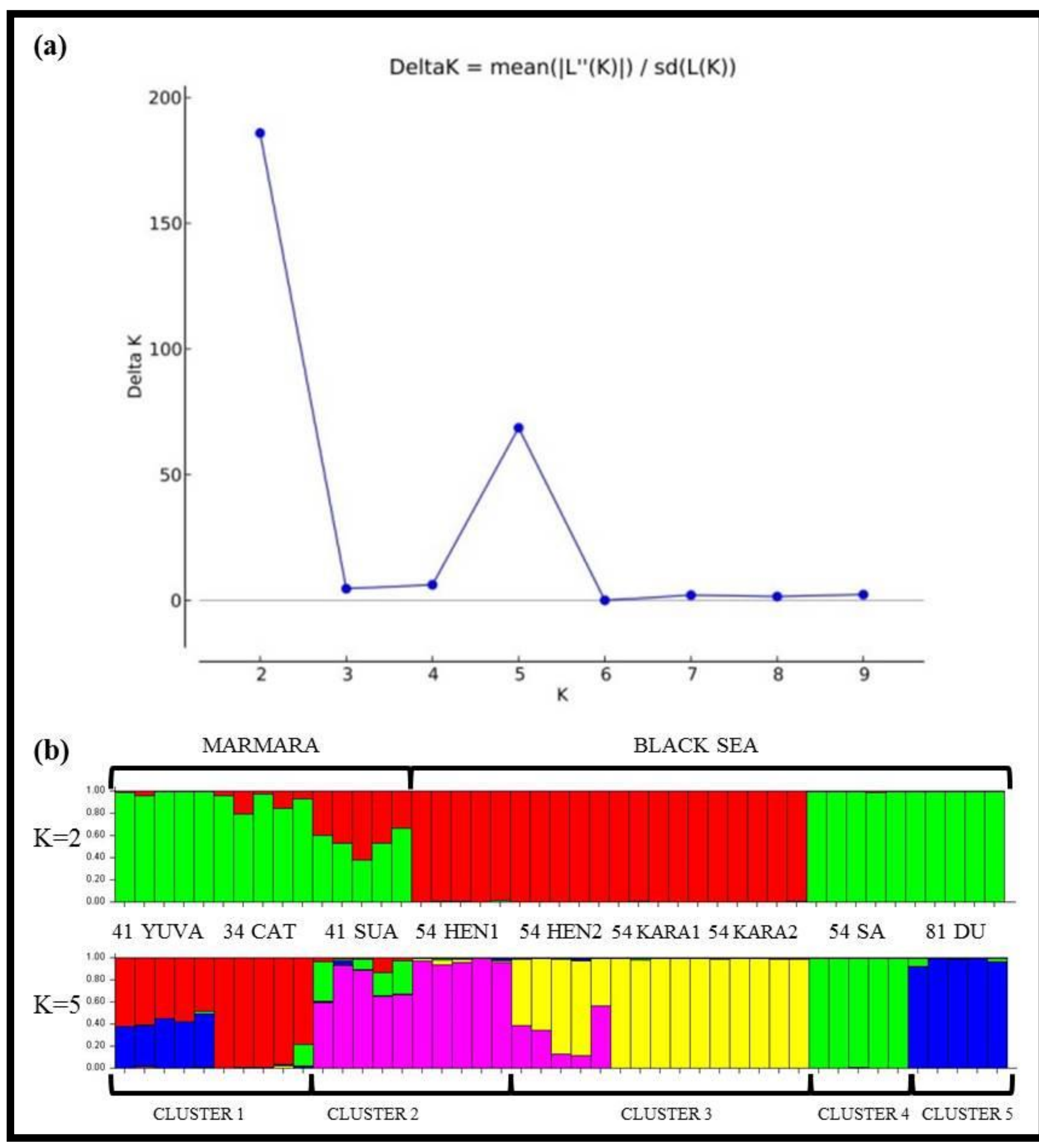

Figure 5.13. Genetic structure of the nine *R. ponticum* L. populations. (a) Plot of Delta K according to K. (b) STRUCTURE clustering results obtained at K = 2 and K = 5. Each individual is represented by one individual column.

Analyses of molecular variance (AMOVA) have been performed in order to evaluate the genetic variance distribution within and among populations of *R. ponticum* L. and *R. luteum* Sweet. The significance of the results was assured by 9999 random permutations of the data. A typical null hypothesis in AMOVA is no difference (H_0 = no genetic differentiation among populations) which means PhiPT = 0. Therefore, H_1 hypothesis suggests there is genetic difference among populations which means PhiPT > 0. Initially,

AMOVA combines populations to a single large random mating pool where there is no genetic differentiation among individuals thus we find minor differences (due to minor sampling effects) and accept H_0. Analysis then shuffles the samples in our data set and calculates the AMOVA for each shuffle resulting in values expected from a single population random mating pool. After high number of shuffles, AMOVA obtains the range of values it would expect if the null hypothesis were true. Finally, the algorithm compares the AMOVA results of each shuffle with initial data set in order to compare our observed value against the outcome of each permutation. If our value is greater than the permutated values at 95% or more of the time, our PhiPT value is significant at 5% level. For analysis with regional parameters, AMOVA shuffles individuals across populations and regions. Resulting PhiPT values represent an estimator of differentiation scaled between 0 and 1. Values above 0.25 represent a very great genetic differentiation whereas values between 0.15 and 0.25 represent moderate genetic differentiation.

AMOVA for *R. ponticum* L and *R. luteum* Sweet populations based on PhiPT displayed high within population variance component 0.51 (p=0.001) and 0.66 (p<0.002) respectively (Table 5.4 and Table 5.5). Among variation component of PhiPT were also high for both species populations with 0.49 (p<0.001) and 0.34 (p<0.002) variance respectively (Table 5.4 and Table 5.5), when populations were grouped into altitudinal clusters suggesting genetic differentiation of individuals belonging to different populations at different altitudes.

Table 5.4. Analysis of molecular variance (AMOVA) based on PhiPT values of *R. ponticum* L. populations combined with regional parameters.

Source	df	SS	MS	Est. Var.	% Variation	P-value
Among Populations	8	992.489	124.061	20.594	0.49	0.001
Within Populations	36	759.200	21.089	21.089	0.51	0.001
Total	44	1751.689	1.254	41.683	1	0.001

Table 5.5. Analysis of molecular variance (AMOVA) based on PhiPT values of *R. luteum* Sweet populations combined with regional parameters.

Source	df	SS	MS	Est. Var.	% Variation	P-value
Among Populations	2	110.754	55.377	8.967	0.34	0.002
Within Populations	10	174.400	17.440	17.440	0.66	0.002
Total	12	285.154	23.763	26.407	1	0.002

Highest genetic differentiation has been observed between 41YUVA and 54SA populations whereas lowest differentiation was between 54HEN2 and 54KARA1 populations (Table 5.6). However, even the lowest PhiPT value still represents a great genetic differentiation between populations. Populations which have similar regional parameters displayed relatively low PhiPT values (0.279) for 54HEN-1 and 54HEN-2 populations which implicate the closer genetic relationships. PhiPT values among *R. luteum* populations displayed high genetic differentiation between 17 CA and 08ARL populations (08ARL1 and 08ARL2) with PhiPT values of 0.424 and 0.438 respectively. On the other hand, there was average genetic differentiation between 08ARL populations (08ARL1 and 08ARL2) with PhiPT value of 0.187 which implies the similar patterns of genetic information between populations with close regional parameters (Table 5.7).

Principal Coordinate Analysis (PCoA) essentially combines the patterns of genetic differentiation with locational information. The results of PCoA analysis generated very close two clusters with low genetic difference among populations collected from Karapurcek and Hendek regions which are both in Sakarya being geographically very close together. Whereas rest of the populations was observed to form individual clusters with distinct distance from each other (Figure 5.14).

For *R. luteum* Sweet populations, samples collected from Artvin and Catalca has been observed to form individual groups with distinct distances from each other. However,

two populations from Artvin region displayed a low density group in which last two members of 08ARL-1 population were observed to group together with 08ARL-2 population which implicates the close genetic compositions within those two populations (Figure 5.15).

Table 5.6. PhiPT values among nine *R. ponticum* L. populations (p-values for all pairs < 0.001).

Population Code	**41 YUVA**	**34 CAT**	**41 SUA**	**54 HEN1**	**54 HEN2**	**54 KARA1**	**54 KARA2**	**54 SA**	**81 DU**
41 YUVA	---								
34 CAT	0.504	---							
41 SUA	0.456	0.366	---						
54 HEN1	0.511	0.507	0.269	---					
54 HEN2	0.531	0.508	0.394	0.279	---				
54 KARA1	0.501	0.490	0.357	0.337	0.260	---			
54 KARA2	0.598	0.537	0.379	0.411	0.426	0.331	---		
54 SA	0.639	0.615	0.426	0.558	0.623	0.579	0.625	---	
81 DU	0.539	0.631	0.508	0.579	0.582	0.560	0.599	0.614	---

Table 5.7. PhiPT values among three *R. luteum* Sweet populations (p-values for all pairs < 0.001).

Population Code	**08 ARL1**	**08 ARL2**	**17 CA**
08 ARL1	---		
08 ARL2	0.187	---	
17 CA	0.424	0.438	---

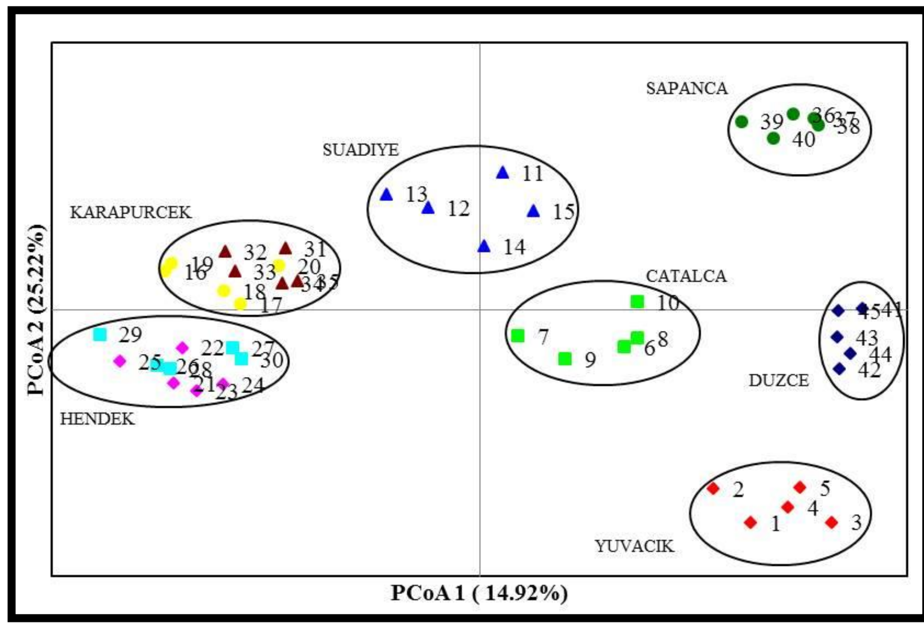

Figure 5.14. PCoA for nine *R. ponticum* L. populations with seven regional groups.

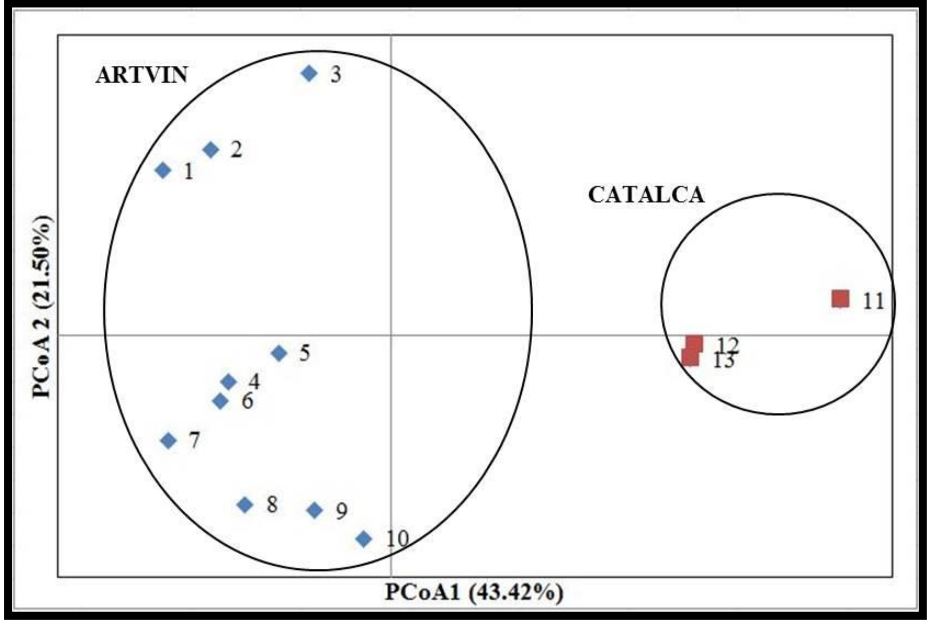

Figure 5.15. PCoA for three *R. luteum* Sweet populations with two regional groups.

6. DISCUSSION AND CONCLUSION

The topic of current extinction crisis mostly focuses on the fates of prominent endangered species with great emphasis on the deliberate exploitations by human beings as well as unavoidable global changes. Rhododendron genus of Turkey has been challenged by both factors for long years. Enlargement of cities and continuous constructions of badly planned roads demolished the natural habitats of these species. Additionally, invasive growth ability and toxicity of these species have been considered bad and accelerated their destruction by villagers sharing the same habitats for agriculture. Ignorance of their ornamental values due to lack of breeding strategies for domesticated cultivars, forces our country to import these cultivars to be used commercially or in landscaping. All known species of Rhododendrons in Turkey were identified and classified by foreign scientist [47] and very limited morphological studies are available in literature [82, 83]. The first comprehensive data on the habitat and morphology of Turkish Rhododendron genus have been collected during doctorate degree studies of Dr. Bahadır Altun [84]. A more detailed study covering all natural habitats of Turkey was conducted by financial support of Tubitak 112O500 project (2013-2016) included collection and recording; tissue culture proliferation; morphological characterization; molecular genetic diversity analysis and *ex-situ* preservation to generate conservation garden. As research partner of this project, the first detailed genetic diversity analysis by molecular marker systems of Turkish Rhododendrons was conducted under the scope of this thesis.

A total of 10 RAPD and 10 ISSR dominant markers as well as a pair of ITS markers were selected to determine genetic diversity and phylogenetic relationships of the 81 plant material out of 147 samples. The material covers all natural habitat of the genus from Marmara region to Black Sea region at altitude ranges 183 - 2289 meters. Both RAPD and ISSR primers have displayed 100% PPB and high value diversity statistics (Table 5.1). The high polymorphic nature of these markers on Rhododendrons was also confirmed by Lanying and Liu in their study using samples from Sichuan and Changbai [68, 69].

The cluster analysis of Rhododendron species, hybrids and variants (total of 23 samples) has indicated high genetic diversity among them with preferential distribution to

location but not to altitudes (Figure 5.9). Although, *R. caucasicum* Pallas (cluster IV), R. *smirnovii* Trautv (cluster V) and R. *luteum* Sweet (cluster VII) were grouped under unique clusters with a genetic distance ranging between 76% - 91.8 %, R. *ponticum* L. and R. *ungernii* Trautv. were grouped together in (cluster VI) in the same cluster with a genetic distance of 82%. Different combinations of these 5 species generate the genetic background of the hybrid species and yet the hybrids 08BO05, 08MU08 and 08MU09 were shown much closer genetic similarity to one of their parental species. For example, although 08BO05 hybrid genetic background comes from parental species, *R. caucasicum* Pallas and *R. ponticum* L, this hybrid has shown only 65.8 % genetic distance to its *R. ponticum* L. parent (Figure B.1). Evolutionary scientist indicated that hybrid species often display higher level of morphological variations then the combined morphological repertoire of both parental species [85]. This has been explained by the mixing of the parental genomes after the formation of F1 generation (segregation) and combined variations of hetero - specific genes (epistasis) [86]. However, segregation and epistasis result in range of hybrid generations with different fitness and fertility between individuals with some hybrids displaying closer genetic similarity to either one of the parents while others showing equal distance to both parents.

The only subspecies of *R. ponticum* L was firstly identified in Turkey by Merev and Yavuz [87] in 2000 as the only natural form with distinct morphological differences as white colored yellow spotted petals and naked ovarium. In our cluster analysis this form (08MU07) has shown very similar genetic distance to the two variants of *R. ponticum* L by being in the same cluster (cluster III, Figure 5.9). Interestingly, these two variants (08DU01 and 08BO01) were also discriminated with their unusually hairy ovarium and lack of yellow spotting in petals from the main species respectively. Thus it could be speculated that these two variants have both genetic and morphological diversity which should be studied further as potential new forms. Especially for the variant 08BO01, our speculation gained more support when results of phylogenetic relationships which were determined by the genetic diversity of ITS regions, has generated a unique cluster III for this variant (Figure 5.10). The rest of the samples in ITS diversity have shown consistent clustering of species with their variants or hybrids. Out of six clusters generated by the ITS sequence based analysis, except the previously mentioned cluster III, all other clusters

were separately gathered unique species with its variants or unique hybrid with its parental species and hybrid variants, indicating similar phylogeny among each cluster.

Population structure of *R. ponticum* L and *R. luteum* Sweet displayed a distinctive diversity among populations dependent to altitudinal and regional changes (Figure 5.11, Figure 5.12 and Figure 5.13). These results therefore indicated a lack gene flow among populations. In sympatric or parapatric populations, lack of genetic interactions was explained by maintenance of certain barriers referred as hybrid zones [88]. These hybrid zones if generated by habitat- independent manner, it has been observed to reflect the incompatibility of genomes to generate a fertile offspring between individuals of populations whereas if it is habitat-dependent, it reflects geographical separation as in altitude or habitat differences [88, 89]. Habitat dependent zones were considered to shape our population structure. Despite the same location, populations from different altitudinal range still preserved a very high genetic difference, as seen for example in individuals of Sakarya populations (54 HEN1,2; 54 KARA1,2 and 54 SA) with an average PhiPT value of 0.443 (Table 5.6). As indicated by other studies, this may be explained by the combined effects of better local genetic adaptations and small-scale abiotic heterogeneity in different altitudes [90]. Additionally, climatic gradients generated by altitudinal changes leading to asynchronous flowering time or disjoint pollinator community could also be another contributing factors limiting the gene flow among populations.

Although existence of species depends on availability of genetic diversity in nature, *in-situ* preservations and *ex-situ* conservation are equally important for the duration of these diversities. Therefore, increase in awareness among Turkish scientists for national treasures requires comprehensive study of endangered species of our country by documenting the genotypes with respect to their habitats, morphological/physiological and genetic characteristics. Our results have contributed the genetic diversity data to the Tubitak 112O500 project which has already documented the other characteristics of Turkish Rhododendron genus and also guide the selection of genotypes in conservation gene garden which was established in 2015 at the slopes of Genya Mountain (1776 m) / Artvin.

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APPENDIX A: PCR AMPLIFICATION RESULTS

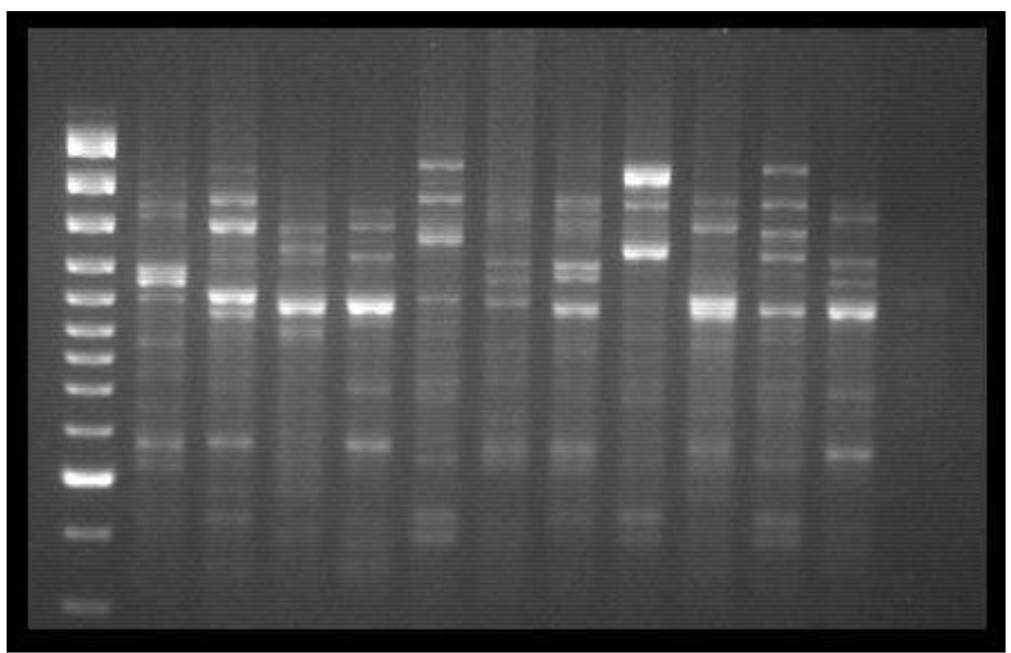

Figure A.1. PCR amplification of 24 Rhododendron species with ISSR 817 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

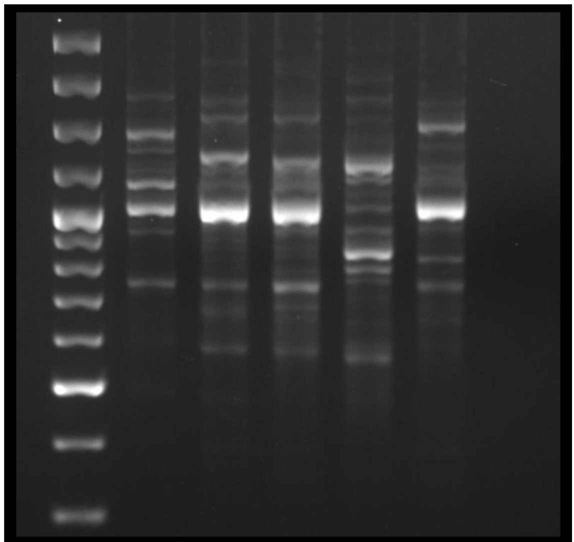

Figure A.2. PCR amplification of 24 Rhododendron species with ISSR 817 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

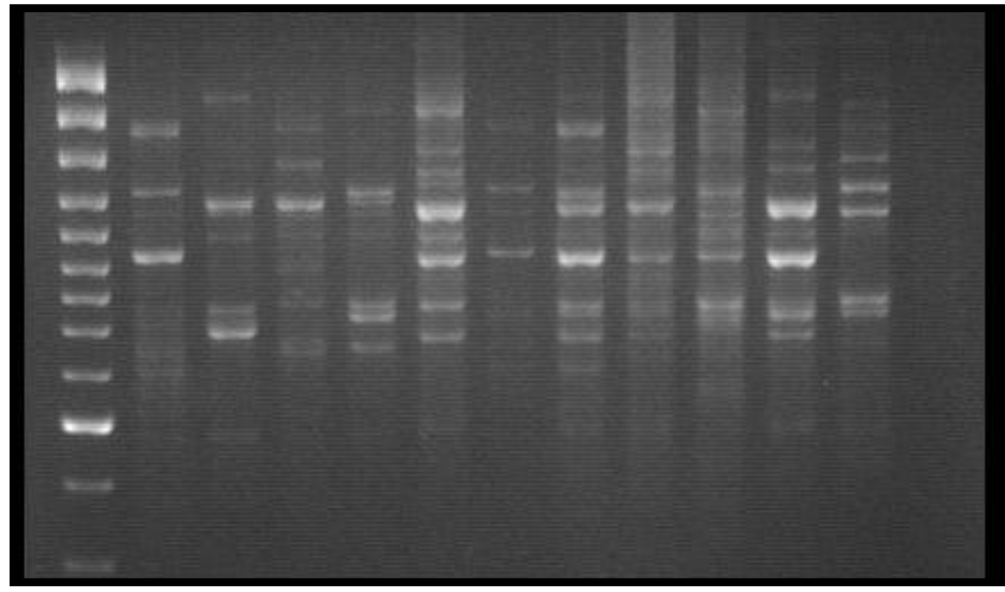

Figure A.3. PCR amplification of 24 Rhododendron species with ISSR 826 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

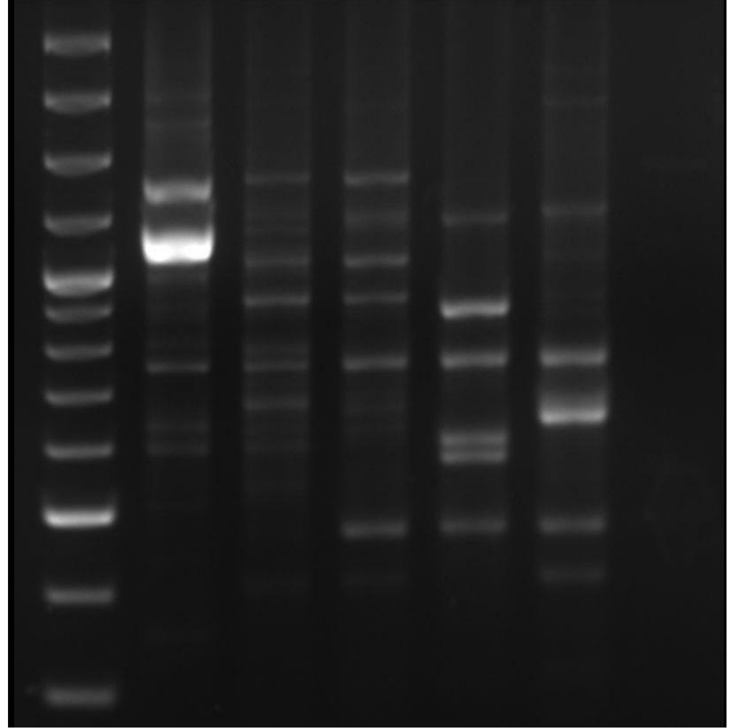

Figure A.4. PCR amplification of 24 Rhododendron species with ISSR 826 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

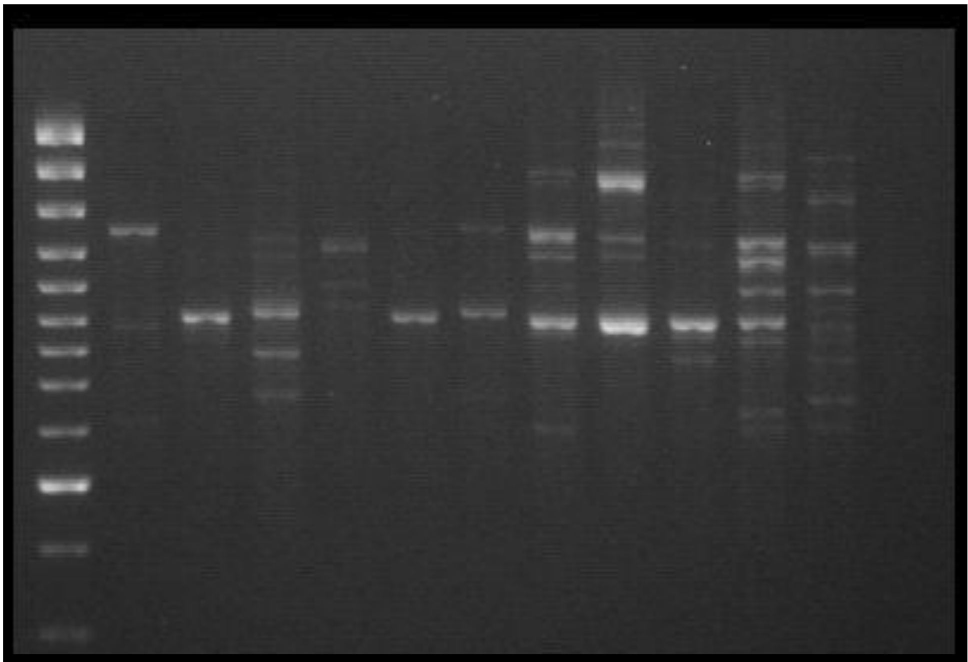

Figure A.5. PCR amplification of 24 Rhododendron species with ISSR 827 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

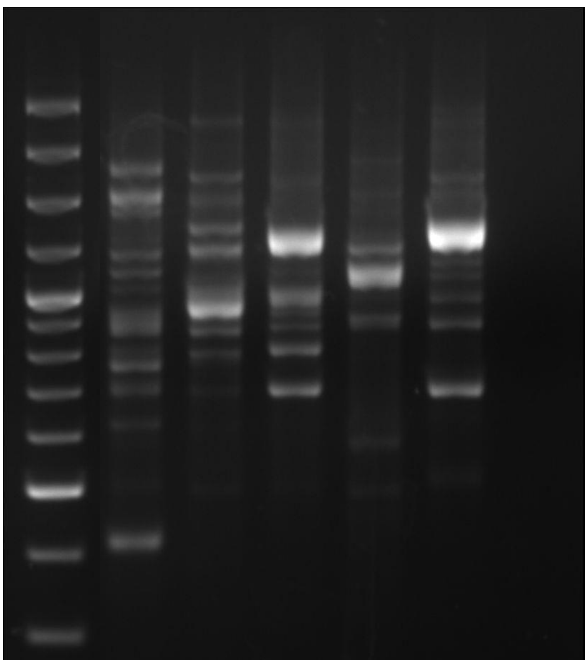

Figure A.6. PCR amplification of 24 Rhododendron species with ISSR 827 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

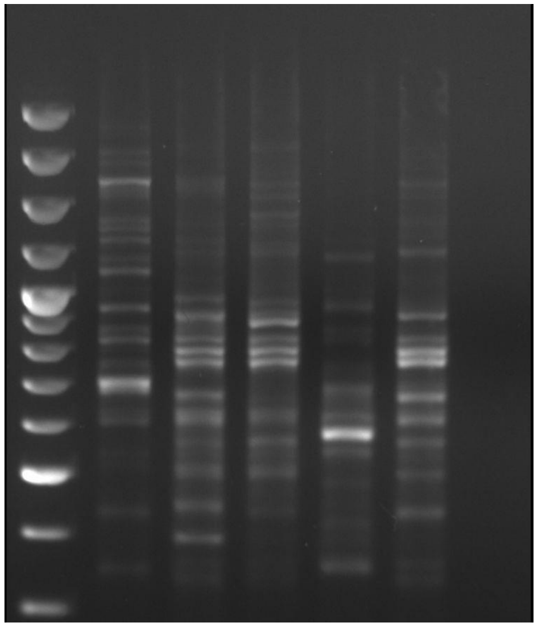

Figure A.7. PCR amplification of 24 Rhododendron species with ISSR 835 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

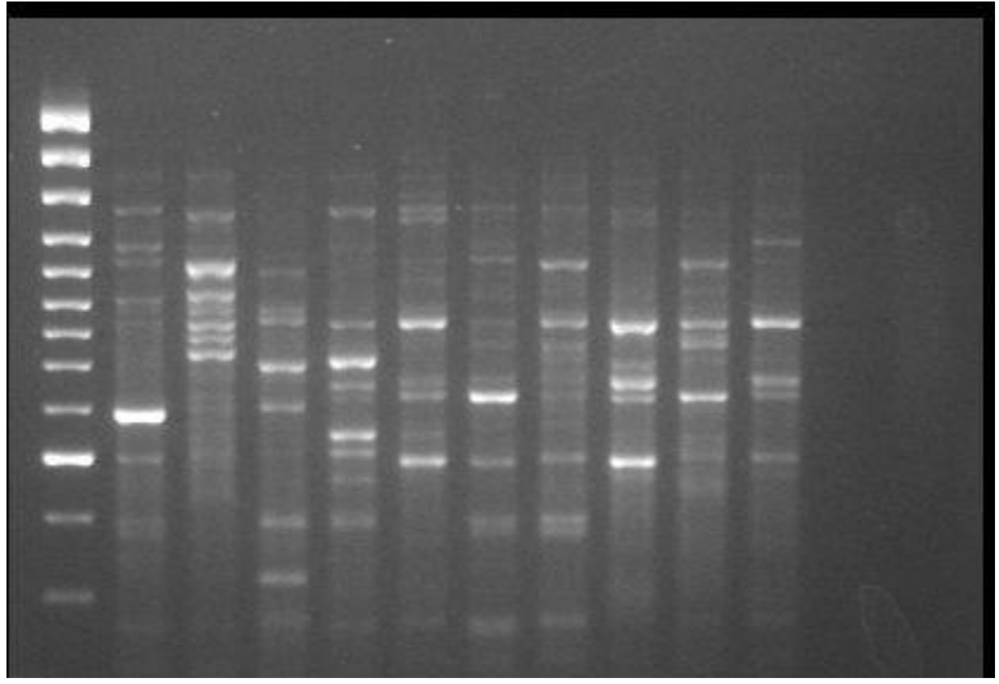

Figure A.8. PCR amplification of 24 Rhododendron species with ISSR 836 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

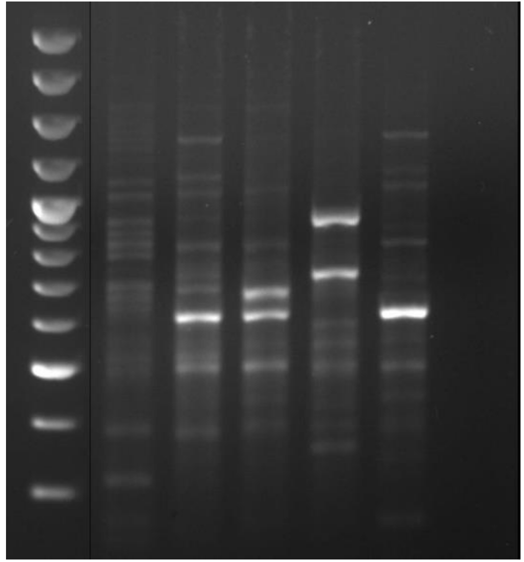

Figure A.9. PCR amplification of 24 Rhododendron species with ISSR 836 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

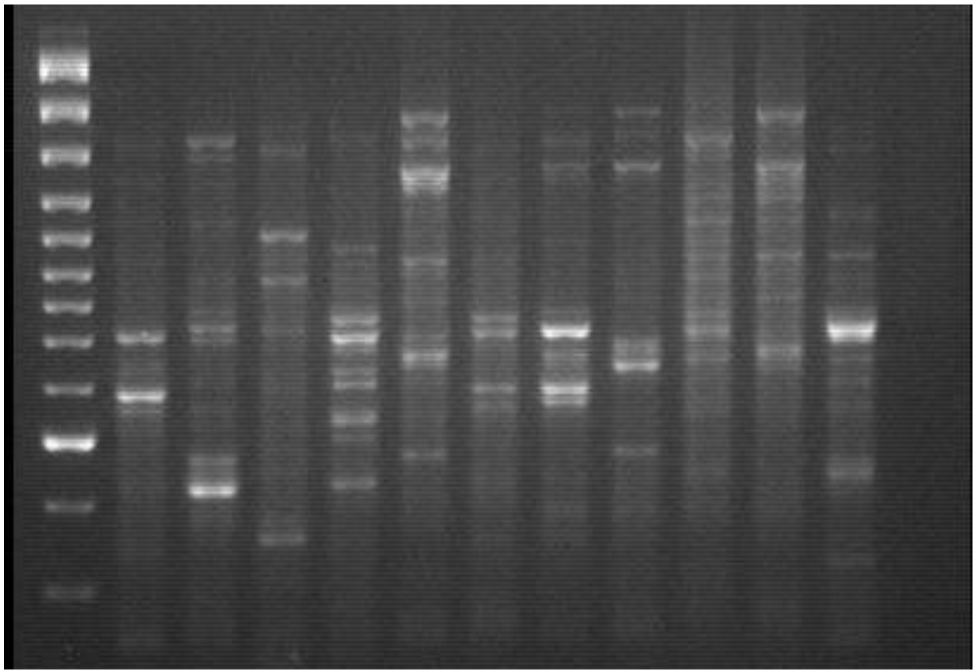

Figure A.10. PCR amplification of 24 Rhododendron species with ISSR 841 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

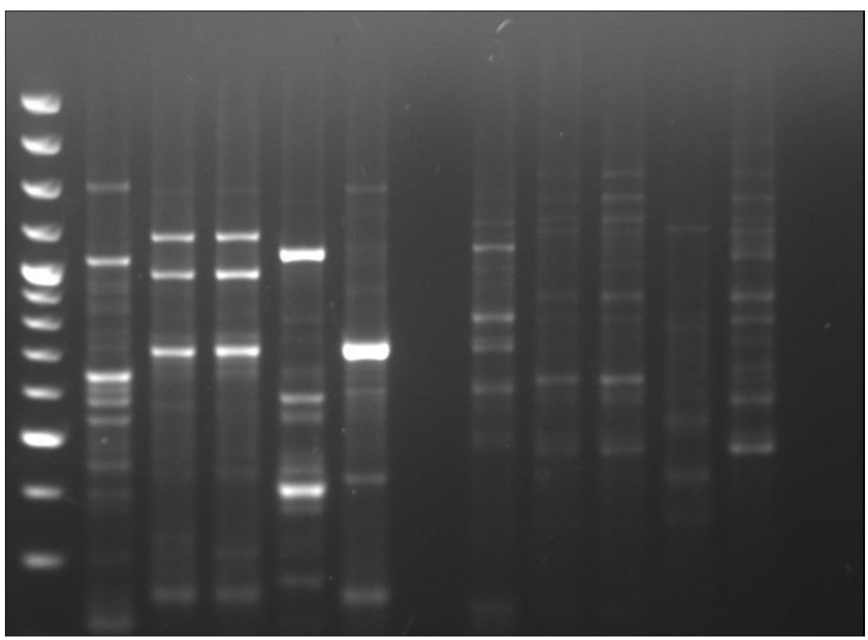

Figure A.11. PCR amplification of 24 Rhododendron species with ISSR 841-842 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

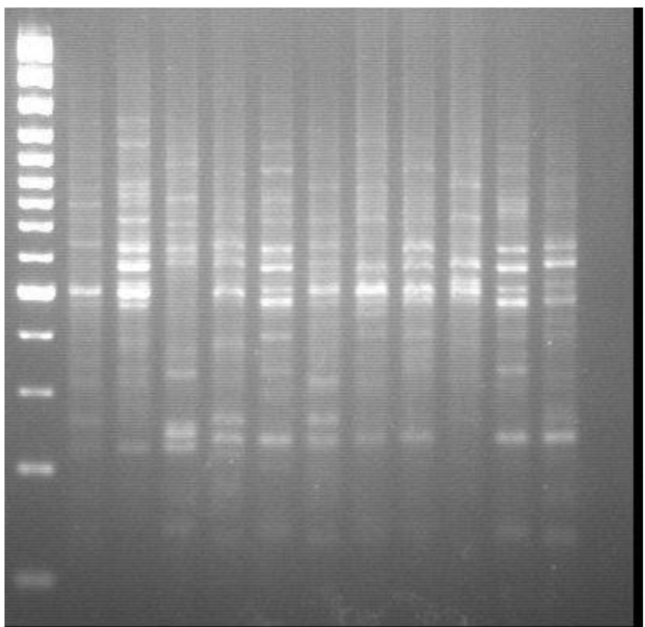

Figure A.12. PCR amplification of 24 Rhododendron species with ISSR 842 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

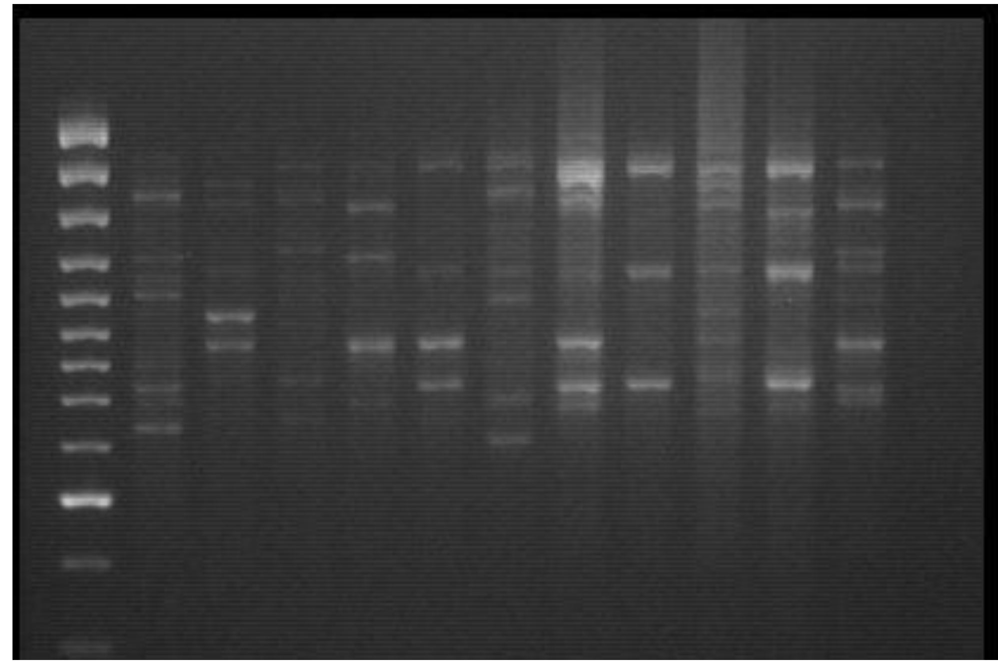

Figure A.13. PCR amplification of 24 Rhododendron species with ISSR 856 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

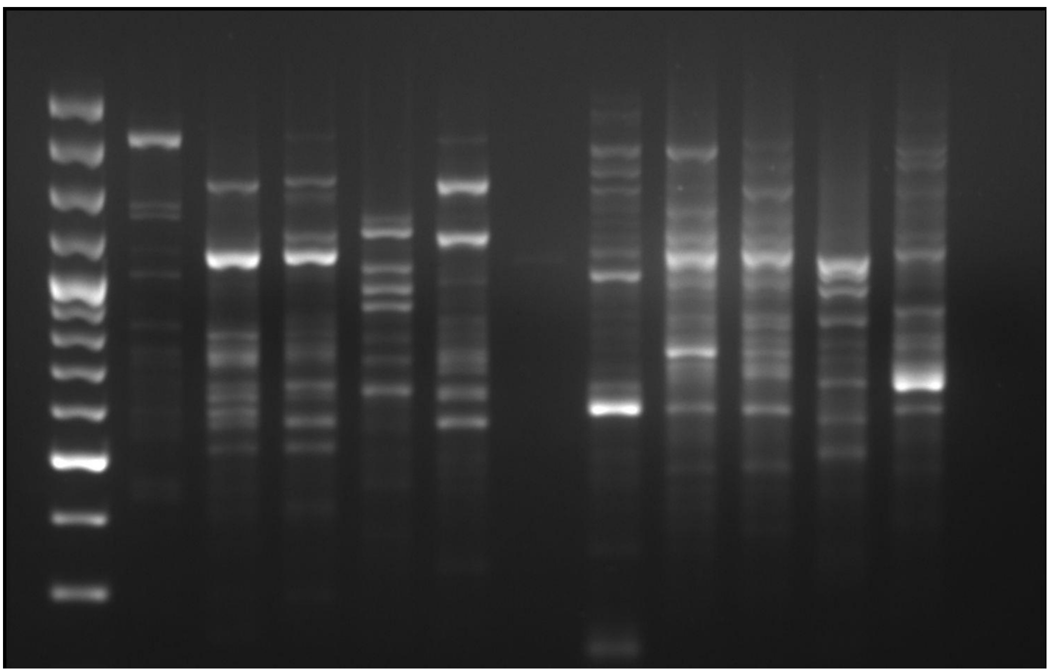

Figure A.14. PCR amplification of 24 Rhododendron species with ISSR 855-856 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

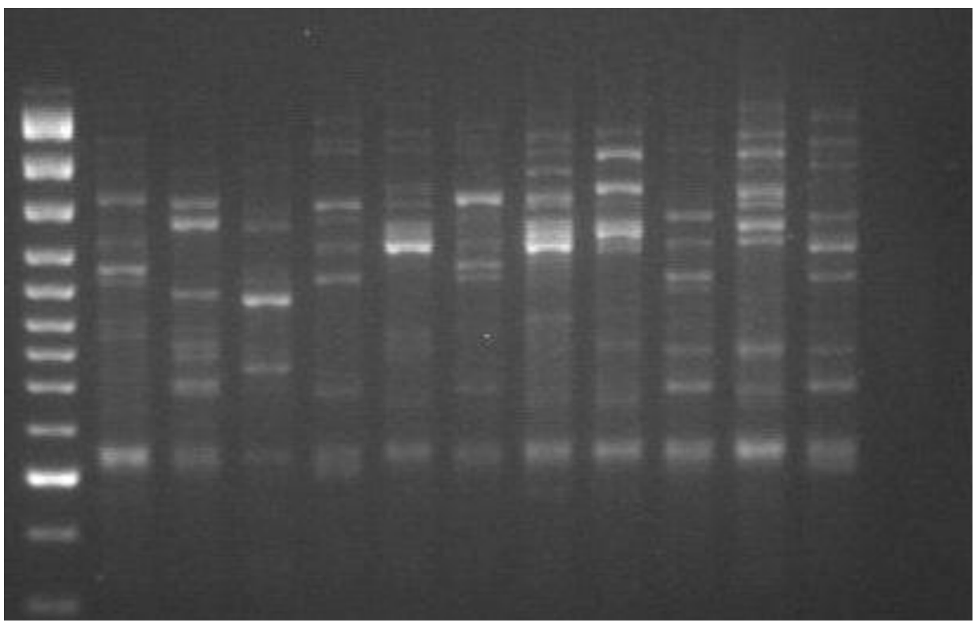

Figure A.15. PCR amplification of 24 Rhododendron species with ISSR 857 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

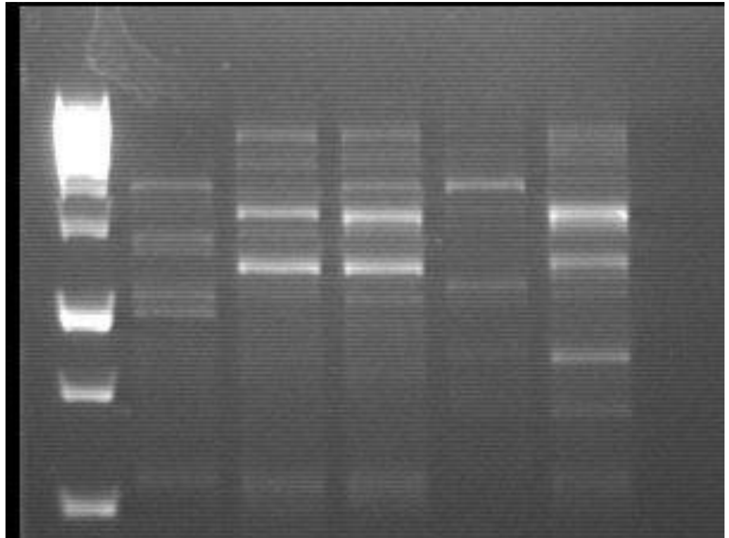

Figure A.16. PCR amplification of 24 Rhododendron species with ISSR 857 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

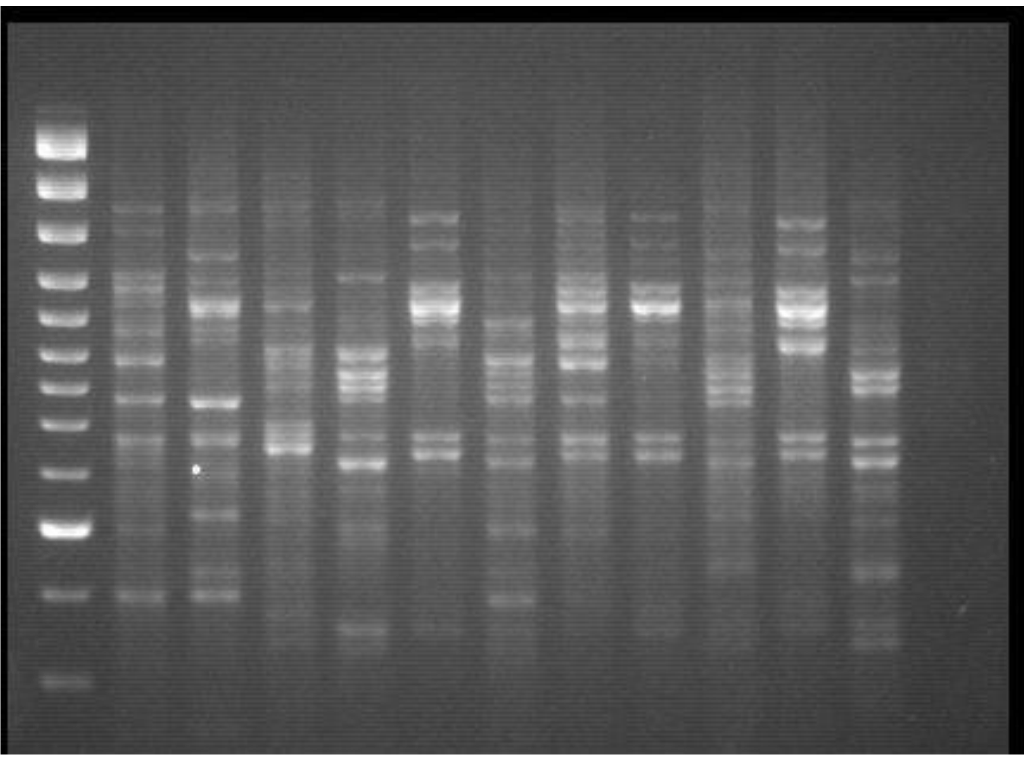

Figure A.17. PCR amplification of 24 Rhododendron species with ISSR 835 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

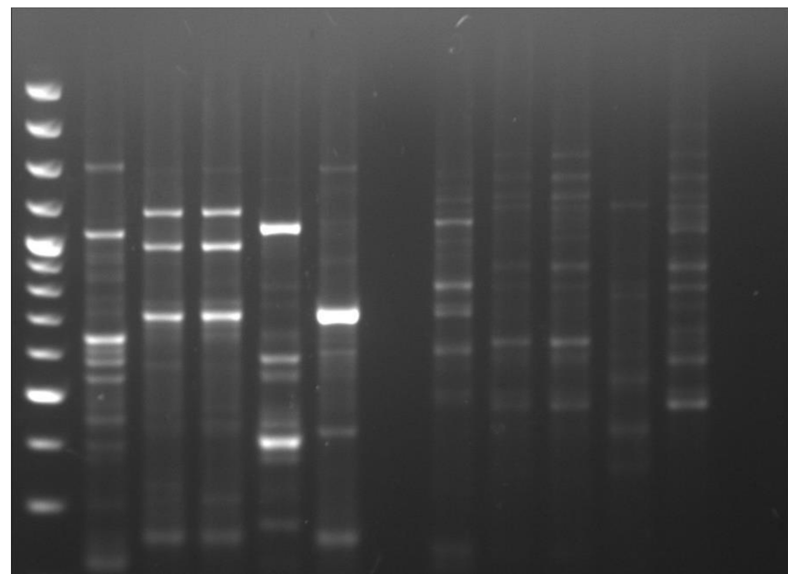

Figure A.18. PCR amplification of 24 Rhododendron species with ISSR 841 - 842 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

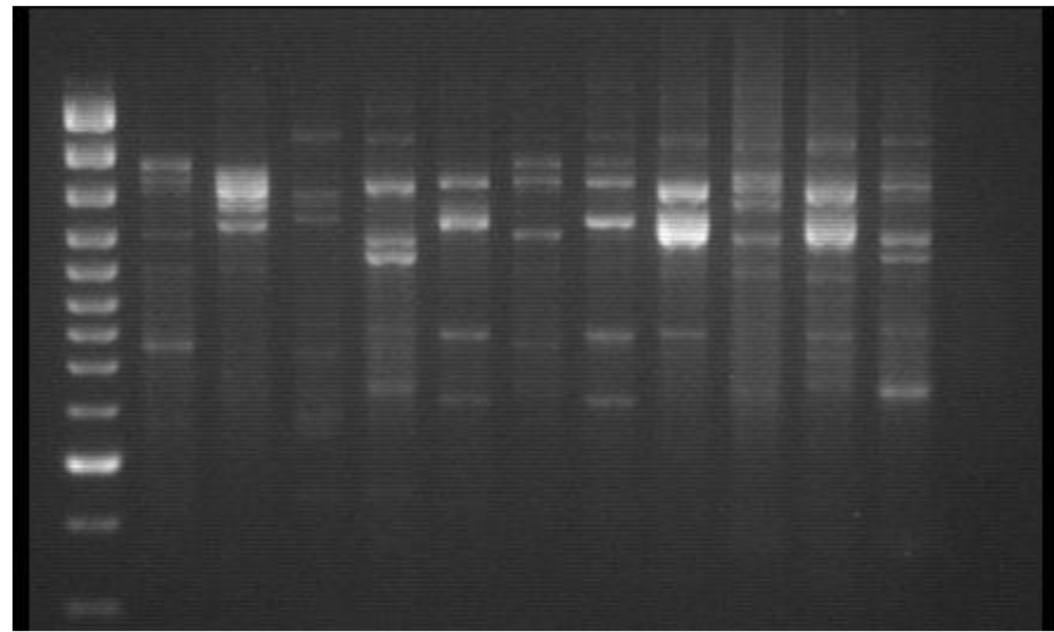

Figure A.19. PCR amplification of 24 Rhododendron species with ISSR 855 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

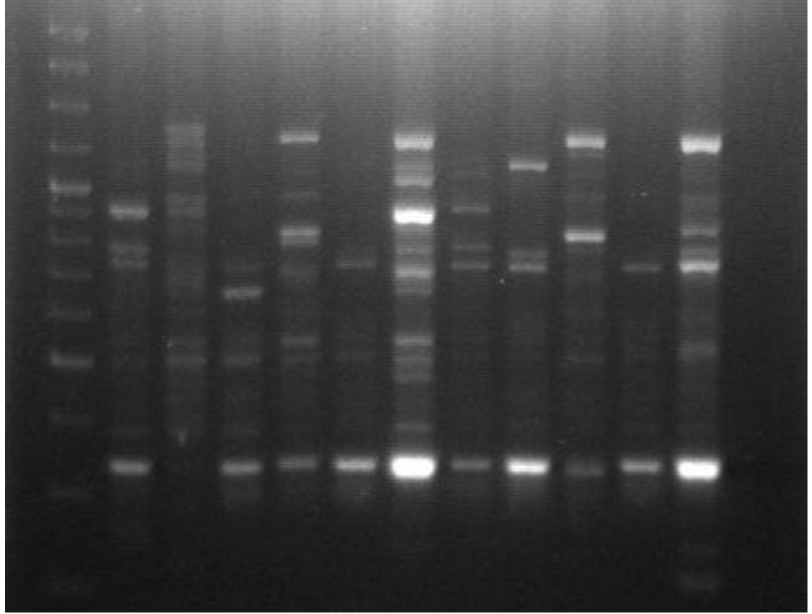

Figure A.20. PCR amplification of 24 Rhododendron species with RAPD F3 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

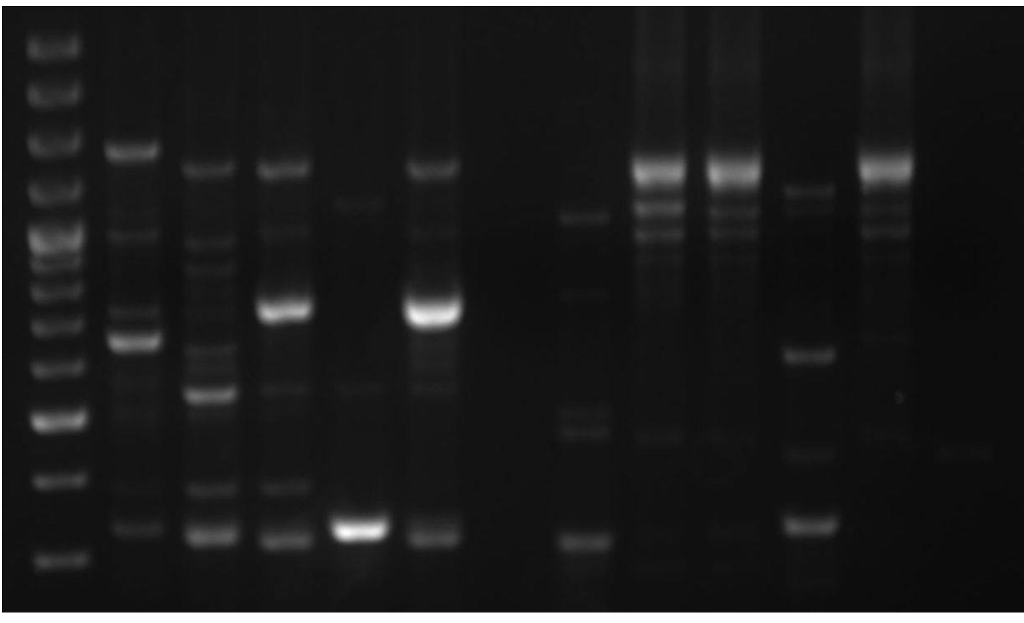

Figure A.21. PCR amplification of 24 Rhododendron species with RAPD F3 – F7 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

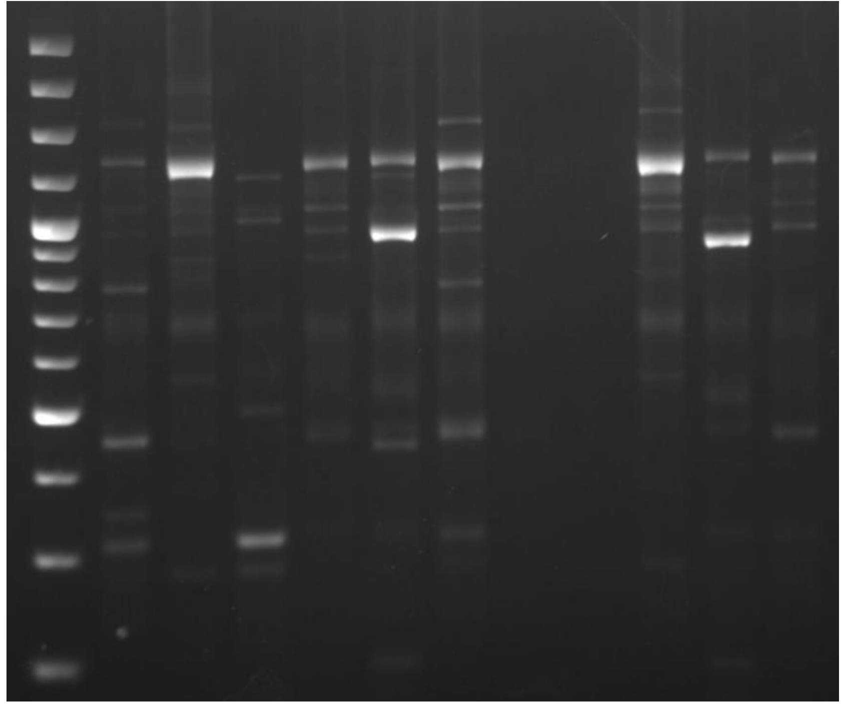

Figure A.22. PCR amplification of 24 Rhododendron species with RAPD F7 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

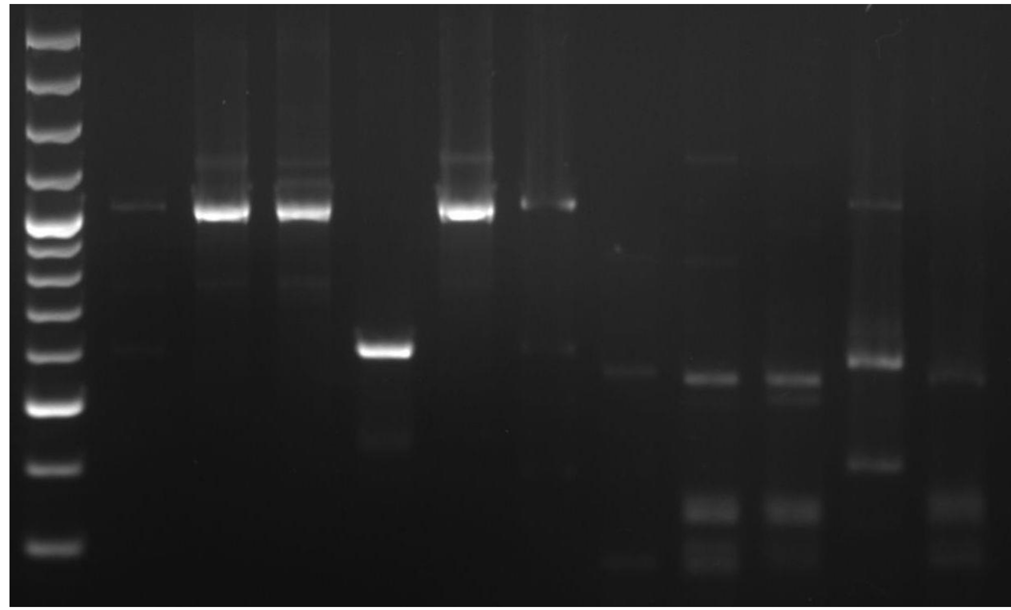

Figure A.23. PCR amplification of 24 Rhododendron species with RAPD F8 – F10 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

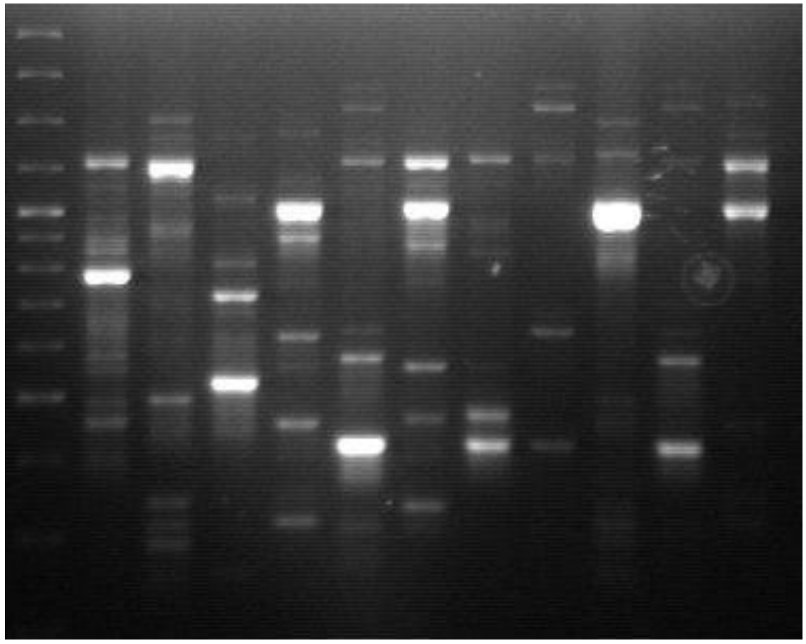

Figure A.24. PCR amplification of 24 Rhododendron species with RAPD F8 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

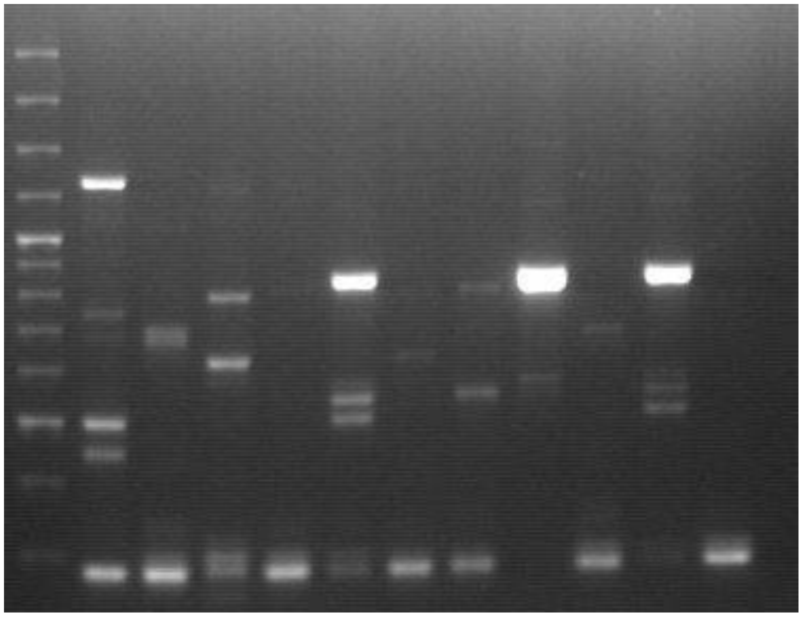

Figure A.25. PCR amplification of 24 Rhododendron species with RAPD F10 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

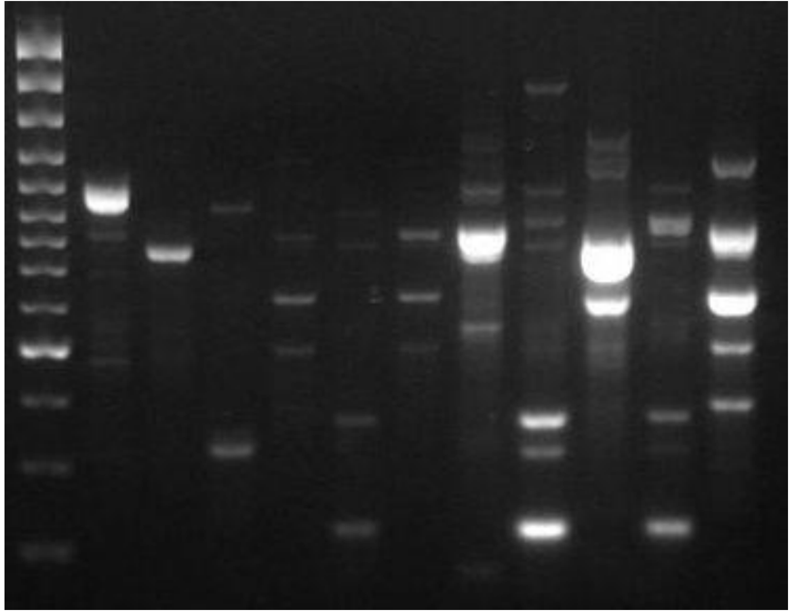

Figure A.26. PCR amplification of 24 Rhododendron species with RAPD F12 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

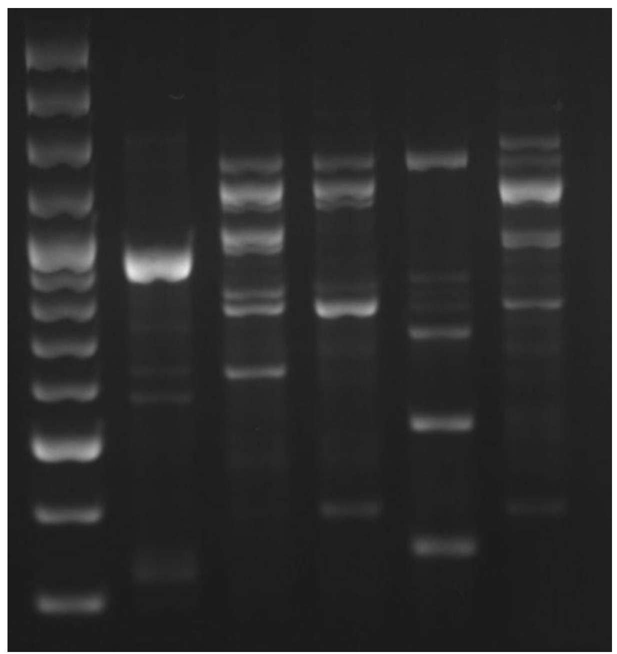

Figure A.27. PCR amplification of 24 Rhododendron species with RAPD F12 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

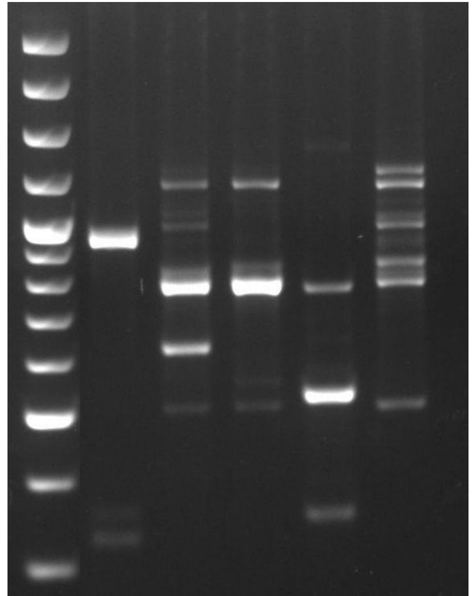

Figure A.28. PCR amplification of 24 Rhododendron species with RAPD K11 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

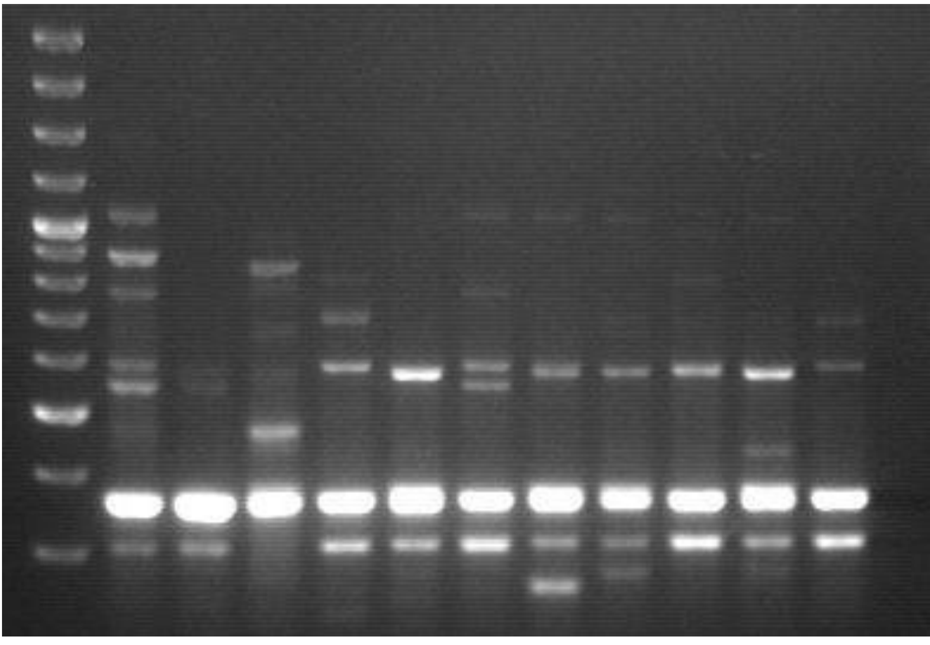

Figure A.29. PCR amplification of 24 Rhododendron species with RAPD F13 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

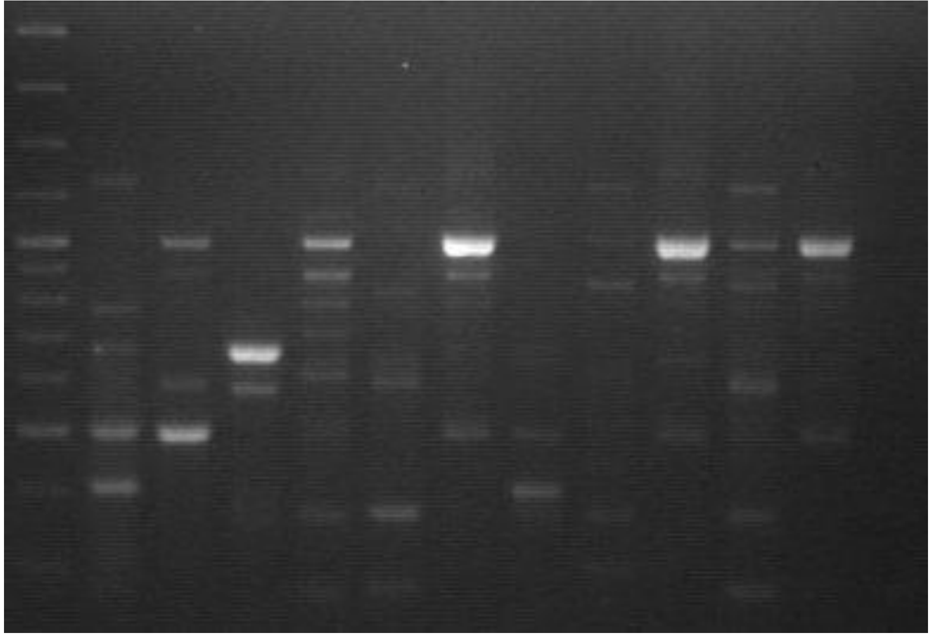

Figure A.30. PCR amplification of 24 Rhododendron species with RAPD F14 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

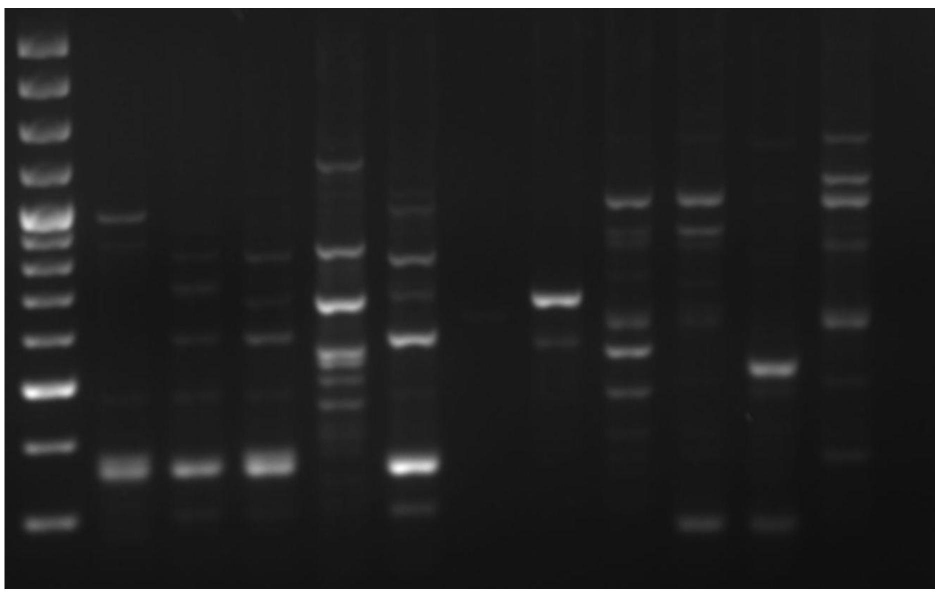

Figure A.31. PCR amplification of 24 Rhododendron species with RAPD F13 – F14 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

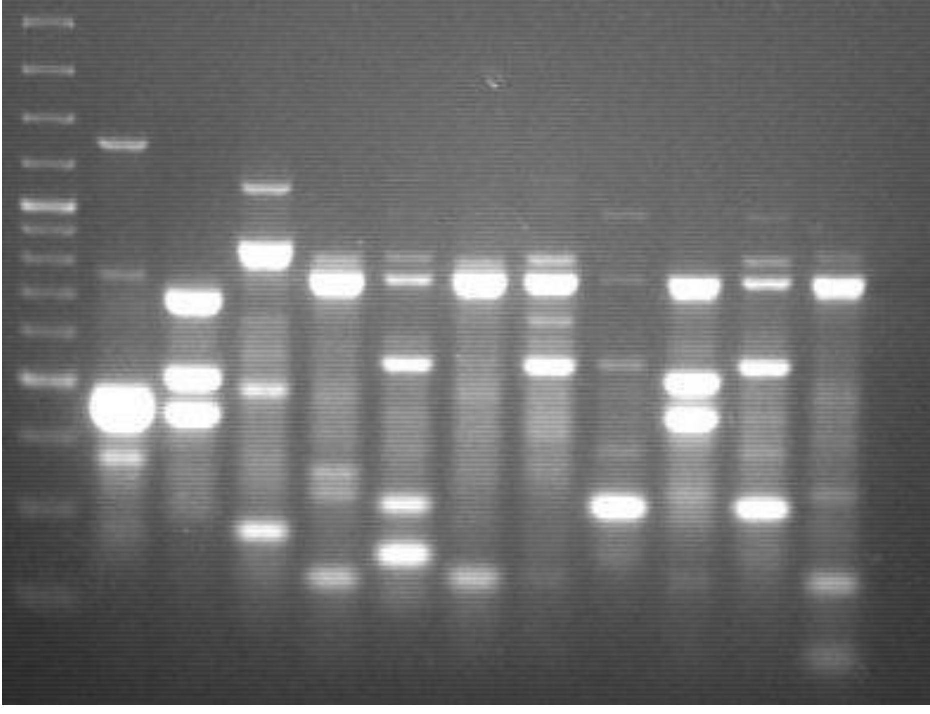

Figure A.32. PCR amplification of 24 Rhododendron species with RAPD G14 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

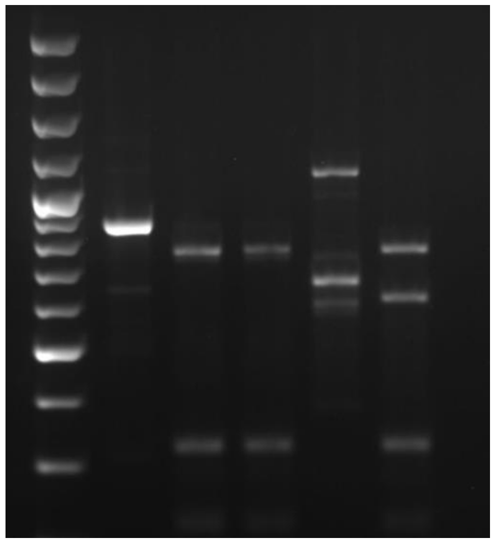

Figure A.33. PCR amplification of 24 Rhododendron species with RAPD G14 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

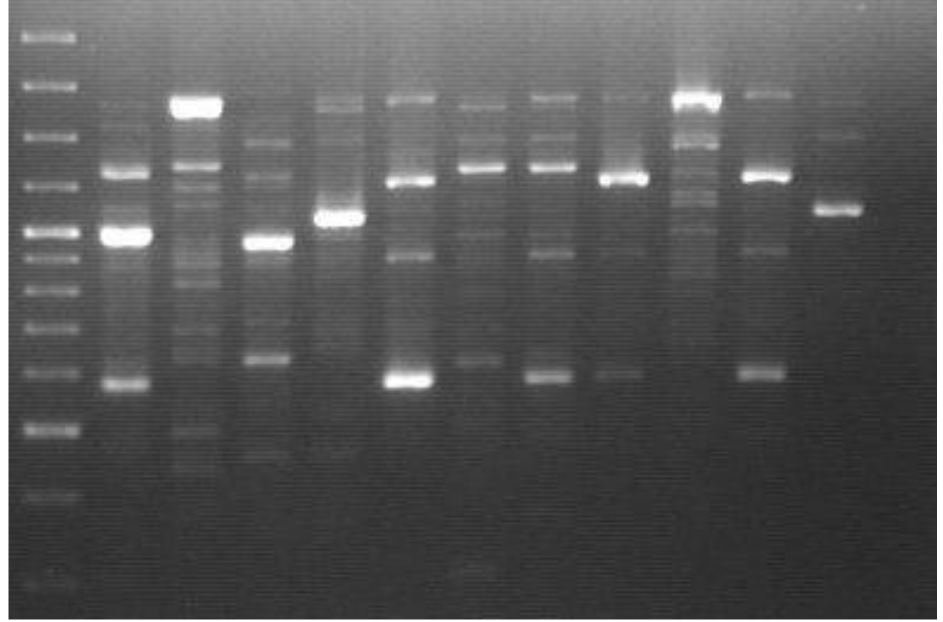

Figure A.34. PCR amplification of 24 Rhododendron species with RAPD K11 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

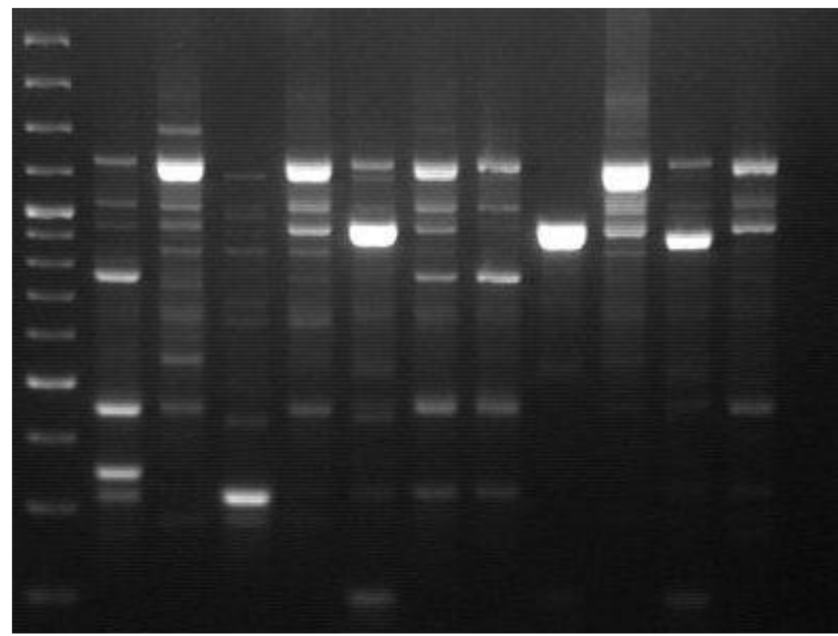

Figure A.35. PCR amplification of 24 Rhododendron species with RAPD M5 primer. M: GeneRuler 100bp plus. Samples 1 – 11: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

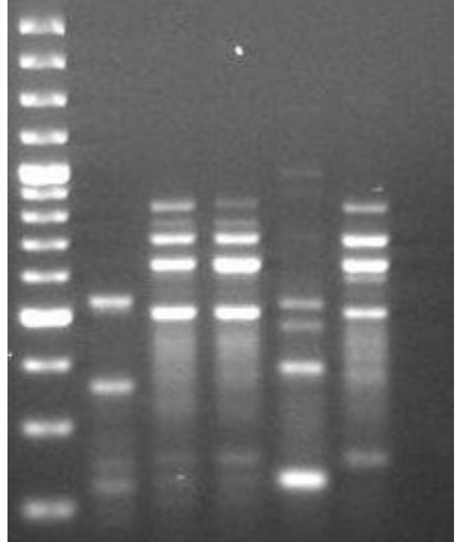

Figure A.36. PCR amplification of 24 Rhododendron species with RAPD M5 primer. M: GeneRuler 100bp plus. Samples 1 – 5: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5.

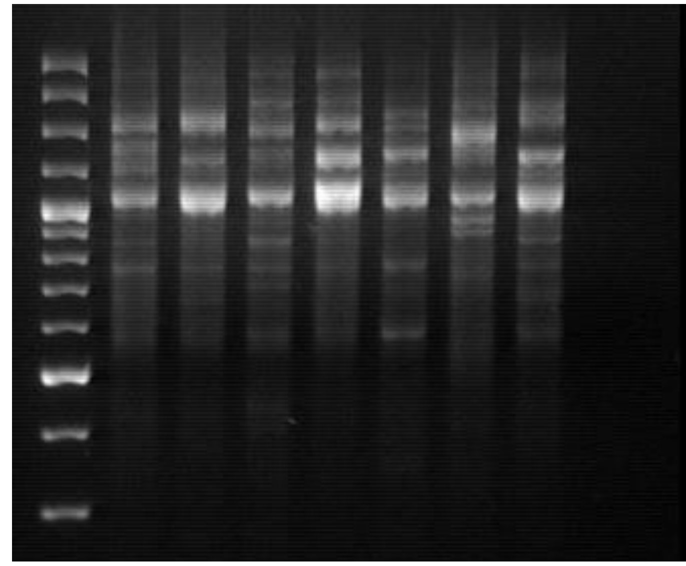

Figure A.37. PCR amplification of 24 Rhododendron species with ISSR 817 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

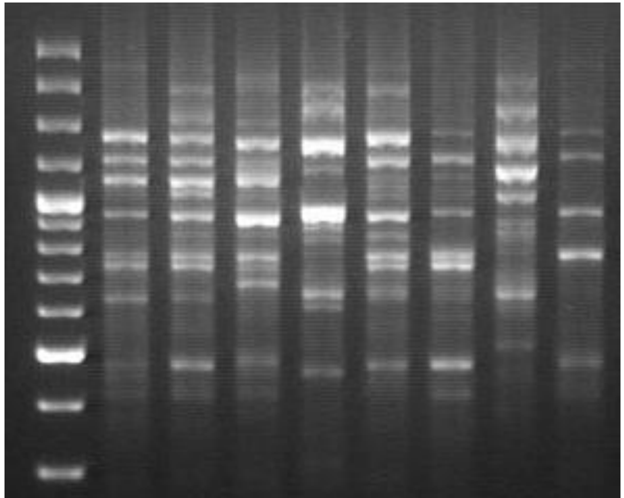

Figure A.38 PCR amplification of 24 Rhododendron species with ISSR 826 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

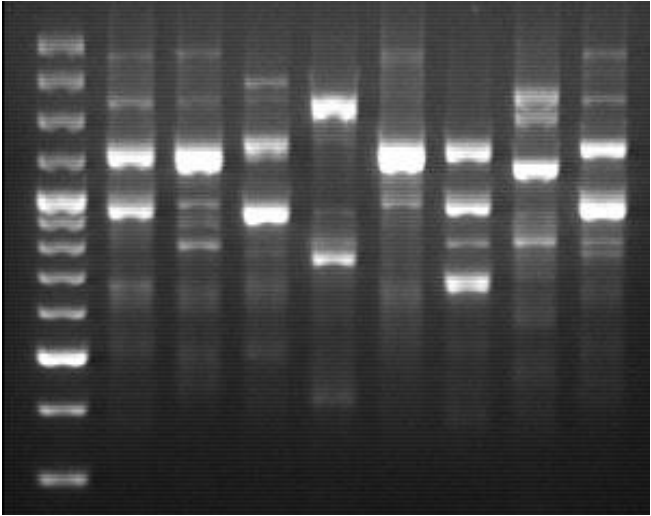

Figure A.39. PCR amplification of 24 Rhododendron species with ISSR 827 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

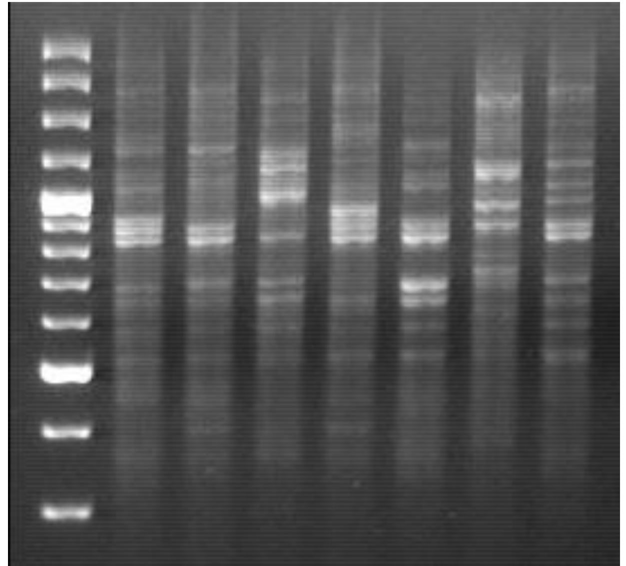

Figure A.40. PCR amplification of 24 Rhododendron species with ISSR 835 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

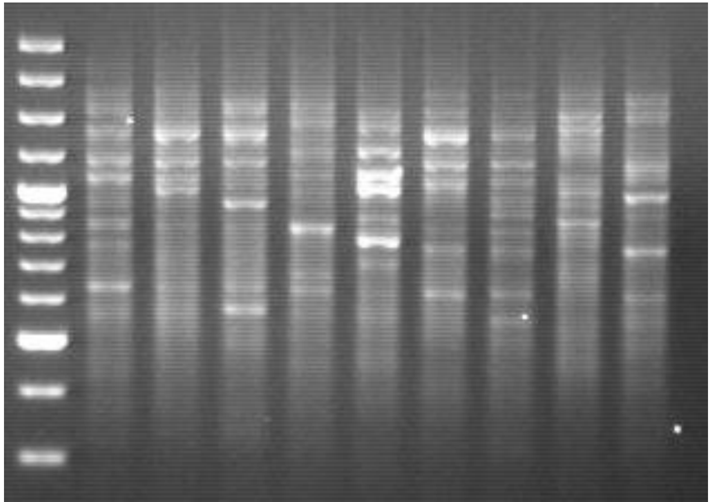

Figure A.41. PCR amplification of 24 Rhododendron species with ISSR 836 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

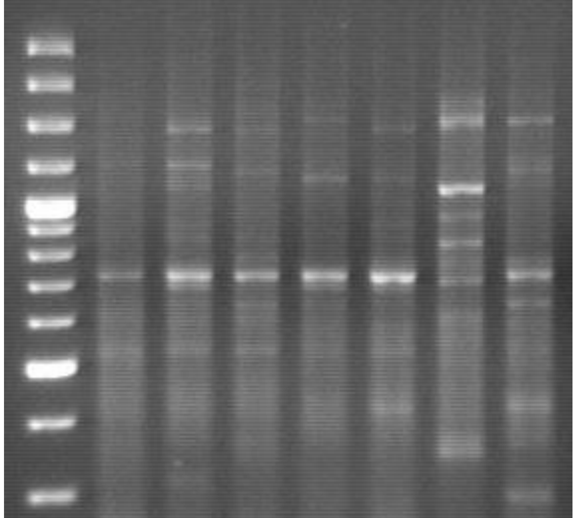

Figure A.42. PCR amplification of 24 Rhododendron species with ISSR 841 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

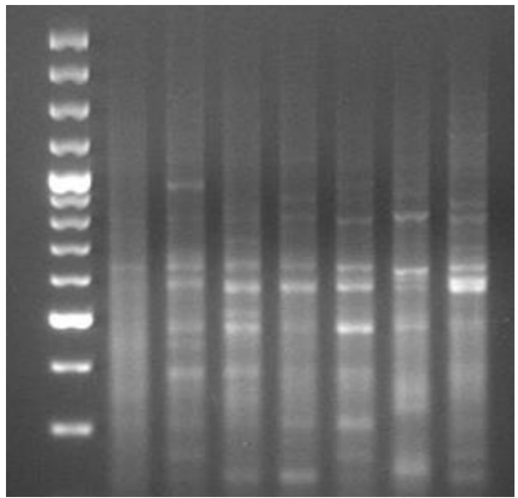

Figure A.43. PCR amplification of 24 Rhododendron species with ISSR 842 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

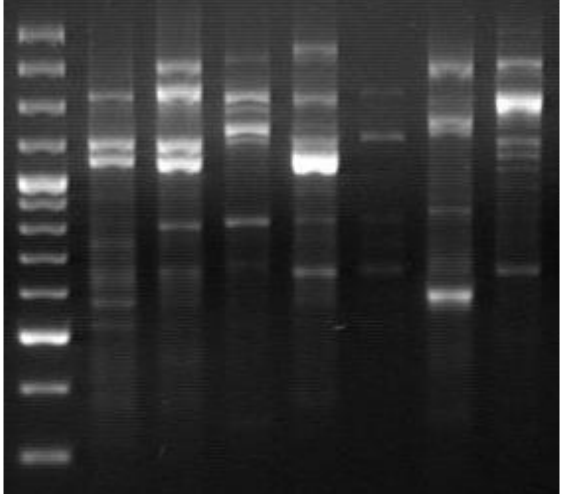

Figure A.44. PCR amplification of 24 Rhododendron species with ISSR 855 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

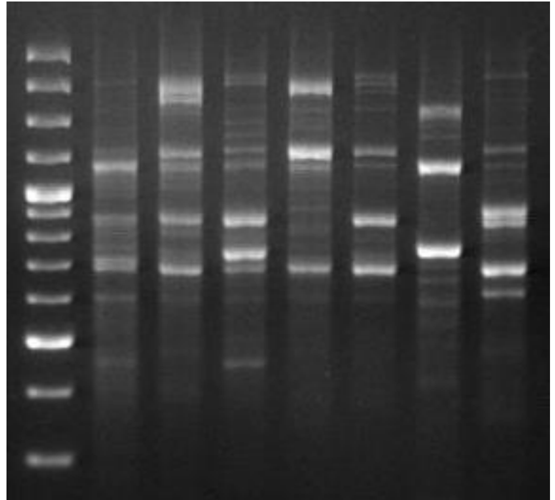

Figure A.45. PCR amplification of 24 Rhododendron species with ISSR 856 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

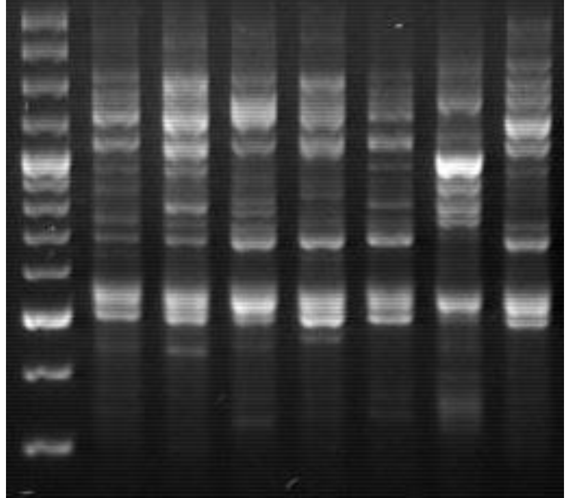

Figure A.46. PCR amplification of 24 Rhododendron species with ISSR 857 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

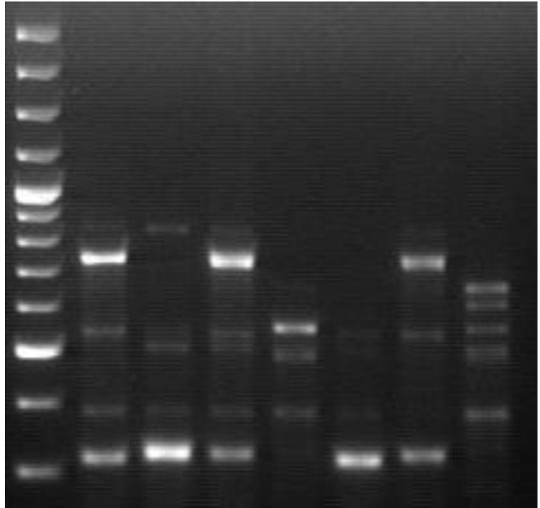

Figure A.47. PCR amplification of 24 Rhododendron species with RAPD F3 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

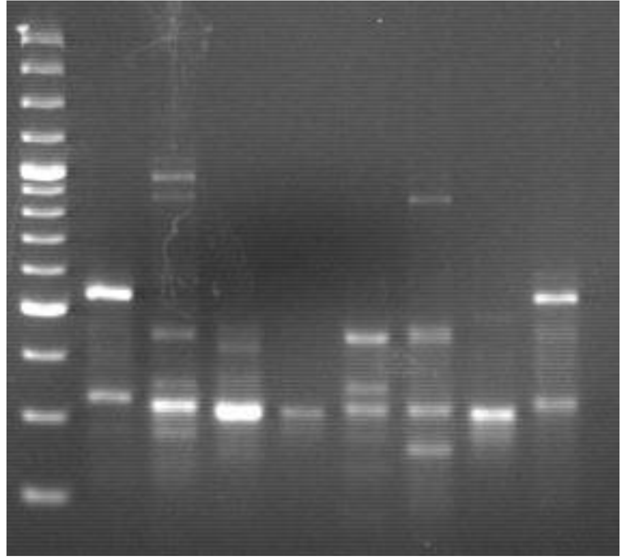

Figure A.48. PCR amplification of 24 Rhododendron species with RAPD F7 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

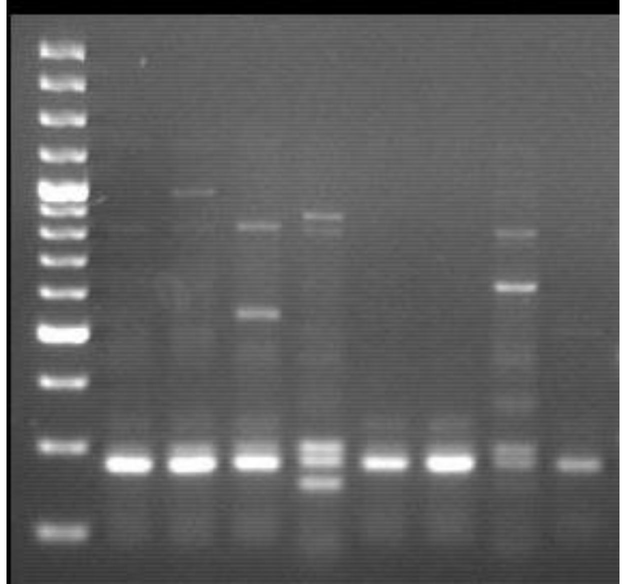

Figure A.49. PCR amplification of 24 Rhododendron species with RAPD F10 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

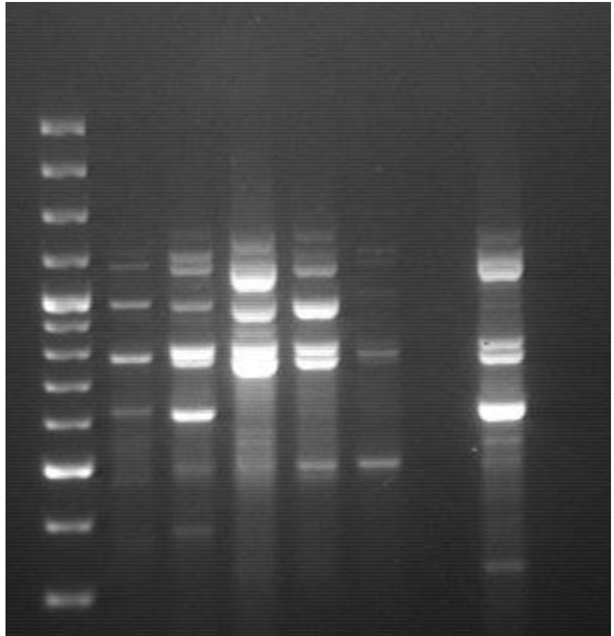

Figure A.50. PCR amplification of 24 Rhododendron species with RAPD F12 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

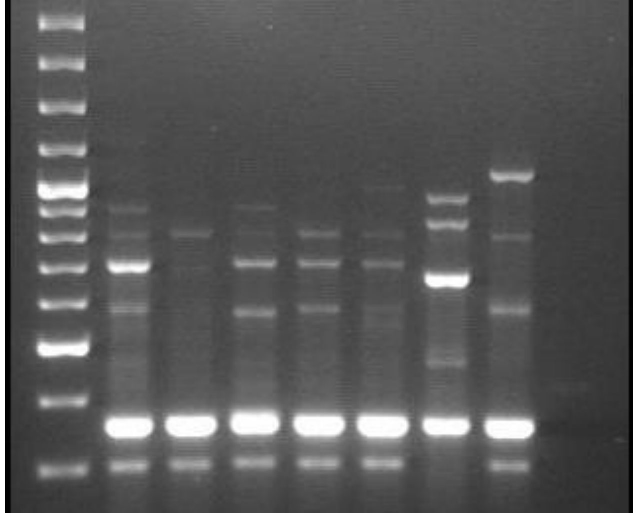

Figure A.51. PCR amplification of 24 Rhododendron species with RAPD F13 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

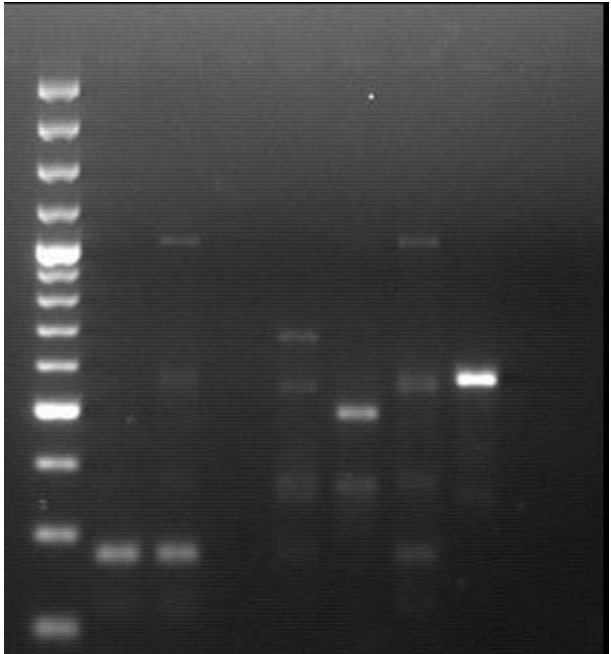

Figure A.52. PCR amplification of 24 Rhododendron species with RAPD F14 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

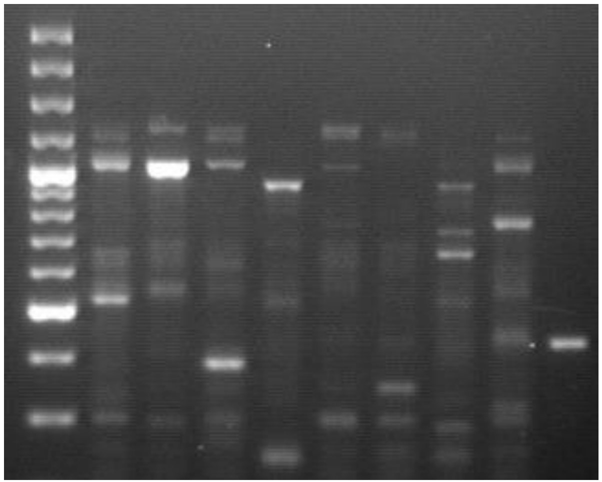

Figure A.53. PCR amplification of 24 Rhododendron species with RAPD G14 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

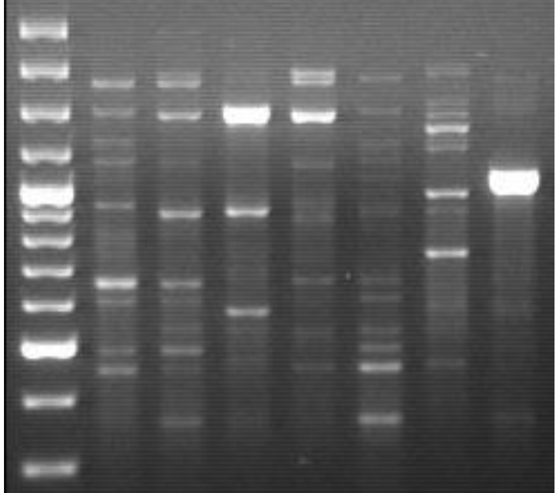

Figure A.54. PCR amplification of 24 Rhododendron species with RAPD K11 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

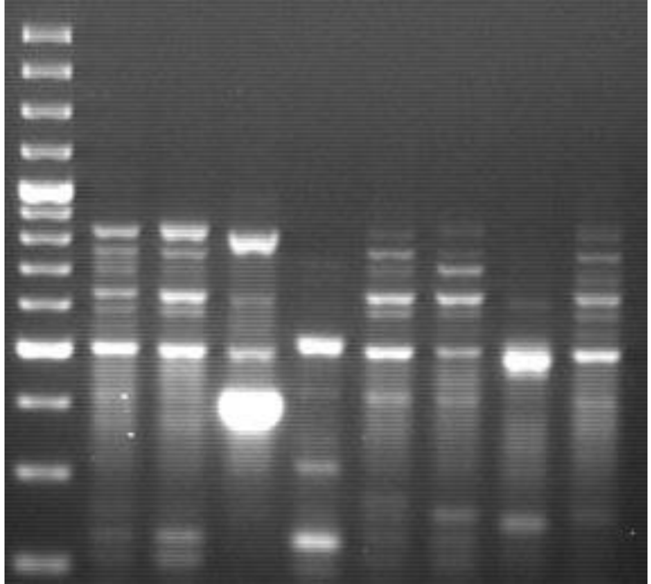

Figure A.55. PCR amplification of 24 Rhododendron species with RAPD M5 primer. M: GeneRuler 100bp plus. Samples 1 – 8: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.6.

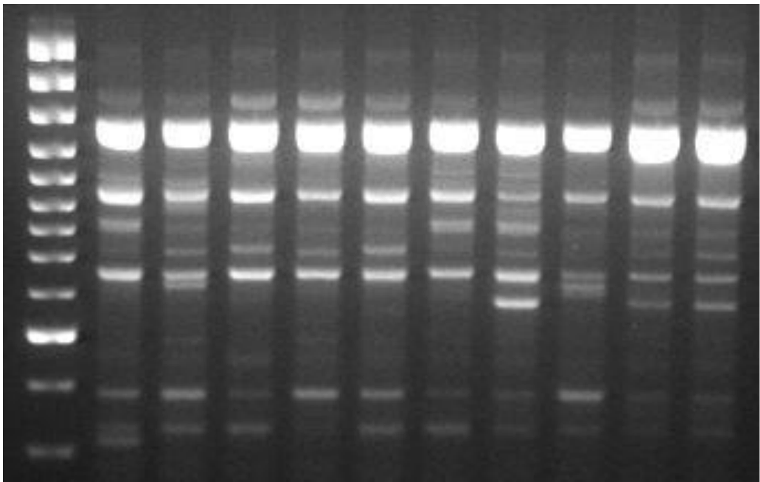

Figure A.56. PCR amplification of Rhododendron population 08ARL with RAPD F3 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 08ARL1-2.

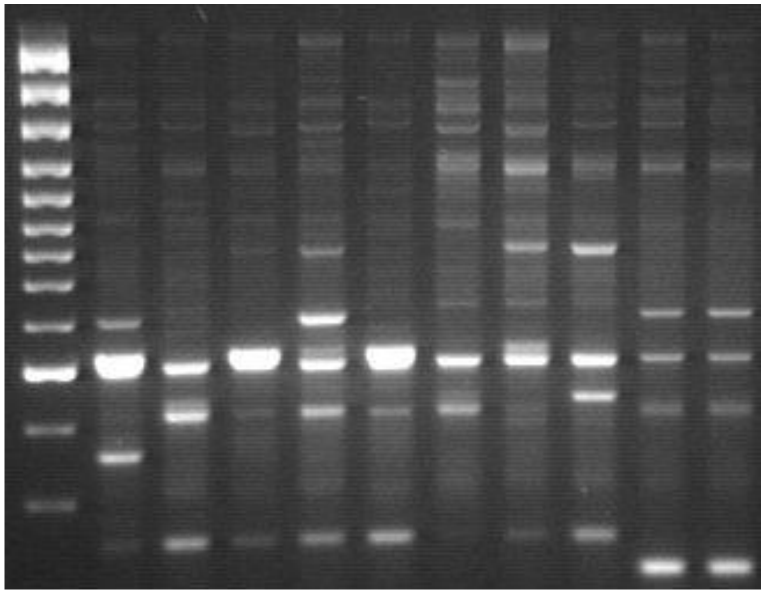

Figure A.57. PCR amplification of Rhododendron population 08ARL with RAPD M5 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 08ARL1-2.

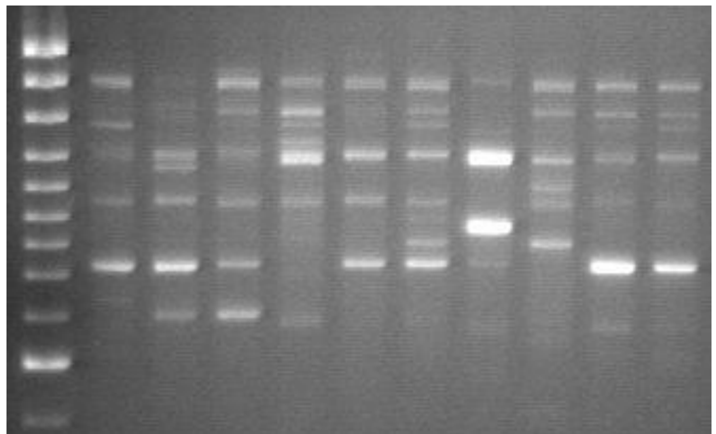

Figure A.58. PCR amplification of Rhododendron population 08ARL with RAPD K11 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 08ARL1-2.

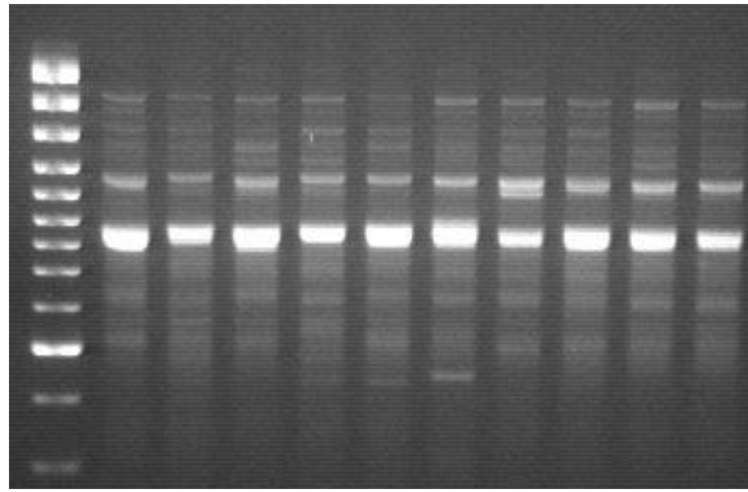

Figure A.59. PCR amplification of Rhododendron population 08ARL with RAPD G14 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 08ARL1-2.

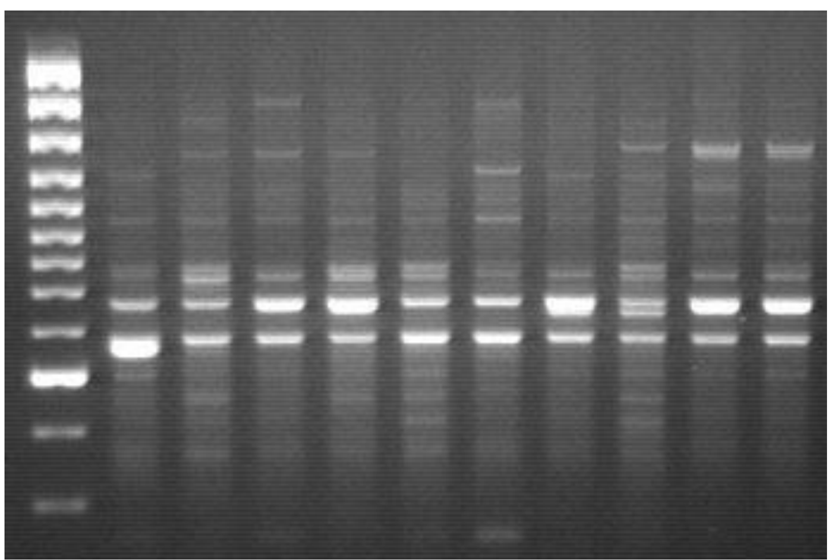

Figure A.60. PCR amplification of Rhododendron population 08ARL with RAPD F14 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 08ARL1-2.

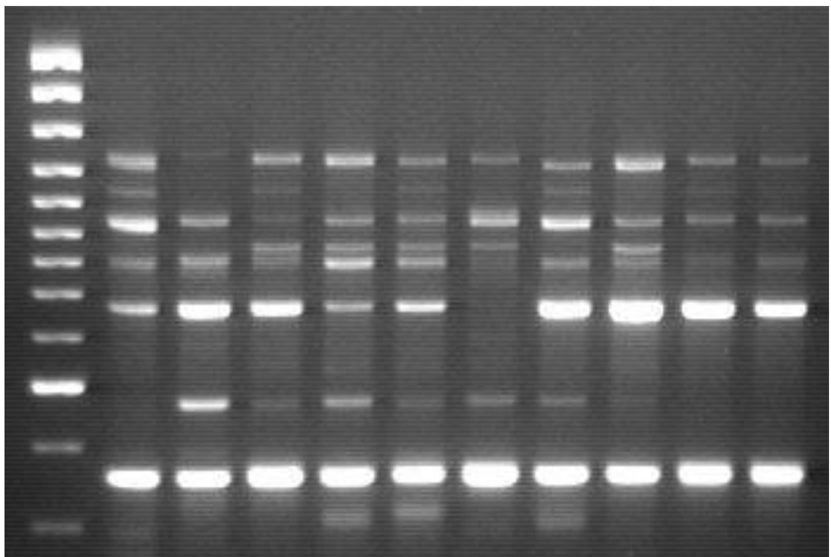

Figure A.61. PCR amplification of Rhododendron population 08ARL with RAPD F13 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 08ARL1-2.

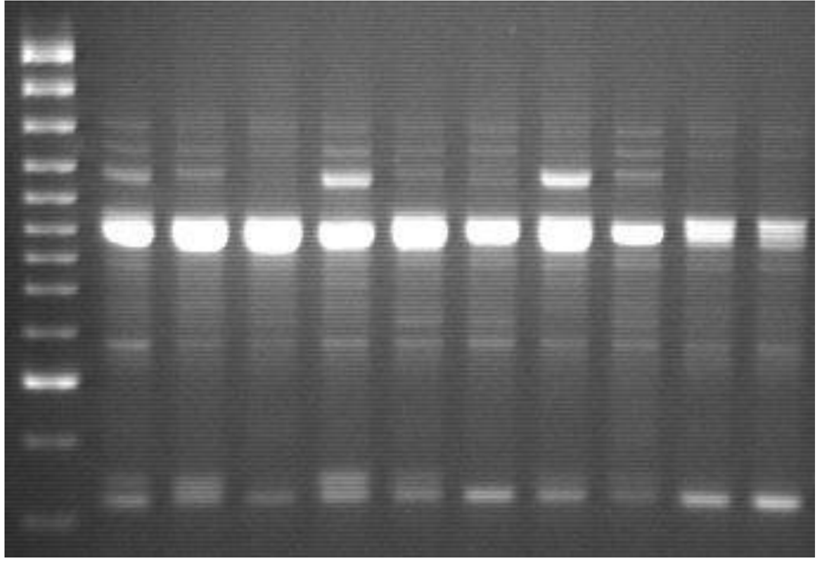

Figure A.62. PCR amplification of Rhododendron population 08ARL with RAPD F12 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 08ARL1-2.

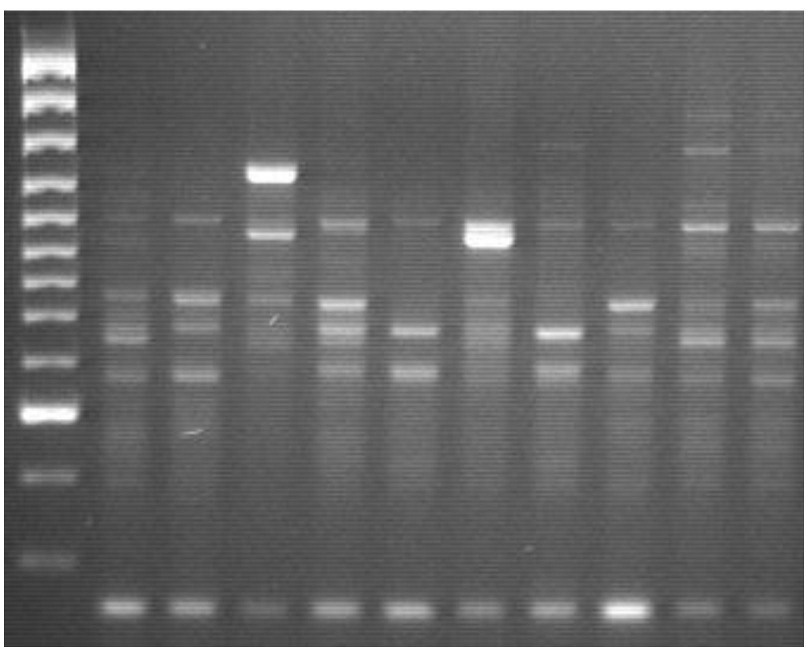

Figure A.63. PCR amplification of Rhododendron population 08ARL with RAPD F8 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 08ARL1-2.

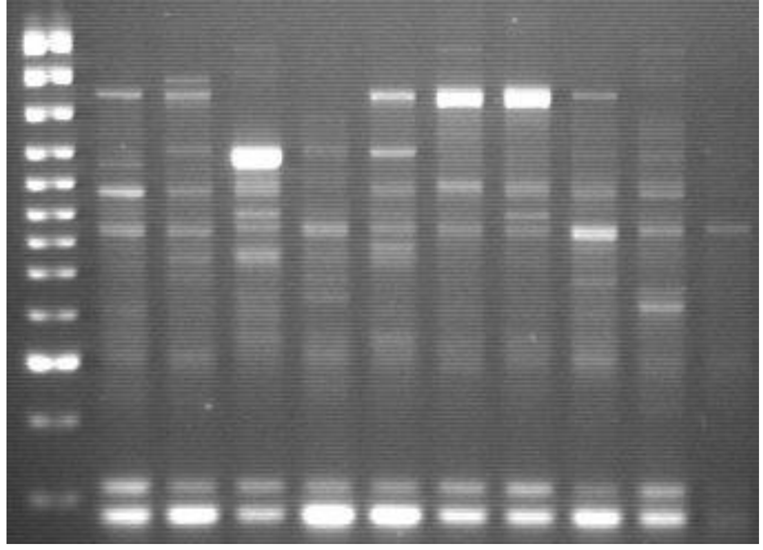

Figure A.64. PCR amplification of Rhododendron population 08ARL with RAPD F7 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 08ARL1-2.

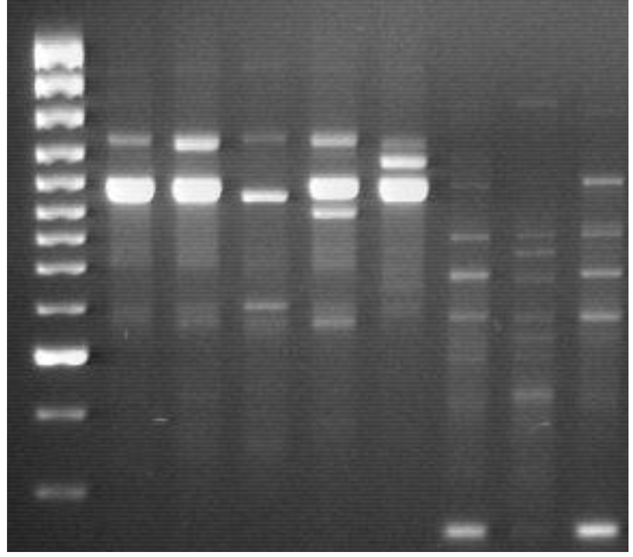

Figure A.65. PCR amplification of Rhododendron populations 34CAT and 17CA with RAPD F8 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

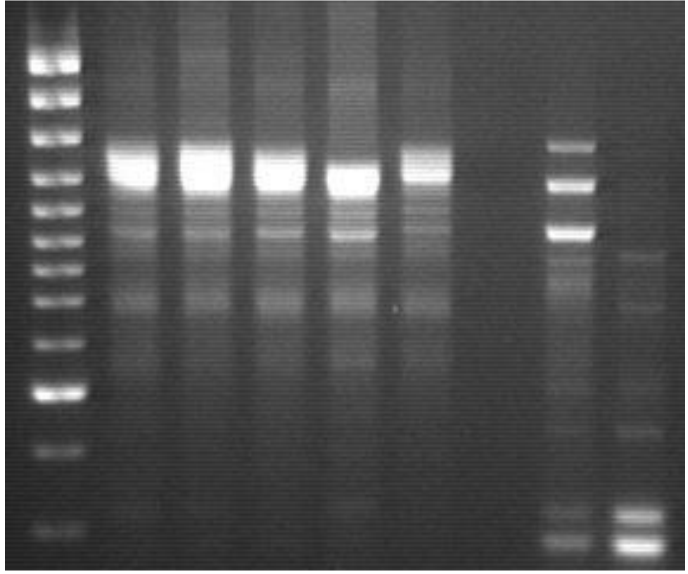

Figure A.66. PCR amplification of Rhododendron populations 34CAT and 17CA with RAPD F7 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

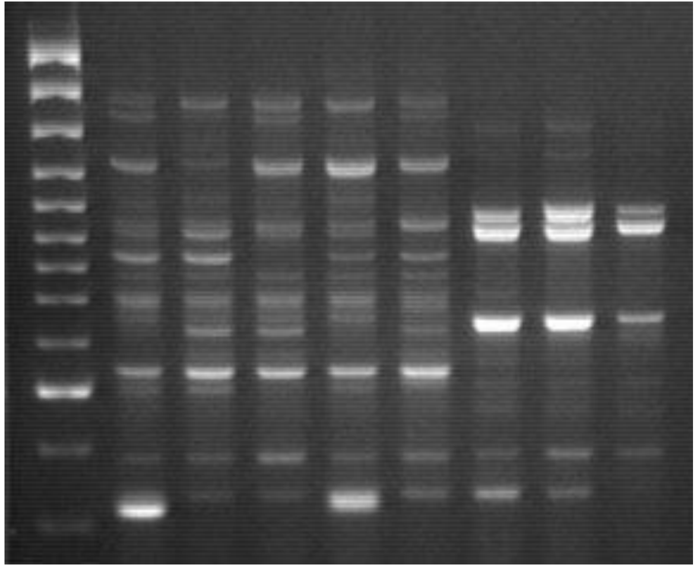

Figure A.67. PCR amplification of Rhododendron populations 34CAT and 17CA with RAPD F3 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

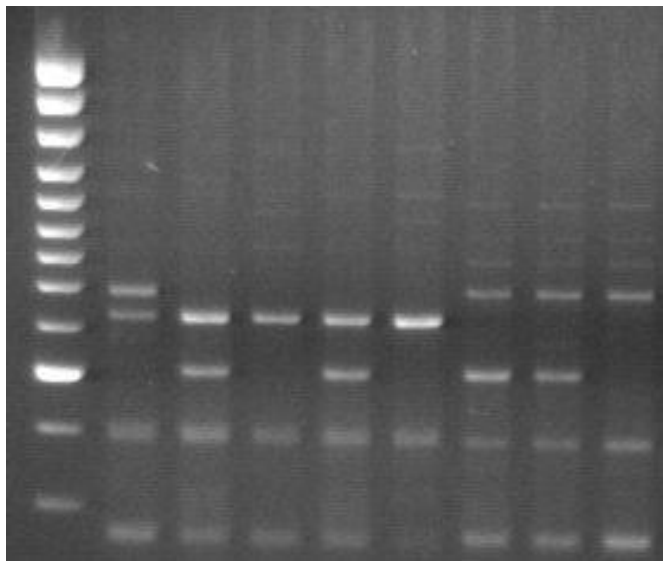

Figure A.68. PCR amplification of Rhododendron populations 34CAT and 17CA with ISSR 836 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

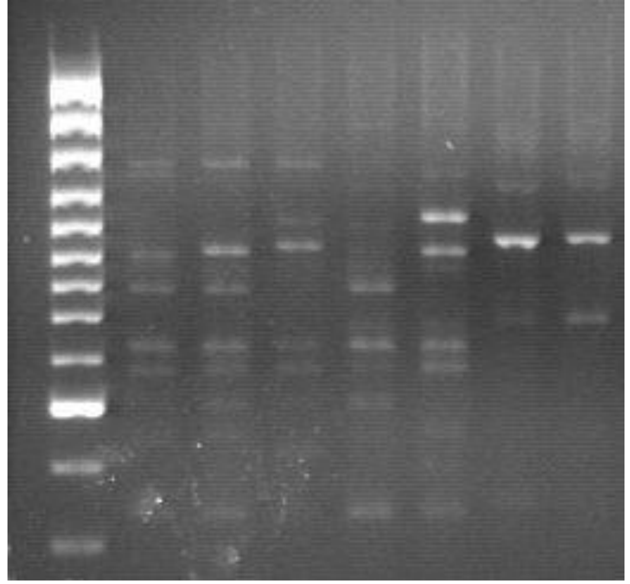

Figure A.69. PCR amplification of Rhododendron populations 34CAT and 17CA with ISSR 835 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

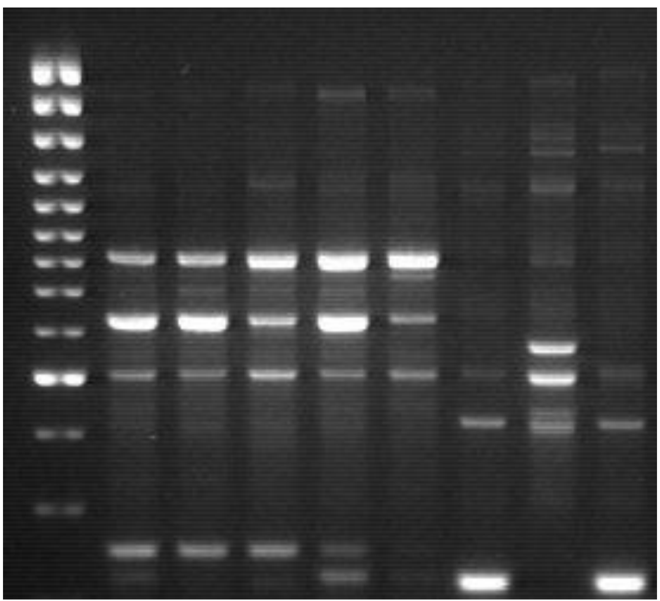

Figure A.70. PCR amplification of Rhododendron populations 34CAT and 17CA with RAPD M5 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

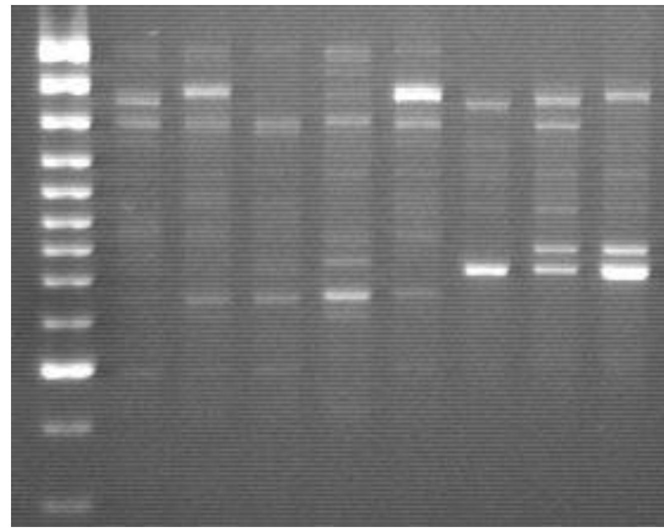

Figure A.71. PCR amplification of Rhododendron populations 34CAT and 17CA with RAPD K11 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

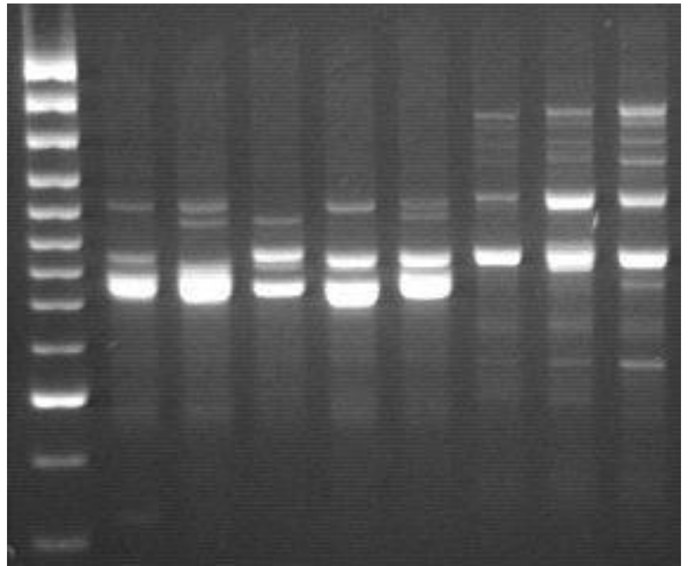

Figure A.72. PCR amplification of Rhododendron populations 34CAT and 17CA with RAPD G14 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

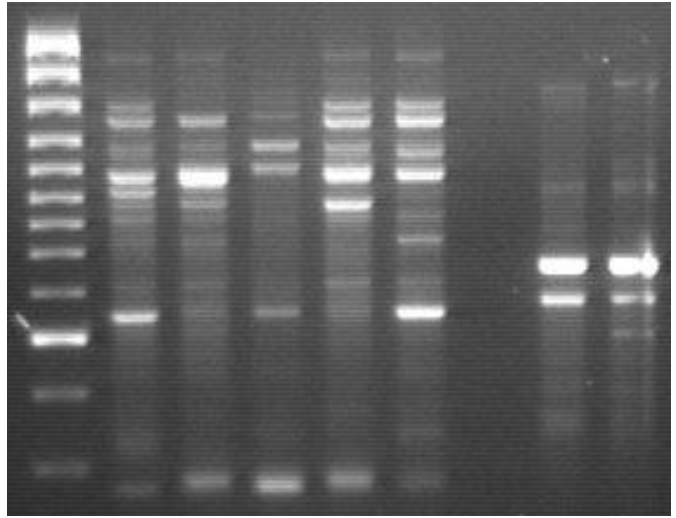

Figure A.73. PCR amplification of Rhododendron populations 34CAT and 17CA with RAPD F14 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

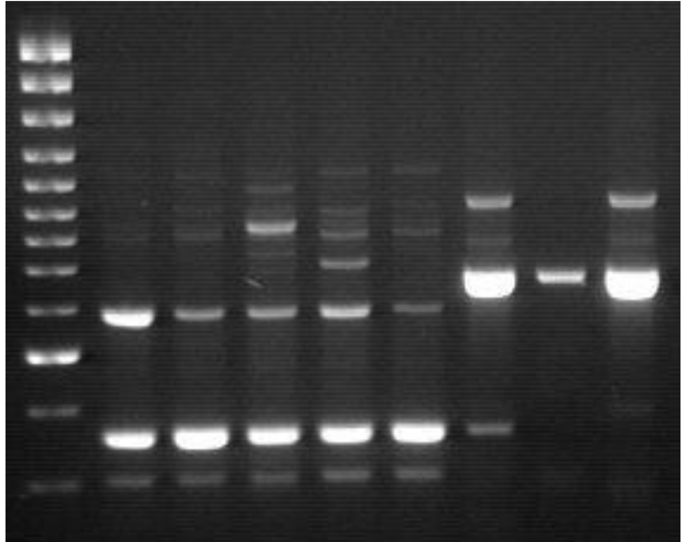

Figure A.74. PCR amplification of Rhododendron populations 34CAT and 17CA with RAPD F13 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

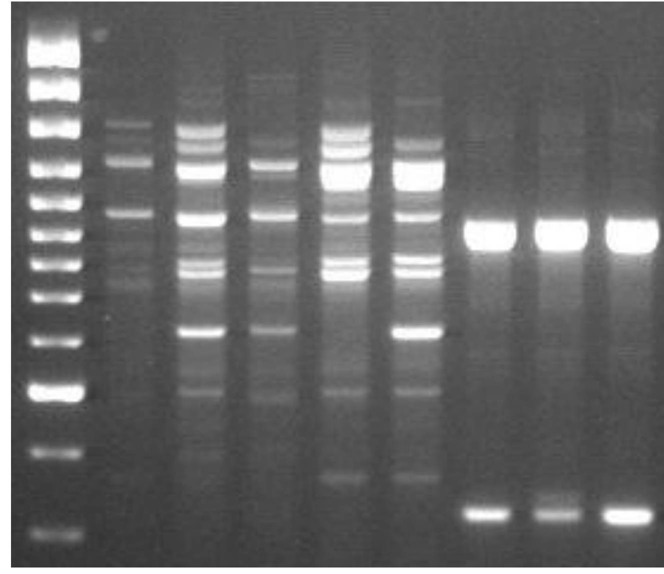

Figure A.75. PCR amplification of Rhododendron populations 34CAT and 17CA with RAPD F12 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 34CAT, 6– 8: Population name 17CA.

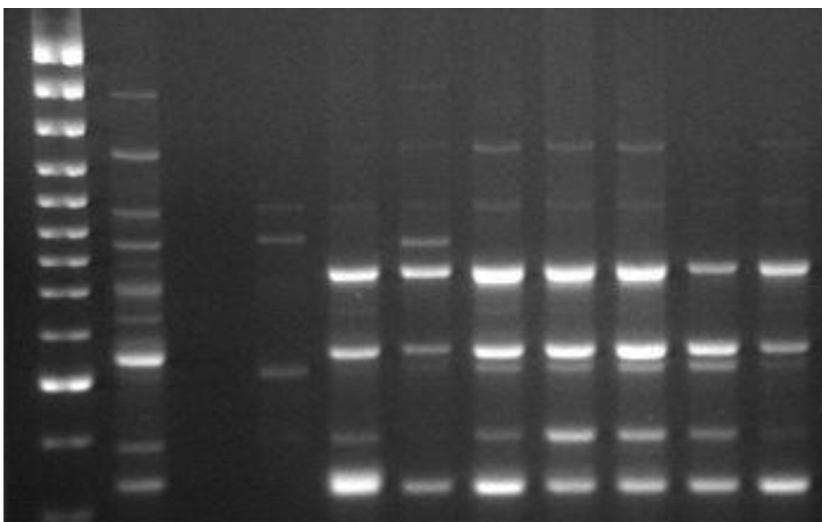

Figure A.76. PCR amplification of Rhododendron populations 41SUA and 54SA with RAPD F3 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

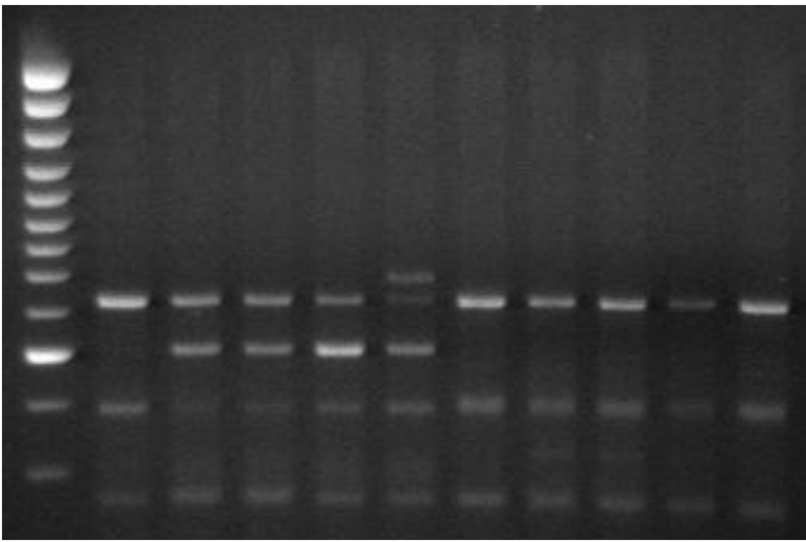

Figure A.77. PCR amplification of Rhododendron populations 41SUA and 54SA with ISSR 836 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

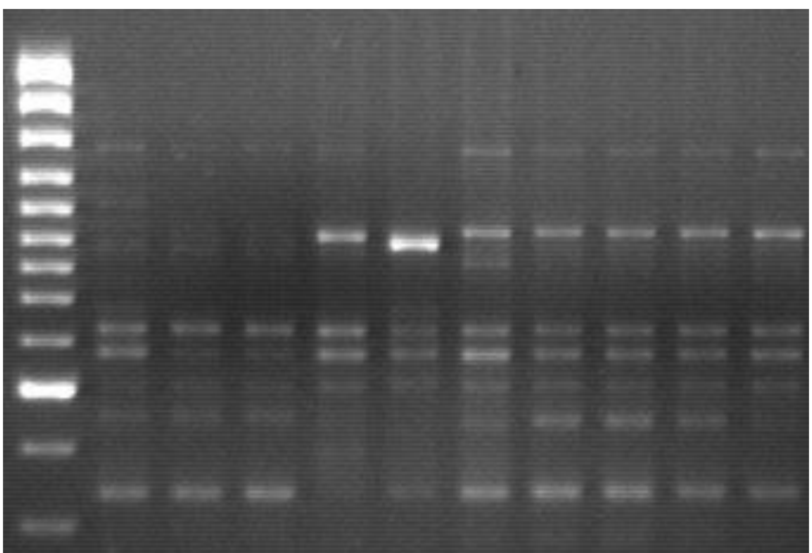

Figure A.78. PCR amplification of Rhododendron populations 41SUA and 54SA with ISSR 835 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

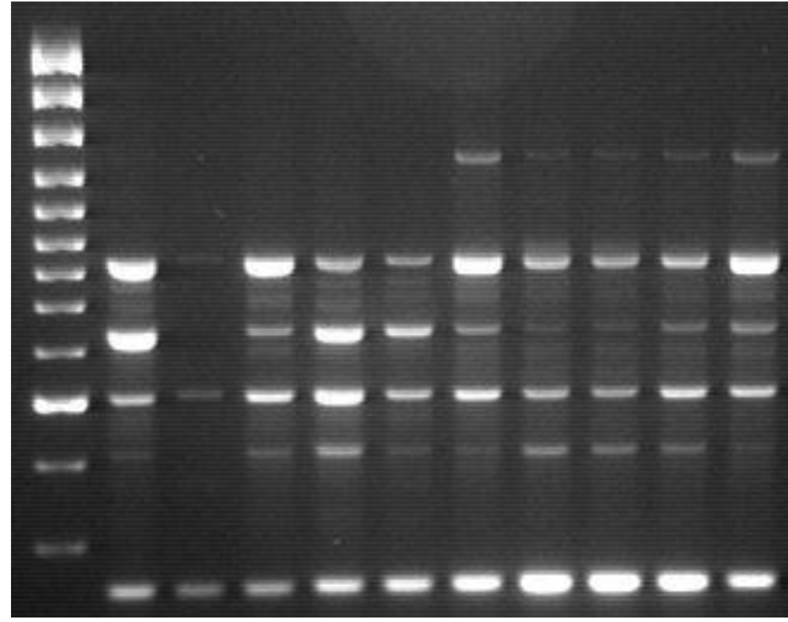

Figure A.79. PCR amplification of Rhododendron populations 41SUA and 54SA with RAPD M5 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

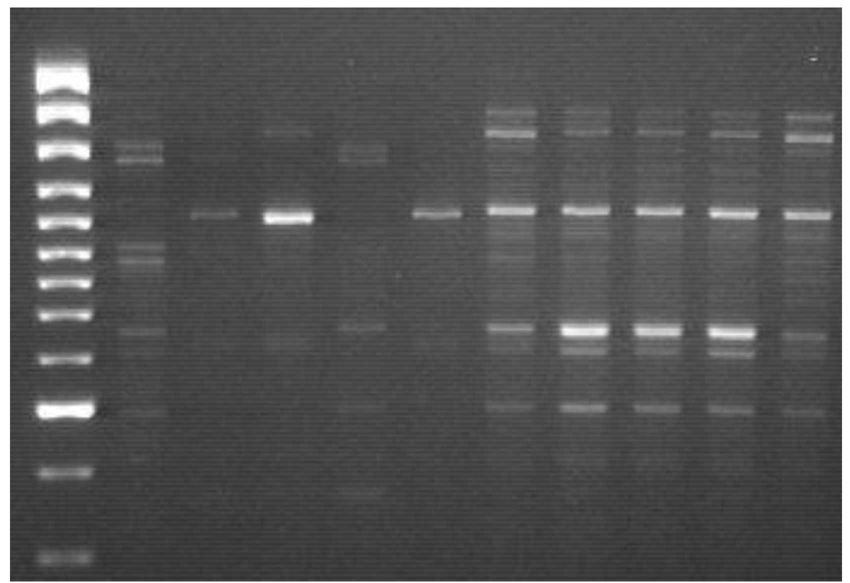

Figure A.80. PCR amplification of Rhododendron populations 41SUA and 54SA with RAPD K11 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

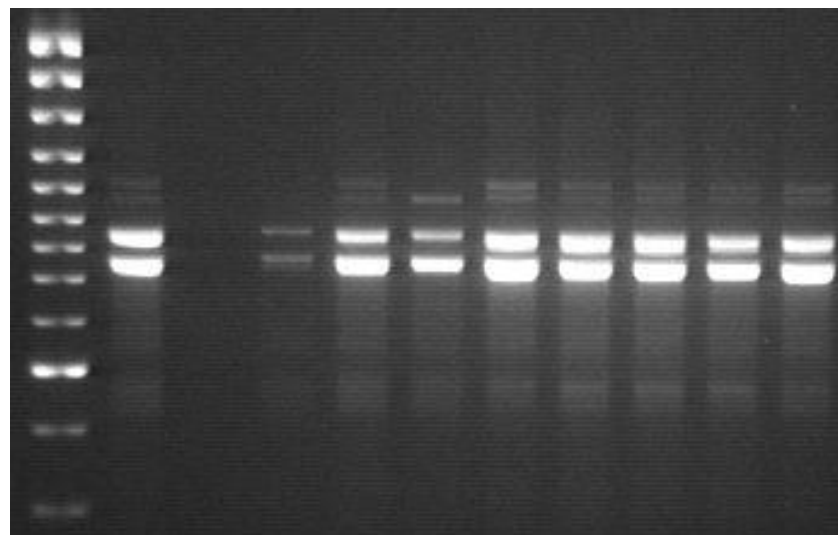

Figure A.81. PCR amplification of Rhododendron populations 41SUA and 54SA with RAPD G14 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

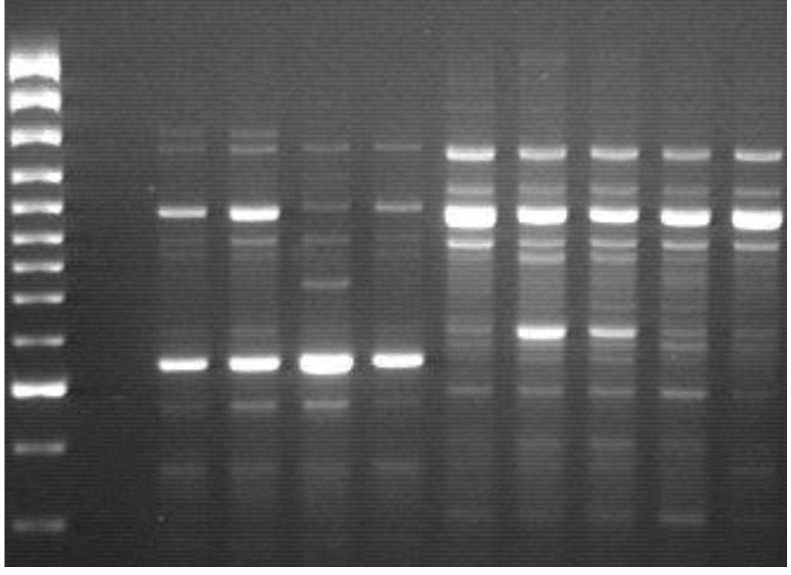

Figure A.82. PCR amplification of Rhododendron populations 41SUA and 54SA with RAPD F14 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

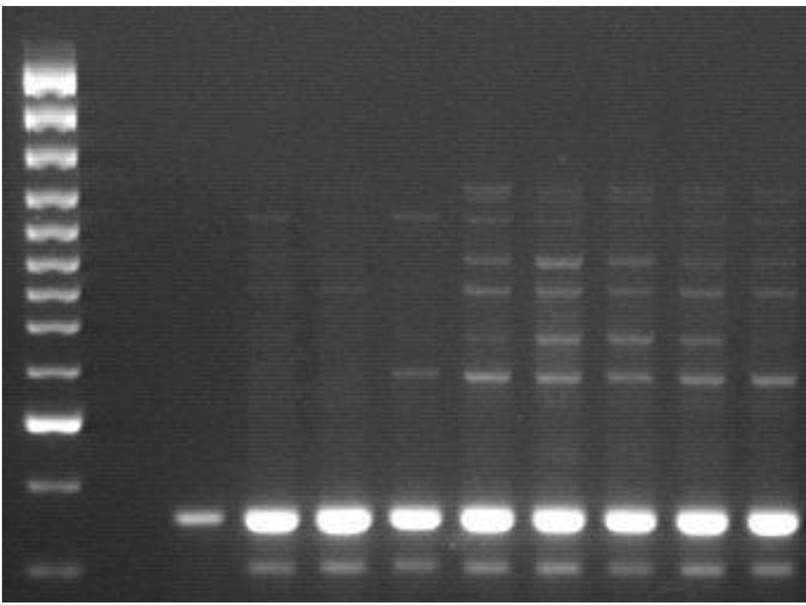

Figure A.83. PCR amplification of Rhododendron populations 41SUA and 54SA with RAPD F13 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

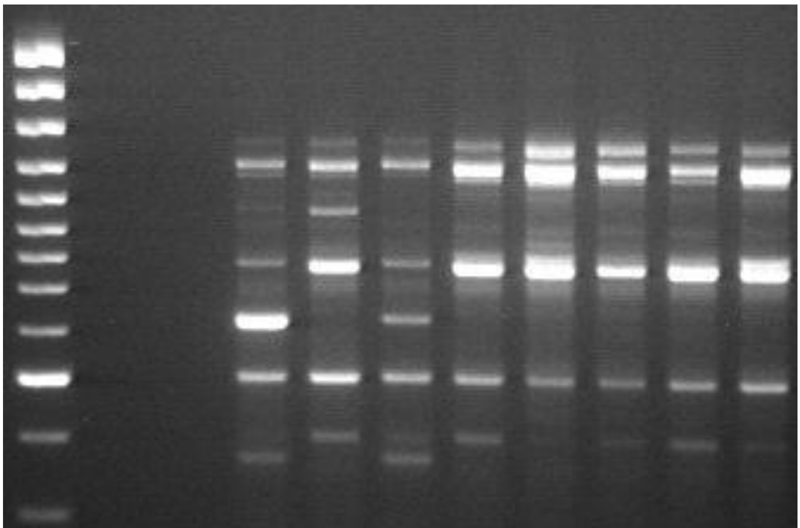

Figure A.84. PCR amplification of Rhododendron populations 41SUA and 54SA with RAPD F12 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

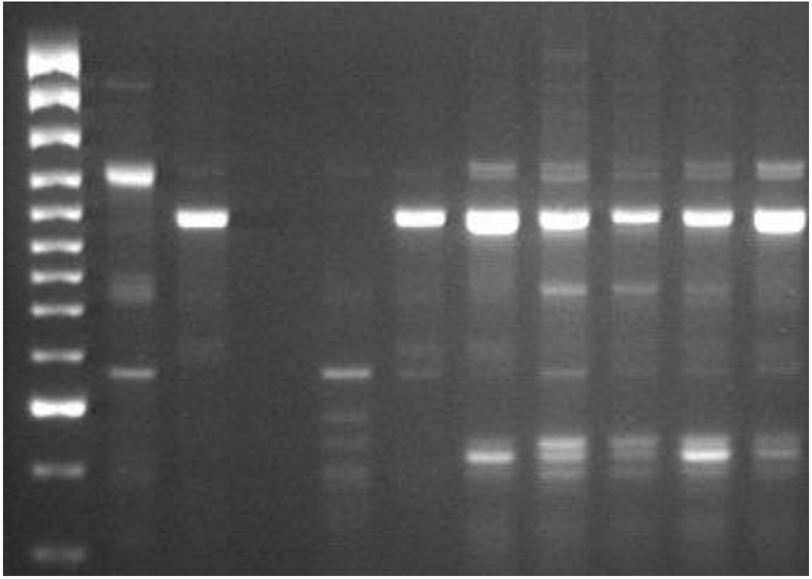

Figure A.85. PCR amplification of Rhododendron populations 41SUA and 54SA with RAPD F8 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41SUA, 6– 10: Population name 54SA.

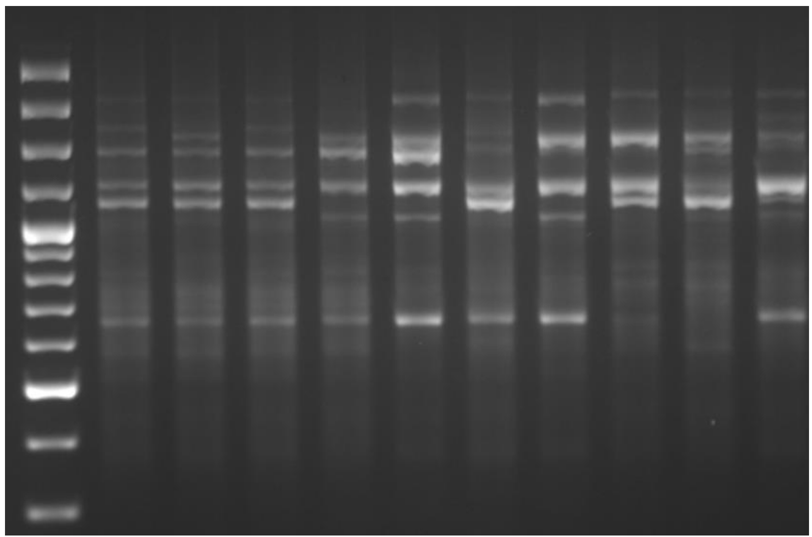

Figure A.86. PCR amplification of Rhododendron populations 41YUVA and 81DU with ISSR 855 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

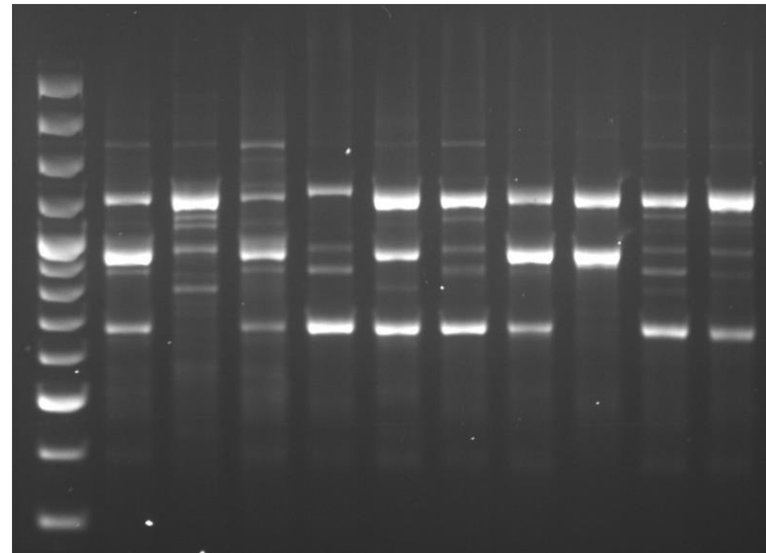

Figure A.87. PCR amplification of Rhododendron populations 41YUVA and 81DU with ISSR 827 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

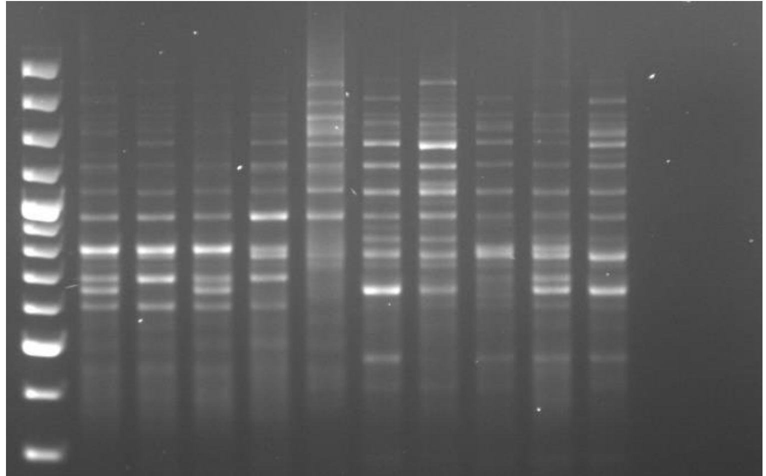

Figure A.88. PCR amplification of Rhododendron populations 41YUVA and 81DU with ISSR 826 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

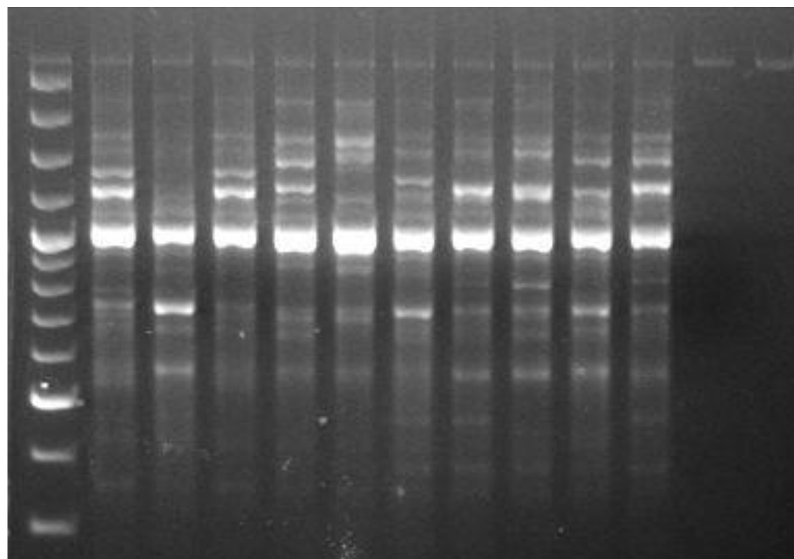

Figure A.89. PCR amplification of Rhododendron populations 41YUVA and 81DU with ISSR 817 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

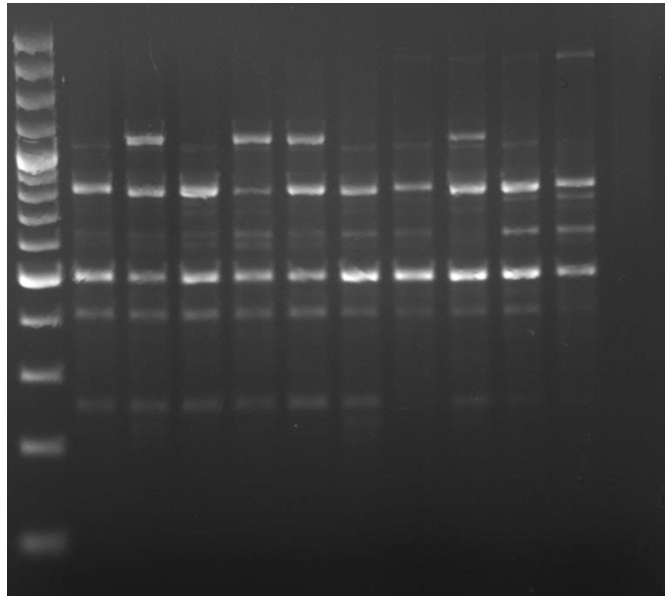

Figure A.90. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD M5 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

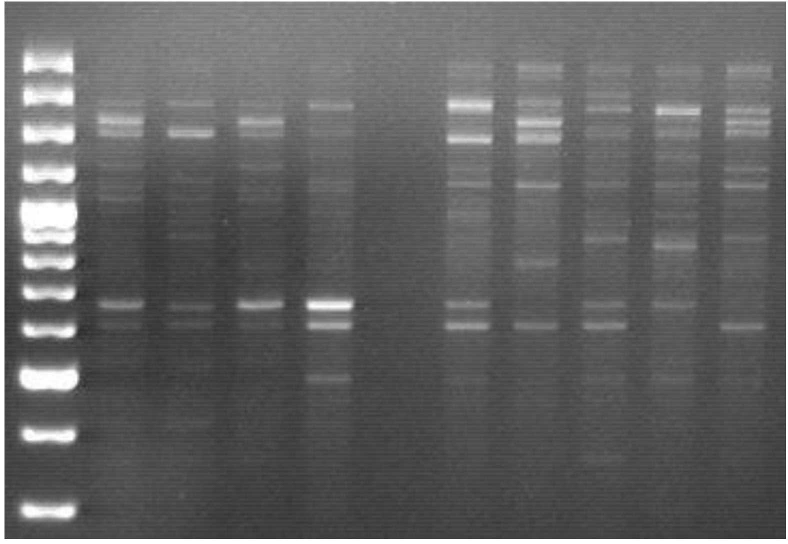

Figure A.91. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD K11 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

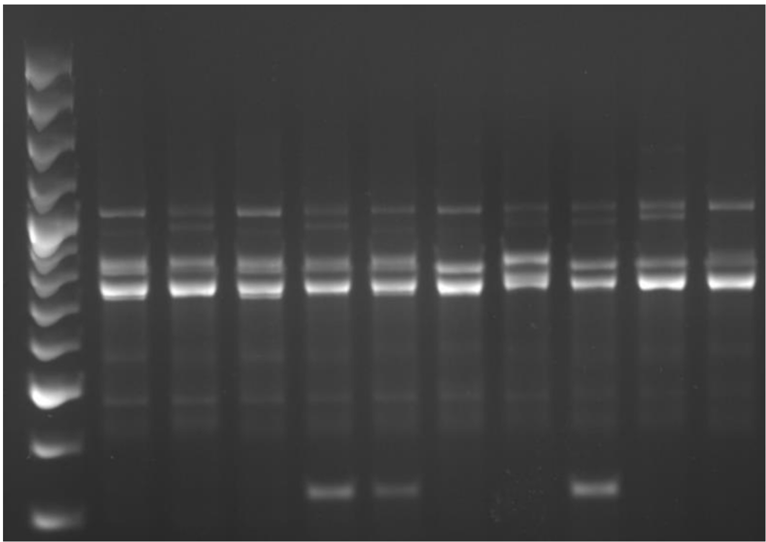

Figure A.92. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD G14 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

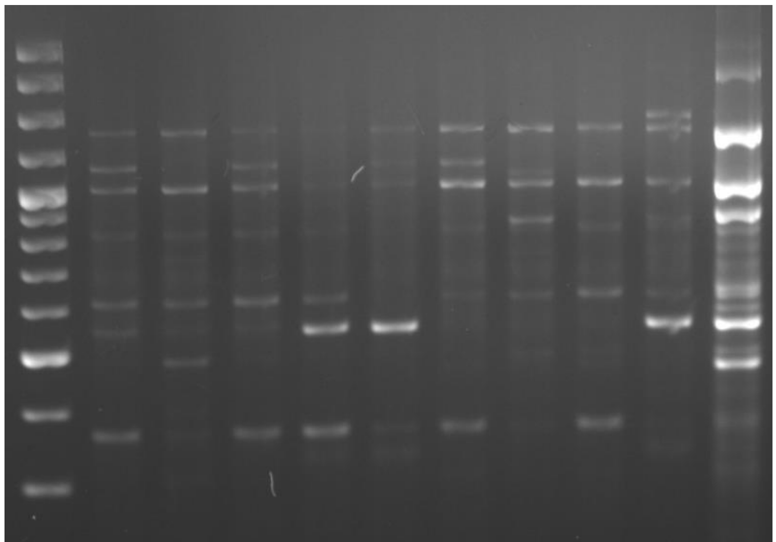

Figure A.93. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD F14 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

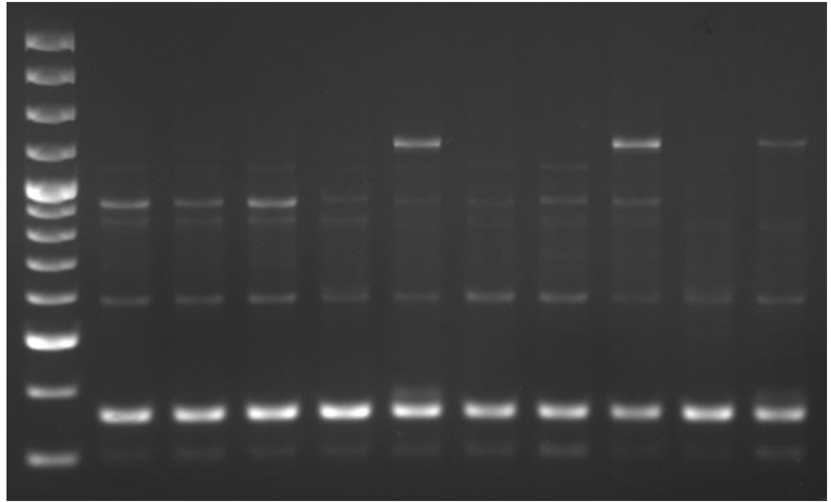

Figure A.94. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD F13 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

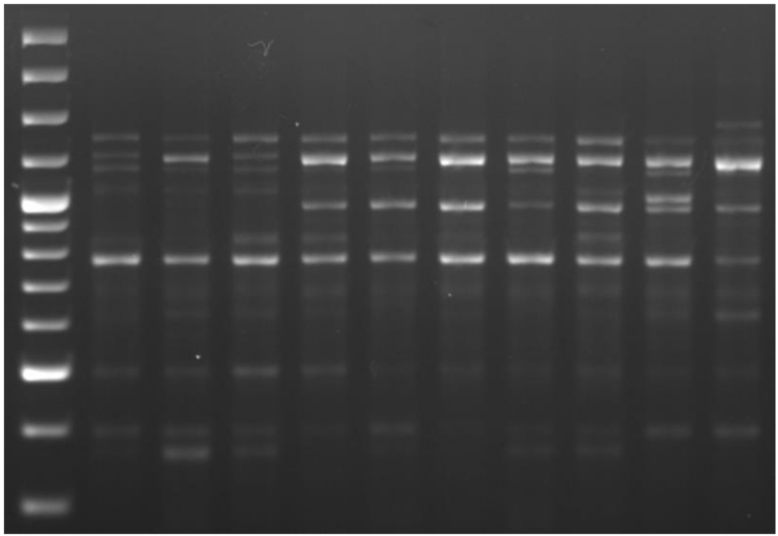

Figure A.95. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD F12 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

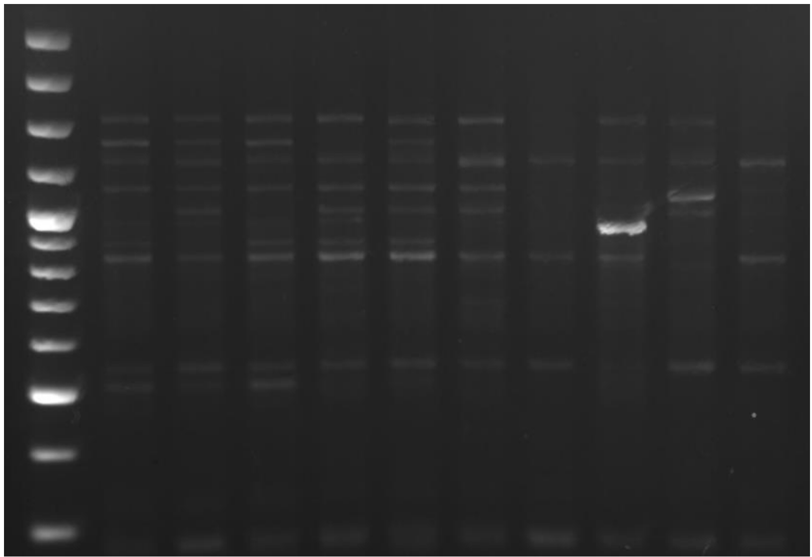

Figure A.96. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD F10 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

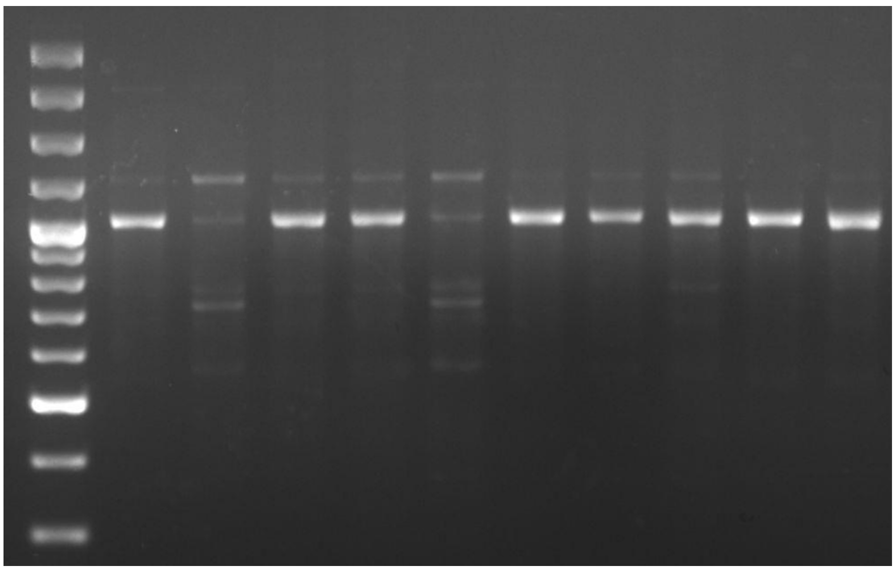

Figure A.97. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD F8 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

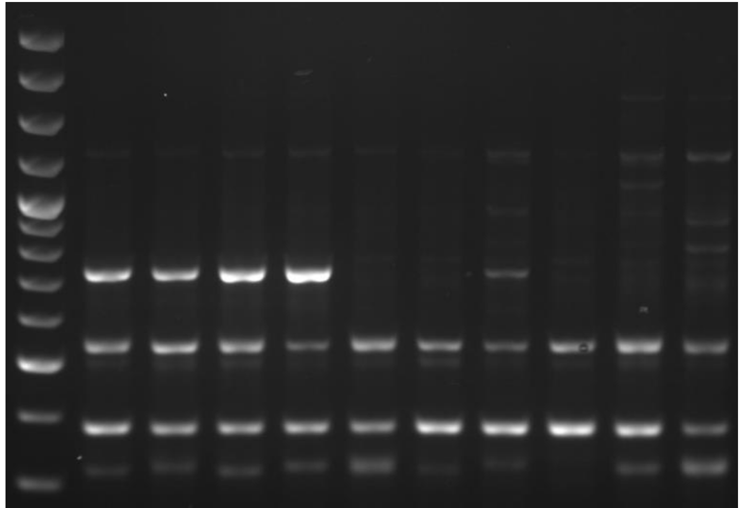

Figure A.98. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD F7 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

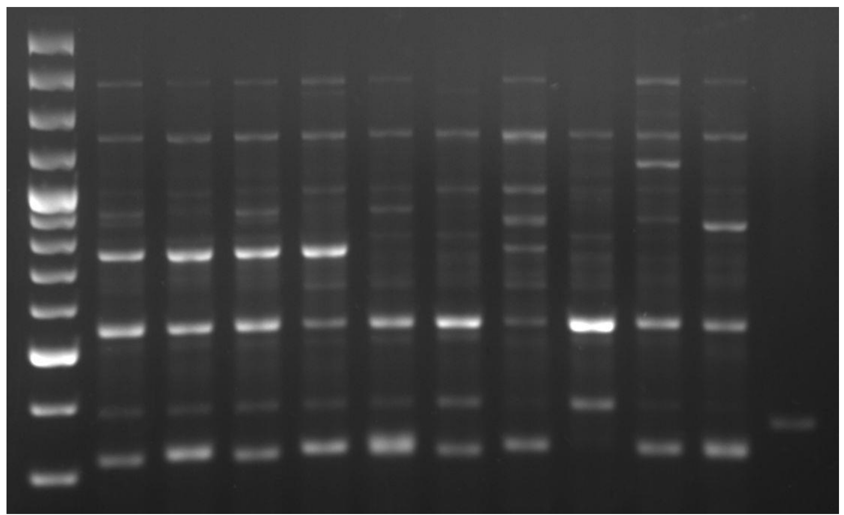

Figure A.99. PCR amplification of Rhododendron populations 41YUVA and 81DU with RAPD F3 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

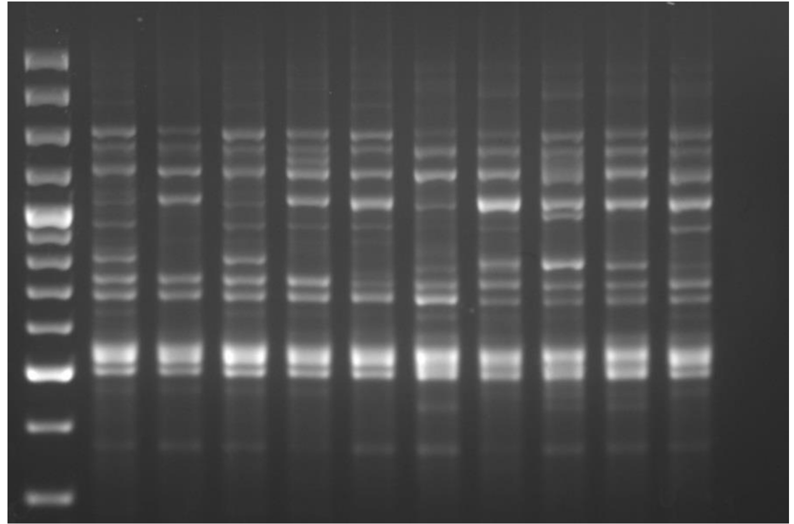

Figure A.100. PCR amplification of Rhododendron populations 41YUVA and 81DU with ISSR 857 primer. M: GeneRuler 100 bp plus. 1 – 5: Population name 41YUVA, 6– 10: Population name 81DU.

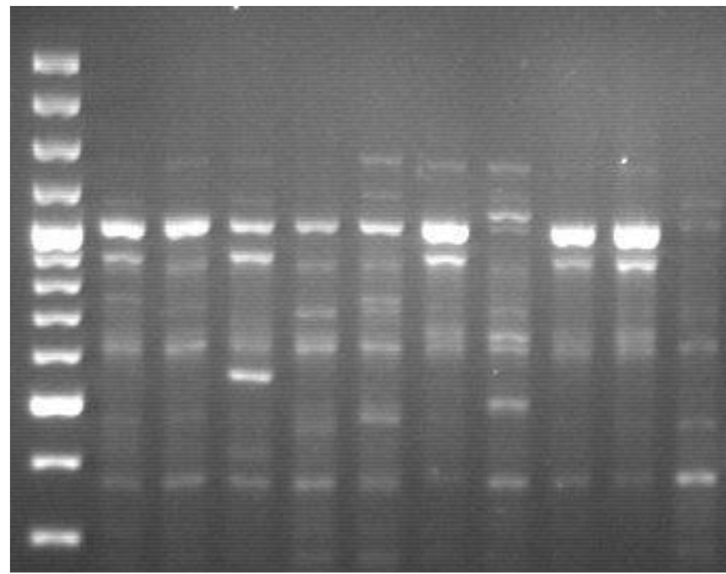

Figure A.101. PCR amplification of Rhododendron population 54HEN with RAPD F14 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

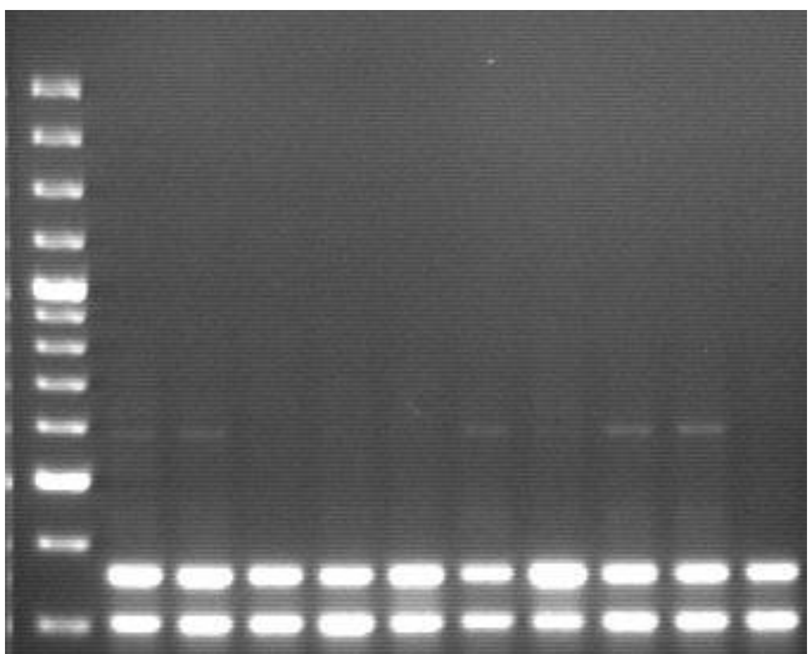

Figure A.102. PCR amplification of Rhododendron population 54HEN with RAPD F13 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

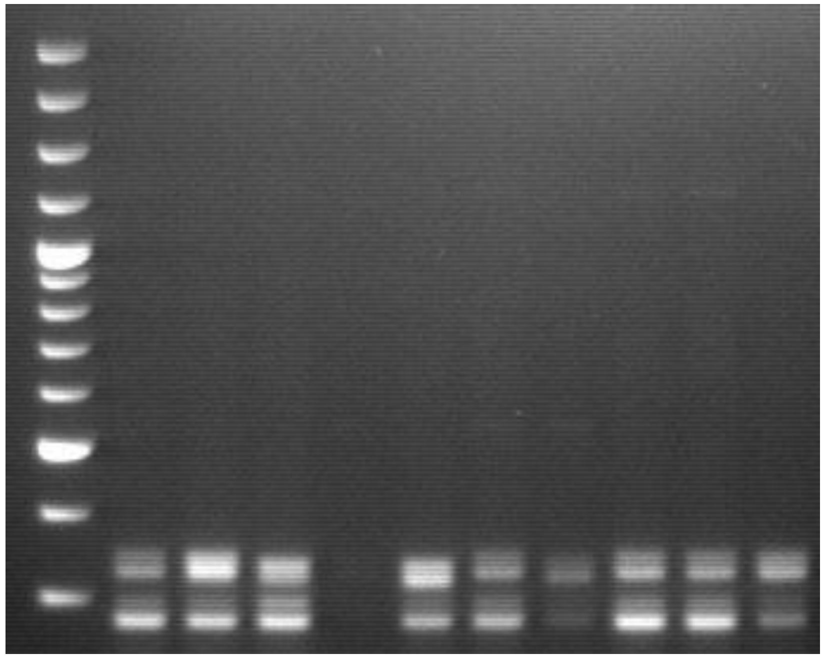

Figure A.103. PCR amplification of Rhododendron population 54HEN with RAPD F10 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

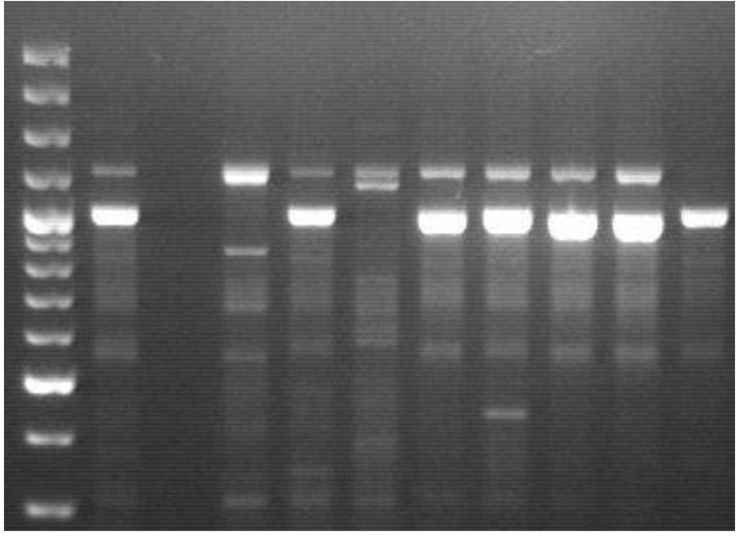

Figure A.104. PCR amplification of Rhododendron population 54HEN with RAPD F8 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

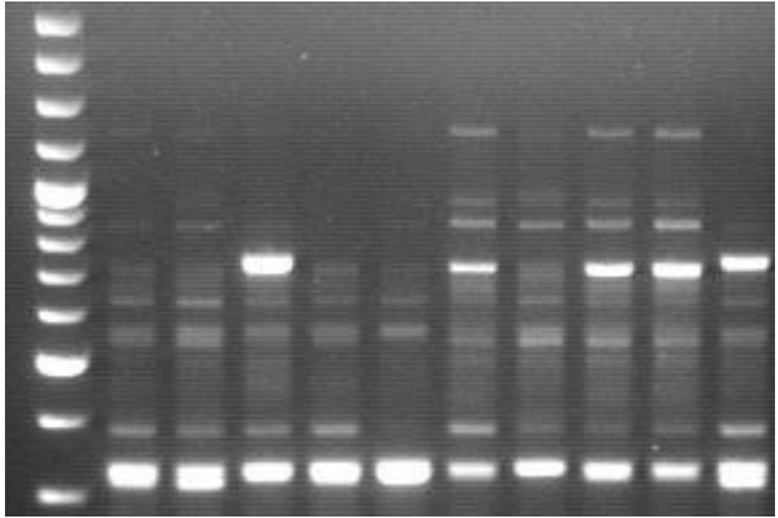

Figure A.105. PCR amplification of Rhododendron population 54HEN with RAPD F3 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

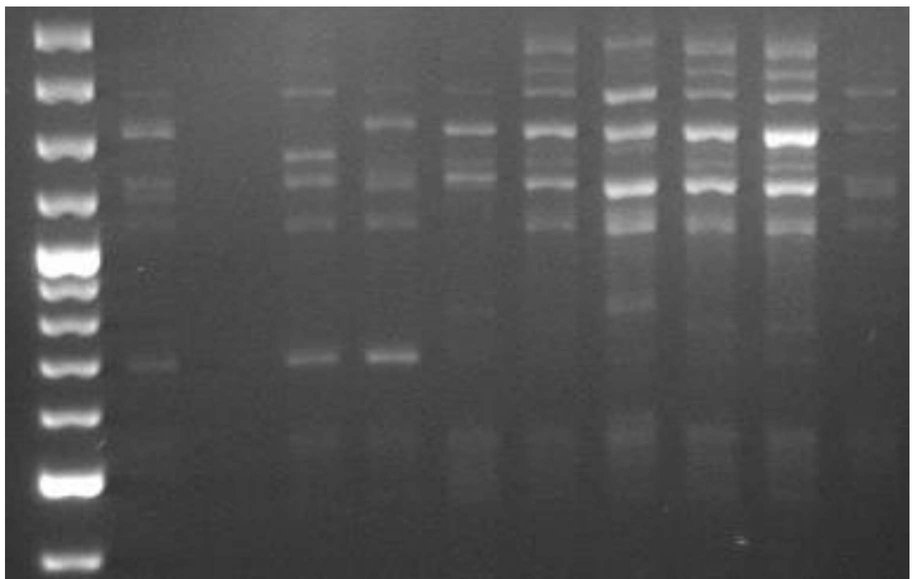

Figure A.106. PCR amplification of Rhododendron population 54HEN with ISSR 857 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

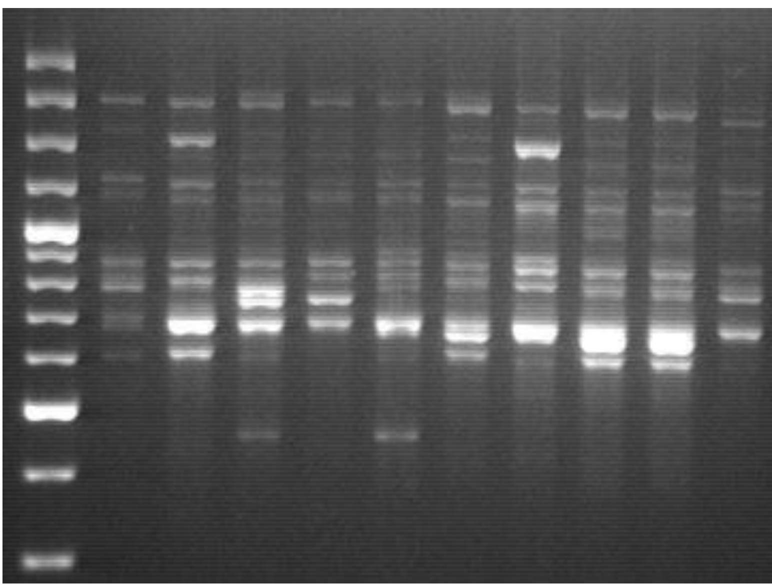

Figure A.107. PCR amplification of Rhododendron population 54HEN with ISSR 856 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

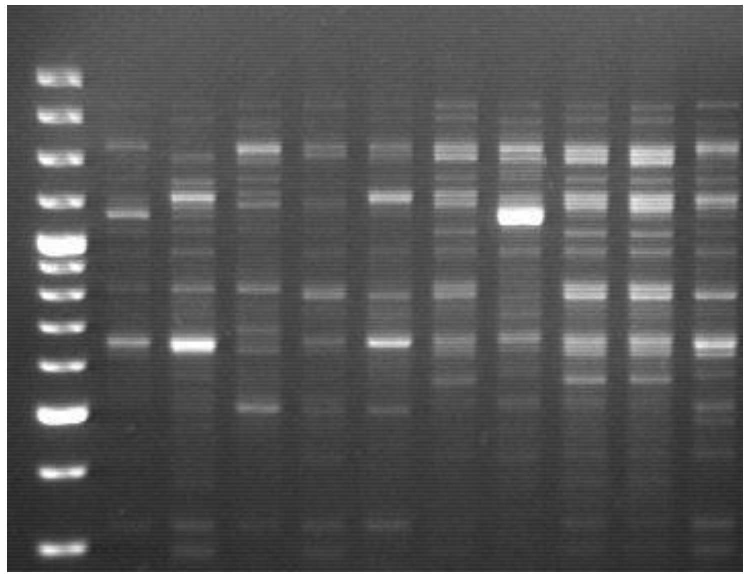

Figure A.108. PCR amplification of Rhododendron population 54HEN with ISSR 855 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

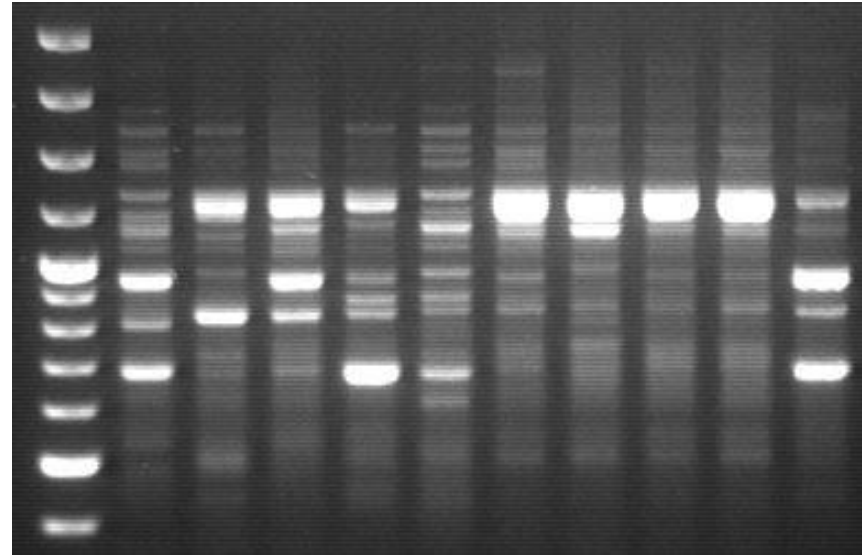

Figure A.109. PCR amplification of Rhododendron population 54HEN with ISSR 827 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

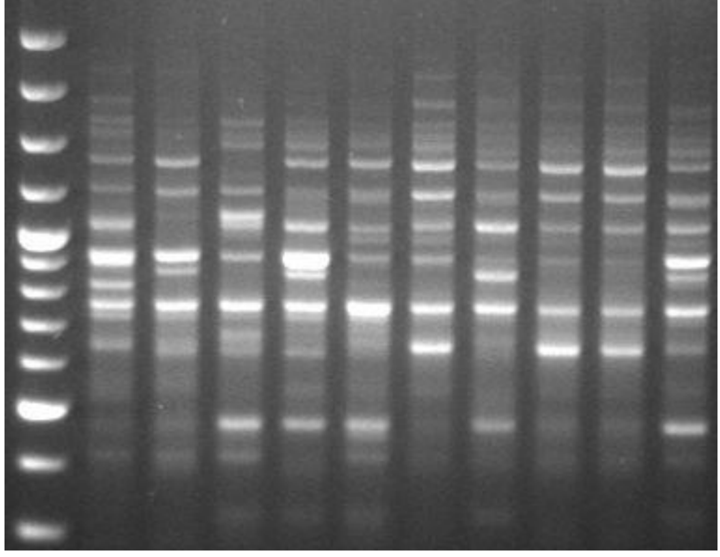

Figure A.110. PCR amplification of Rhododendron population 54HEN with ISSR 826 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

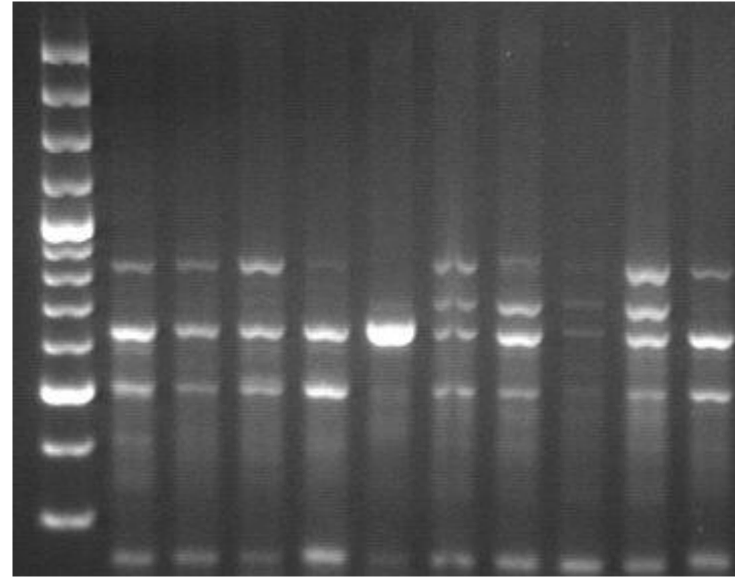

Figure A.111. PCR amplification of Rhododendron population 54HEN with RAPD M5 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

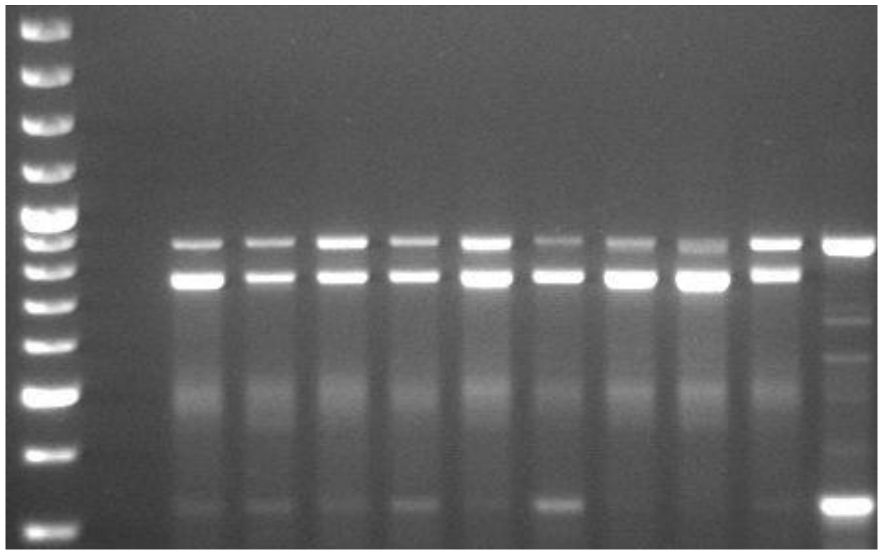

Figure A.112. PCR amplification of Rhododendron population 54HEN with RAPD G14 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54HEN1-2.

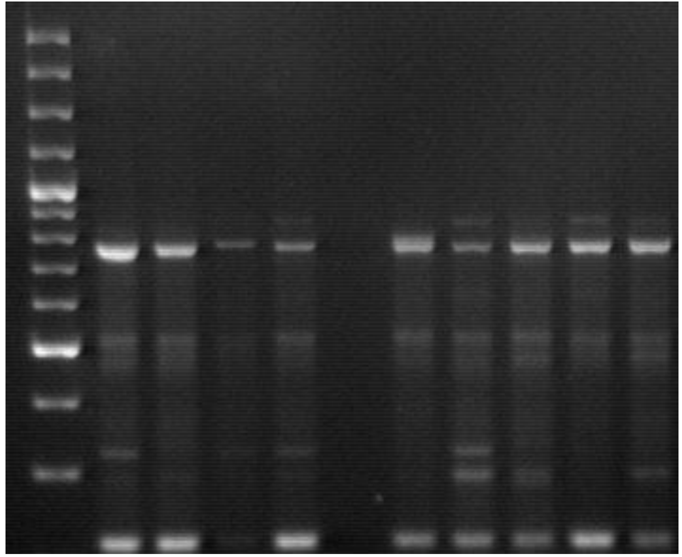

Figure A.113. PCR amplification of Rhododendron population 54KARA with RAPD G14 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54KARA1-2.

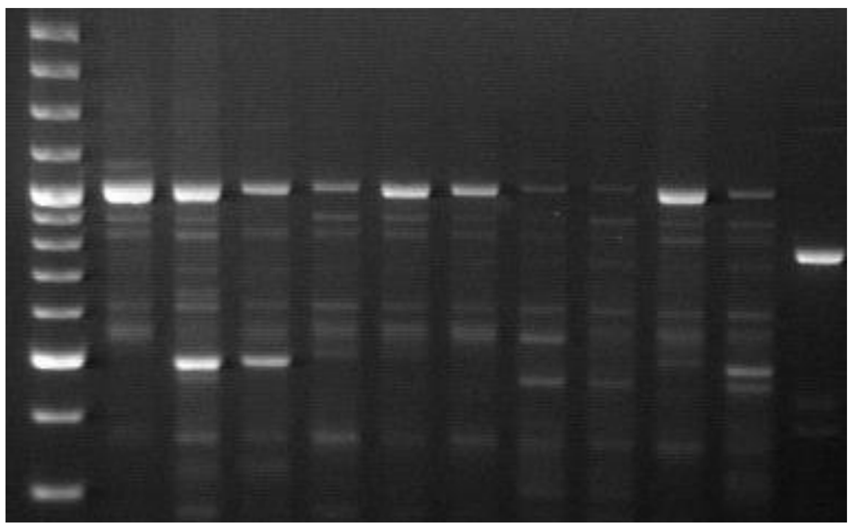

Figure A.114. PCR amplification of Rhododendron population 54KARA with RAPD F14 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54KARA1-2.

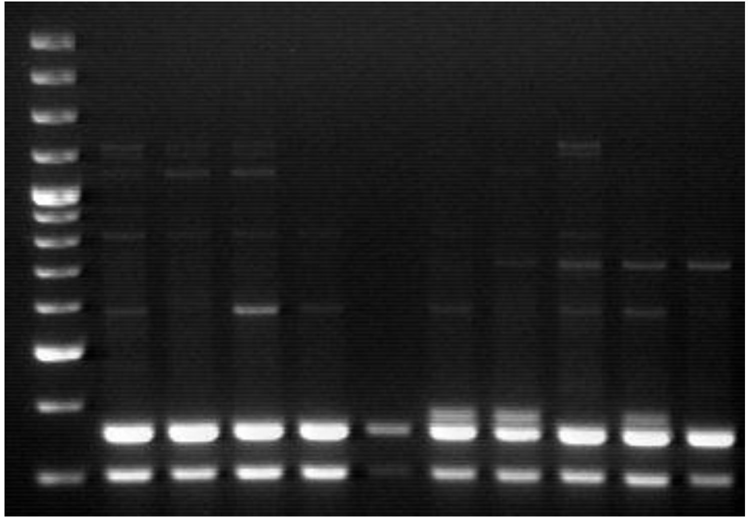

Figure A.115. PCR amplification of Rhododendron population 54KARA with RAPD F13 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54KARA1-2.

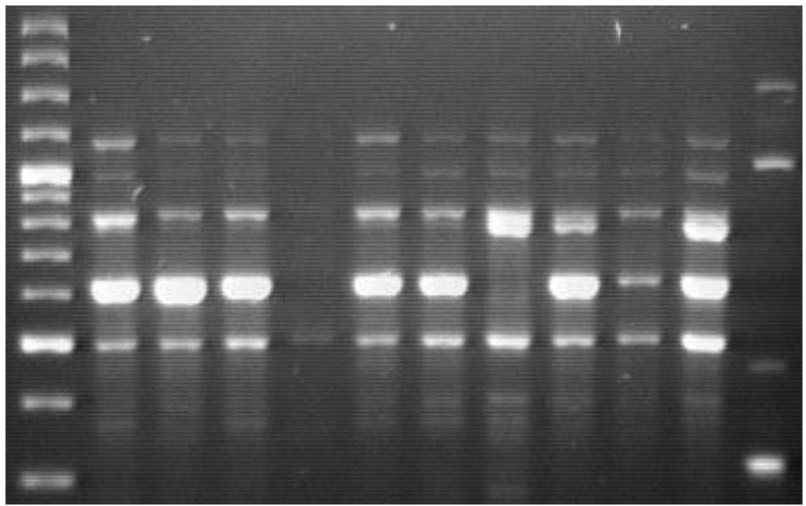

Figure A.116. PCR amplification of Rhododendron population 54KARA with RAPD F12 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54KARA1-2.

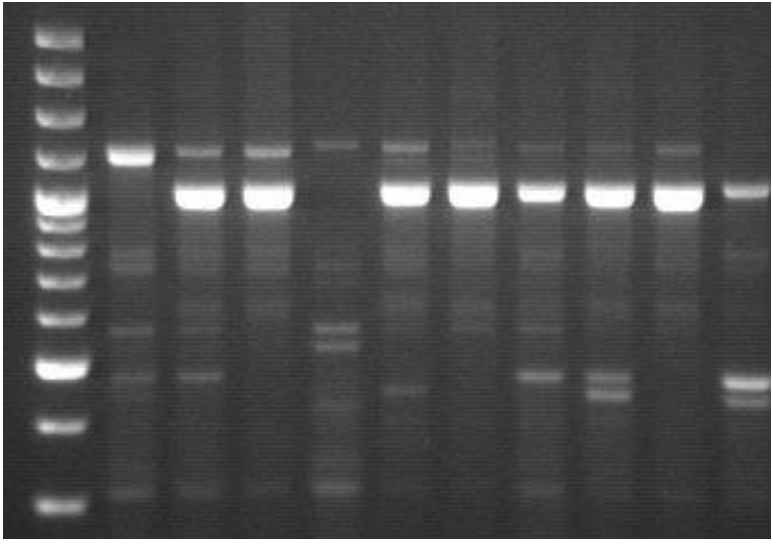

Figure A.117. PCR amplification of Rhododendron population 54KARA with RAPD F8 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54KARA1-2.

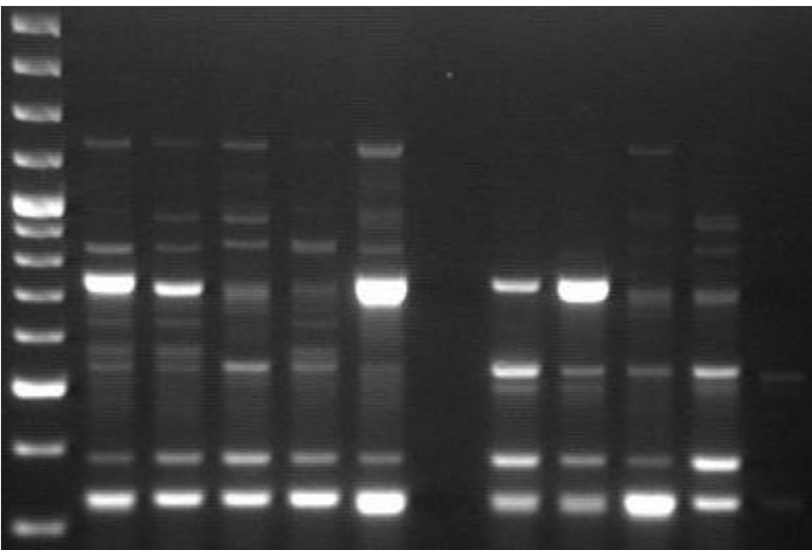

Figure A.118. PCR amplification of Rhododendron population 54KARA with RAPD F3 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54KARA1-2.

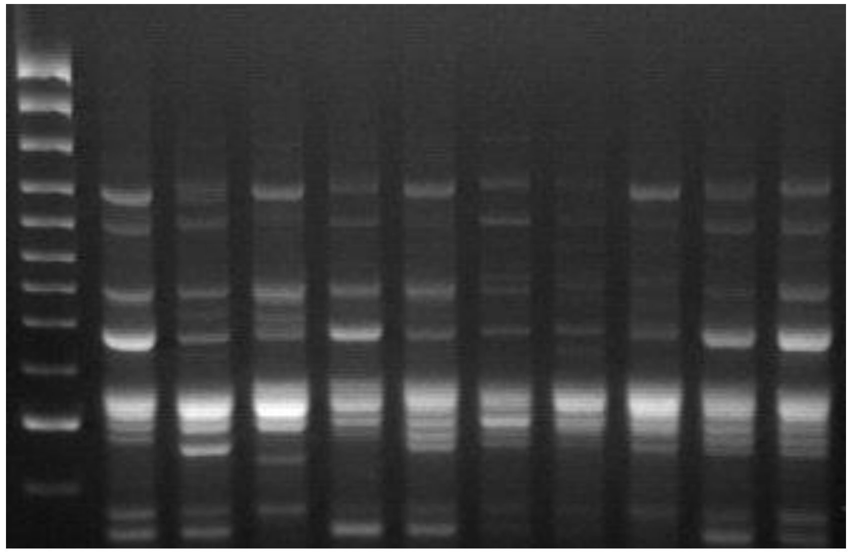

Figure A.119. PCR amplification of Rhododendron population 54KARA with ISSR 857 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54KARA1-2.

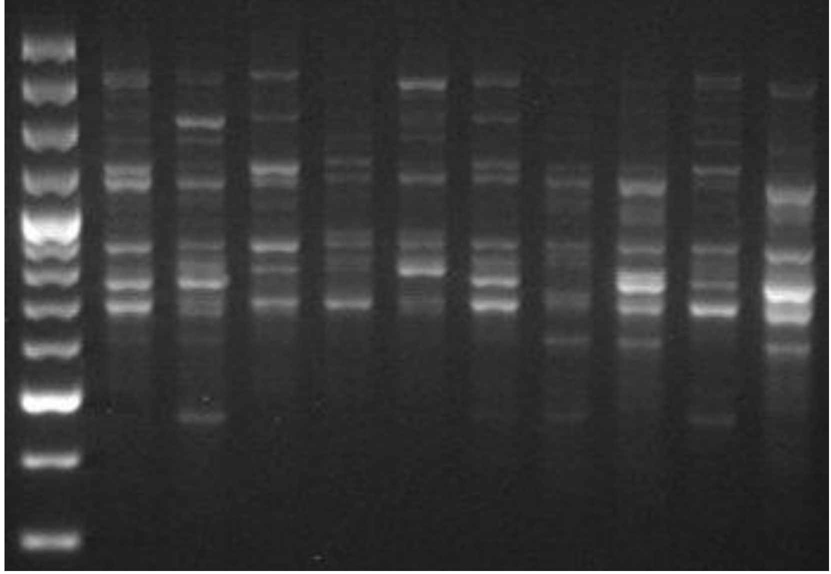

Figure A.120. PCR amplification of Rhododendron population 54KARA with ISSR 856 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54KARA1-2.

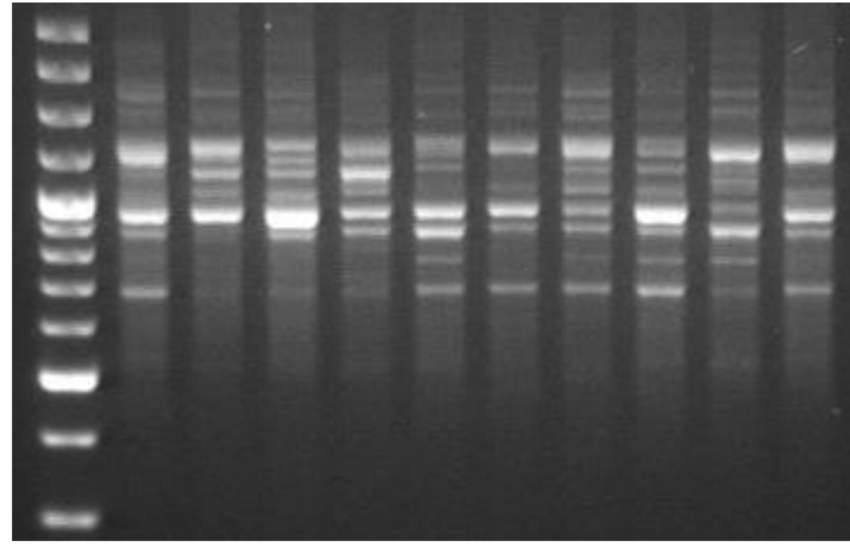

Figure A.121. PCR amplification of Rhododendron population 54KARA with ISSR 827 primer. M: GeneRuler 100 bp plus. 1 – 10: Population name 54KARA1-2.

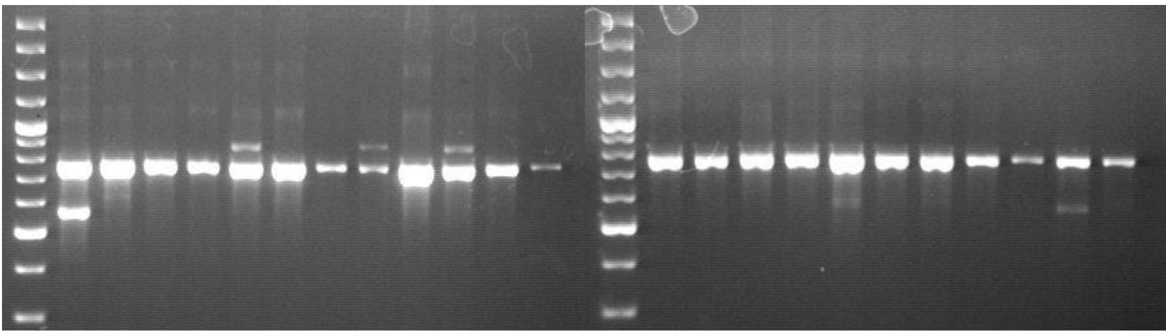

Figure A.122 PCR amplification of 23 Rhododendron species with ITS 4-ITS5 primers. M: GeneRuler 100bp plus. Samples 1 – 23: Rhododendron genus and outlier *A. unedo* in the order as given in Table 3.5 and Table 3.6.

APPENDIX B: GENETIC DISTANCE MATRICES

Table B.1. Genetic dissimilarity (distance) matrix among 23 Rhododendron genus and A. unedo using Jaccard's correlation coefficient.

	08 ART 03	08 ART 01	08 MU 01	08 MU 02	08 MU 05	08 BO 05	08 MU 08	08 MU 09	08 MU 10	08 MU 11	08 MU 03	08 SA 02	81 DU 01	08 BO 01	A. unedo	08 MU 07	67 AL 01	08 AR 01	08 AR 02	17 CA 01	39 DE 01	28 GO 01	52 KU 01
08 ART 01	0.791	0.00																					
08 MU 01	0.836	0.817	0.000																				
08 MU 02	0.741	0.770	0.778	0.000																			
08 MU 05	0.793	0.728	0.818	0.748	0.000																		
08 BO 05	0.617	0.717	0.797	0.638	0.775	0.000																	
08 MU 08	0.695	0.757	0.779	0.712	0.656	0.669	0.000																
08 MU 09	0.827	0.814	0.852	0.816	0.564	0.839	0.623	0.000															
08 MU 10	0.796	0.704	0.807	0.735	0.750	0.722	0.707	0.803	0.000														
08 MU 11	0.807	0.831	0.803	0.794	0.597	0.833	0.628	0.504	0.799	0.000													
08 MU 03	0.776	0.778	0.859	0.555	0.776	0.654	0.685	0.813	0.674	0.766	0.000												
08 SA 02	0.829	0.869	0.803	0.830	0.883	0.848	0.837	0.876	0.867	0.848	0.874	0.000											
81 DU 01	0.806	0.835	0.837	0.786	0.843	0.817	0.825	0.854	0.812	0.829	0.801	0.801	0.000										
08 BO 01	0.837	0.833	0.812	0.795	0.804	0.783	0.797	0.845	0.784	0.819	0.773	0.843	0.484	0.000									
A. unedo	0.901	0.878	0.897	0.866	0.894	0.899	0.861	0.862	0.892	0.864	0.902	0.866	0.852	0.851	0.000								
08 MU 07	0.829	0.832	0.842	0.795	0.826	0.806	0.799	0.837	0.792	0.796	0.844	0.812	0.582	0.504	0.841	0.000							
67 AL 01	0.856	0.846	0.785	0.836	0.883	0.875	0.854	0.830	0.844	0.840	0.819	0.878	0.815	0.786	0.857	0.833	0.000						
08 AR 01	0.847	0.829	0.854	0.799	0.800	0.845	0.856	0.832	0.772	0.880	0.776	0.875	0.820	0.825	0.862	0.846	0.634	0.000					
08 AR 02	0.815	0.850	0.831	0.788	0.826	0.830	0.765	0.795	0.824	0.783	0.812	0.878	0.824	0.821	0.856	0.765	0.677	0.659	0.000				
17 CA 01	0.833	0.882	0.873	0.859	0.803	0.851	0.823	0.895	0.855	0.831	0.860	0.833	0.890	0.897	0.919	0.892	0.807	0.767	0.825	0.000			
39 DE 01	0.827	0.846	0.790	0.765	0.831	0.825	0.829	0.853	0.803	0.862	0.736	0.929	0.835	0.809	0.899	0.824	0.742	0.654	0.607	0.839	0.000		
28 GO 01	0.867	0.796	0.806	0.788	0.789	0.800	0.783	0.806	0.785	0.821	0.770	0.897	0.833	0.799	0.871	0.791	0.654	0.614	0.579	0.821	0.595	0.000	
52 KU 01	0.843	0.887	0.818	0.822	0.880	0.872	0.831	0.853	0.876	0.846	0.828	0.826	0.842	0.890	0.926	0.923	0.831	0.791	0.775	0.789	0.780	0.870	0.000
52 UL 01	0.833	0.827	0.794	0.785	0.800	0.824	0.775	0.820	0.779	0.824	0.786	0.871	0.824	0.815	0.860	0.814	0.769	0.731	0.687	0.882	0.658	0.672	0.873

Table B.2. Genetic dissimilarity (distance) matrix of ITS4-ITS5 region among 21 Rhododendron genus and A. unedo using Kimura's two parameter method.

	08 ART 01	08 MU 01	08 MU 02	08 MU 05	08 BO 05	08 MU 08	08 MU 09	08 MU 10	08 MU 11	08 MU 03	08 SA 02	81 DU 01	08 BO 01	A. unedo	08 MU 07	67 AL 01	08 AR 01	08 AR 02	39 DE 01	28 GO 01	52 KU 01	52 UL 01
08 ART 01	0.000																					
08 MU 01	0.016	0.000																				
08 MU 02	0.009	0.016	0.000																			
08 MU 05	0.002	0.016	0.009	0.000																		
08 BO 05	0.009	0.016	0.003	0.009	0.000																	
08 MU 08	0.002	0.016	0.009	0.000	0.009	0.000																
08 MU 09	0.005	0.016	0.009	0.005	0.009	0.005	0.000															
08 MU 10	0.009	0.016	0.003	0.003	0.001	0.009	0.009	0.000														
08 MU 11	0.002	0.016	0.009	0.000	0.009	0.000	0.005	0.009	0.000													
08 MU 03	0.009	0.016	0.002	0.009	0.003	0.009	0.009	0.003	0.009	0.000												
08 SA 02	0.016	0.000	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.000											
81 DU 01	0.009	0.016	0.000	0.009	0.003	0.009	0.009	0.003	0.009	0.002	0.016	0.000										
08 BO 01	0.009	0.016	0.006	0.009	0.006	0.009	0.009	0.006	0.009	0.006	0.016	0.006	0.000									
A. unedo	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.129	0.000								
08 MU 07	0.009	0.016	0.000	0.009	0.003	0.009	0.009	0.003	0.009	0.002	0.016	0.000	0.006	0.129	0.000							
67 AL 01	0.009	0.016	0.000	0.009	0.003	0.009	0.009	0.003	0.009	0.002	0.016	0.000	0.006	0.129	0.000	0.000						
08 AR 01	0.009	0.016	0.000	0.009	0.003	0.009	0.009	0.003	0.009	0.002	0.016	0.000	0.006	0.129	0.000	0.000	0.000					
08 AR 02	0.002	0.016	0.009	0.002	0.009	0.002	0.005	0.009	0.002	0.009	0.016	0.009	0.009	0.129	0.009	0.009	0.009	0.000				
39 DE 01	0.009	0.016	0.000	0.009	0.003	0.009	0.009	0.003	0.009	0.002	0.016	0.000	0.006	0.129	0.000	0.000	0.000	0.009	0.000			
28 GO 01	0.009	0.016	0.000	0.009	0.003	0.009	0.009	0.003	0.009	0.002	0.016	0.000	0.006	0.129	0.000	0.000	0.000	0.009	0.000	0.000		
52 KU 01	0.016	0.000	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.000	0.016	0.016	0.129	0.016	0.016	0.016	0.016	0.016	0.016	0.000	
52 UL 01	0.009	0.016	0.001	0.009	0.002	0.009	0.009	0.002	0.009	0.002	0.016	0.001	0.006	0.129	0.001	0.001	0.001	0.009	0.001	0.001	0.016	0.000

APPENDIX C: SEQUENCE COMPARISIONS

Figure C.1. Sequence comparison of the ITS4 and ITS5 regions from 21 taxa of the genus Rhododendron and *A. unedo*. Dots (.) indicate the same nucleotides and gaps (-) are introduced to maximize homology (cont.).

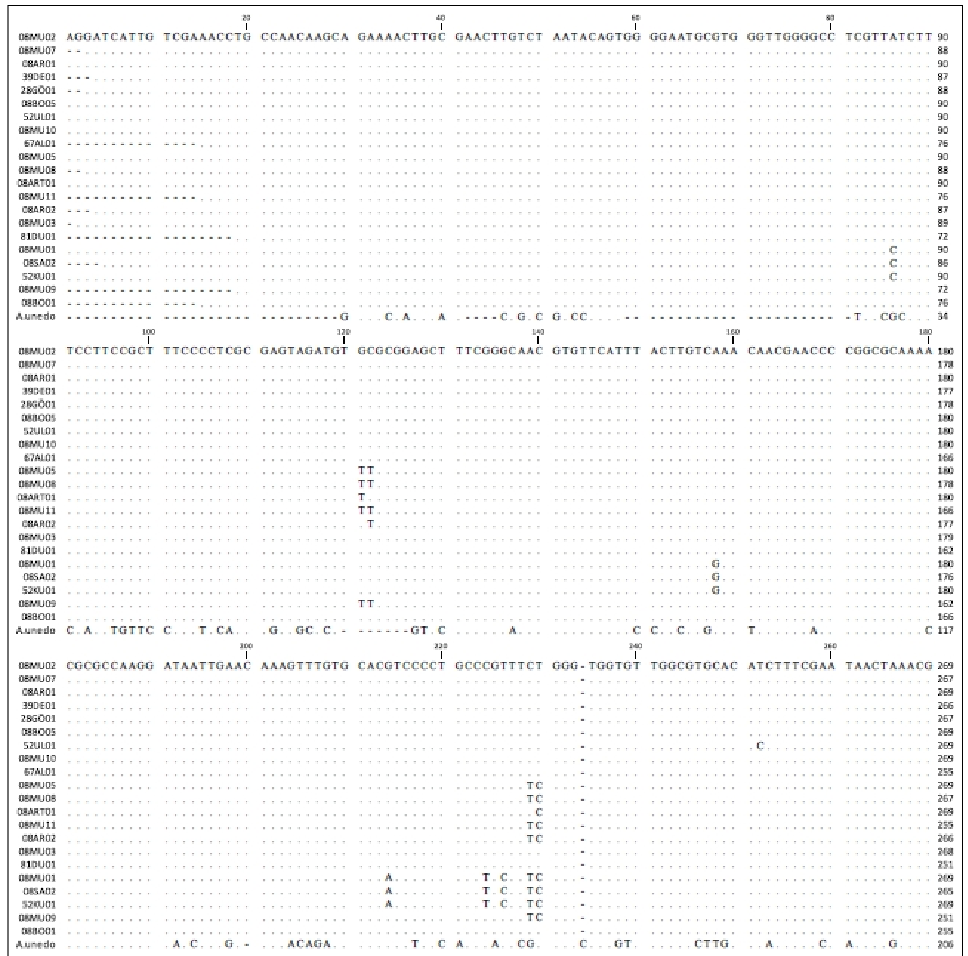

Figure C.1. Sequence comparison of the ITS4 and ITS5 regions from 21 taxa of the genus Rhododendron and A. unedo. Dots (.) indicate the same nucleotides and gaps (-) are introduced to maximize homology (cont.).

```
                  280        300        320        340        360
                    |          |          |          |          |
08MU02  ACTCTCGGCA ACGGATATCT CGGCTCTTGC ATCGATGAAG AACGTAGCGA AATGCGATAC TTGGTGTGAA TTGCAGAATC CCGTGAACCA 359
08MU07  .......... .......... .......... .......... .......... .......... .......... .......... .......... 357
08AR01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 359
39DE01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 356
28GÖ01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 357
08BO05  .......... .......... .......... .......... .......... .......... .......... .......... .......... 359
52UL01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 359
08MU10  .......... .......... .......... .......... .......... .......... .......... .......... .......... 359
67AL01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 345
08MU05  .......... .......... .......... .......... .......... .......... .......... .......... .......... 359
08MU08  .......... .......... .......... .......... .......... .......... .......... .......... .......... 357
08ART01 .......... .......... .......... .......... .......... .......... .......... .......... .......... 359
08MU11  .......... .......... .......... .......... .......... .......... .......... .......... .......... 345
08AR02  .......... .......... .......... .......... .......... .......... .......... .......... .......... 356
08MU03  .......... .......... .......... .......... .......... .......... .......... .......... .......... 358
81DU01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 341
08MU01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 359
08SA02  .......... .......... .......... .......... .......... .......... .......... .......... .......... 355
52KU01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 359
08MU09  .......... .......... .......... .......... .......... .......... .......... .......... .......... 341
08BO01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 345
A.unedo .......... .......... .......... .......... .......... .......... .......... .......... .......... 296

                             380        400        420        440
                               |          |          |          |
08MU02  TCGAGTCTTT GAACGCAAGT TGCGCCTGAA GCCATTAGGT TGAAGGCACG TCTGCCTGGG CGTCACGCAT TGCGTCACCC ACTCACCCCG 449
08MU07  .......... .......... .......... .......... .......... .......... .......... .......... .......... 447
08AR01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 449
39DE01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 446
28GÖ01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 447
08BO05  .......... .......... .......... .......... .......... .......... .......... .......... .......... 449
52UL01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 449
08MU10  .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 449
67AL01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 435
08MU05  .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 449
08MU08  .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 447
08ART01 .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 449
08MU11  .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 435
08AR02  .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 446
08MU03  .......... .......... .......... .......... .......... .......... .......... .......... .......... 448
81DU01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 431
08MU01  .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 449
08SA02  .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 445
52KU01  .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 449
08MU09  .......... .......... .......... .......... .......... .......... .......... .......T.. .......... 431
08BO01  .......... .......... .......... .......... .......... .......... .......... .......... .......... 435
A.unedo ..C....... .......... ......C... .......... .......... .......... .......... CCG....... G.CAG..T.. 386

                  460        480        500        520        540
                    |          |          |          |          |
08MU02  TGCC--TTAT CGACGGGTAA GTGTGTGGGT GGATATTGGC CCCCCGTTCA CATTCGTGCT CGGTCGGCCT AAAAATGACG GTCCCCGATG 537
08MU07  ....--.... .......... .......... .......... .......... .......... .......... .......... .......... 535
08AR01  ....--.... .......... .......... .......... .......... .......... .......... .......... .......... 537
39DE01  ....--.... .......... .......... .......... .......... .......... .......... .......... .......... 534
28GÖ01  ....--.... .......... .......... .......... .......... .......... .......... .......... .......... 535
08BO05  ....---C.. .......... .........C .......... .......... .......... .......... .......... .......... 537
52UL01  ....--.... .......... .......... .......... .......... .......... .......... .......... .......... 537
08MU10  ....---C.. .......... .........C .......... .......... .......... .......... .......... .......... 537
67AL01  ....--.... .......... .......... .......... .......... .......... .......... .......... .......... 523
08MU05  ....---C.. .......... .........C .......... .......... ...C...... ....T..... .......... .......... 537
08MU08  ....---C.. .......... .........C .......... .......... ...C...... ....T..... .......... .......... 535
08ART01 ....---C.. .......... .........C .......... .......... ...C...... ....T..... .......... .......... 537
08MU11  ....---C.. .......... .........C .......... .......... ...C...... ....T..... .......... .......... 523
08AR02  ....---C.. .......... .........C .......... .......... ...C...... .......... .......... .......... 534
08MU03  ....--.... .......... .......... .......... .......... .......... .......... .......... .......... 536
81DU01  ....--.... .......... .......... .......... .......... .......... .......... .......... .......... 519
08MU01  ....---C.. ..G....... ...C.....C .......... .......... .......... .......... .......G.. .......... 537
08SA02  ....---C.. ..G....... ...C.....C .......... .......... .......... .......... .......G.. .......... 533
52KU01  ....---C.. ..G....... ...C.....C .......... .......... .......... .......... .......G.. .......... 537
08MU09  ....---C.. .......... .........C .......... .......... ...C...... ....T..... .......... .......... 519
08BO01  ....--.... .......... .......... .......... ......C... .......... ........A. .......... .......... 523
A.unedo .T..GTCCGC G.G.A..... A..C...... .......... .T.....G.. .G........ T..CT..... .....C.-.A ....T...C. 475
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Figure C.1. Sequence comparison of the ITS4 and ITS5 regions from 21 taxa of the genus Rhododendron and A. unedo. Dots (.) indicate the same nucleotides and gaps (-) are introduced to maximize homology (cont.).

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                                      560                   580                   600                   620
                                        |                     |                     |                     |
08MU02  ATGGACATCA CGGCAAGTGG TGGTTGCCAA ACCGTCGCGT CATGTCGTGC ATGCCAT--T CTTTGTCGCG GGCTG--GCT CAT-TGACCC 622
08MU07  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-...... 620
08AR01  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-...... 622
39DE01  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-...... 619
28GÖ01  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-...... 620
08BO05  .......... .......... .......... .......... .......... .......--. .......... .....--... ...C...... 623
52UL01  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-...... 622
08MU10  .......... .......... .......... .......... .......... .......--. .......... .....--... ...C...... 623
67AL01  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-...... 608
08MU05  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-C..... 622
08MU08  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-C..... 620
08ART01 .......... .......... .......... .......... .......... .......--. .......... .....--... ...-C..... 622
08MU11  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-C..... 608
08AR02  .......... .......... .......... .......... .......... .......--. .......... .....--... ...-C..... 619
08MU03  .......... .......... .......... .......... .......A.. .......--. .......... .....--... ...-...... 621
81DU01  .......... .......... .......... .......... .......... .......--. .......... .....--... ...C...... 605
08MU01  .......... ..A....... .......... .......... .......... .......--. .C........ .....--... ...CGAC..T 623
08SA02  .......... ..A....... .......... .......... .......... .......--. .C........ .....--... ...CGAC..T 619
52KU01  .......... ..A....... .......... .......... .......... .......--. .C........ .....--... ...CGAC..T 623
08MU09  .......... .......... .......... .......... .......... .......--. .......... .....--... ...CGAC..T 605
08BO01  .......... .......... .......... ........A. .......A.. .C....C--. ...------- ---------- ---------- 584
A.unedo .C........ .AA.CG.... ......ATC. ..T..T...C G.A..T.... ...T.G.CG. .G....G.T. .....TT... .C.TC..... 565

                         640                   660
                           |                     |
08MU02  TTAAGTACCA TCAACTGTGG TAC-CTCAAC TGCGACCCCA 661
08MU07  .......... .......... ...-...... .......... 659
08AR01  .......... .......... ...-...... .......... 661
39DE01  .......... .......... ...-...... .......... 658
28GÖ01  .......... .......... ...-...... .......... 659
08BO05  .......... .......... ...-...... .......... 662
52UL01  .......... .......... ...-...... .......... 661
08MU10  .......... .......... ...-...... .......... 662
67AL01  .......... .......... ...-...... .......... 647
08MU05  .......... .......... ...-...... .......... 661
08MU08  .......... .......... ...-...... .......... 659
08ART01 .......... .......... ...-...... .......... 661
08MU11  .......... .......... ...-...... .......... 647
08AR02  .......... .......... ...-...... .......... 658
08MU03  .......... .......... G..-...... ..-------- 652
81DU01  .......... ........-- ---------- ---------- 623
08MU01  CA........ .......... ...T...... .......... 663
08SA02  CA........ .......... ...-...... .......... 658
52KU01  CA........ .......... ...-...... ........-- 660
08MU09  .--------- ---------- ---------- ---------- 606
08BO01  ---------- ---------- ---------- ---------- 584
A.unedo AA.....--- ---------- -..G.G.TCT ..TT.----- 586
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