Genetic diversity analysis in *Gaultheria fragrantissima* Wall. from the two biodiversity hotspots in India using molecular markers

A thesis submitted to the University of Pune for the Degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

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CERTIFICATE

*Certified that the work incorporated in the thesis "**Genetic diversity analysis in Gaultheria fragrantissima Wall. from the two biodiversity hotspots in India using molecular markers**" submitted by Mrs. Gauri Salil Apte was carried out by her under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.*

Date:

Dr (Mrs) V. S. Gupta
(Research Guide)

DECLARATION

I hereby declare that the thesis entitled **“Genetic diversity analysis in *Gaultheria fragrantissima* Wall. from the two biodiversity hotspots in India using molecular markers”** submitted for Ph.D degree at University of Pune has not been submitted by me for a degree at any other University.

Date:

Mrs. Gauri Salil Apte

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Dedicated to my parents

Leaf material used for thesis work was collected with the help of:

Botanical Survey of India

(Western circle, Eastern circle, Southern circle, Sikkim- Himalayan circle and Northern circle)

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and

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Gauri Apte

List of abbreviations

AFLP: Amplified Fragment Length Polymorphism

AMOVA: Analysis of Molecular Variance

B.P: Before Present

bp: base pair

BSI: Botanical Survey of India

CAPS: Cleaved Amplified Polymorphic Sequence

CBD: Convention on Biological Diversity

cpDNA: chloroplast DNA

CTAB: hexadecyl-trimethyl-ammonium bromide

°C: degree celcius

DNA: Deoxy Ribonucleic Acid

dNTPs: Deoxy Ribonucleotide Tri Phosphate

EDTA: Ethylene Diamine Tetra Acetic acid

ESU: Evolutionary Significant Unit

g: gram

GC: Gas Chromatography

h: hour

ha: hectar

Hg: mercury

IAA: Iso-Amyl Alcohol

ISSR: Inter Simple Sequence Repeat

kb: kilobase pair

KCl: Potassium Chloride

kg: kilogram

km^2: Square kilometer

µg: microgram

µl: microlitre

µM: micromolar

m: meter

M: molar

$MgCl_2$: Magnesium Chloride

min: minute

ml: milliliter

mm: milimeter

mM: milimolar

MoEF: Ministry of Environment and Forest

MSD: Mean Squared Deviation

mtDNA: mitochondrial DNA

MW: Molecular Weight

mya: million years ago

NaCl : Sodium Chloride

NE: Northeast

ng : nanogram

NTSYSpc: Numerical Taxanomy System

pA: pico ampere

PCO: Principle Coordinate Analysis

PCR: Polymerase Chain Reaction

PIC: Polymorphism Information Content

PPB: Percentage of Polymorphic Bands

RAPD: Random Amplified Polymorphic DNA

RFLP: Restriction Fragment Length Polymorphism

rpm: revolution per minute

s: Second

SSCP: Single Strand Conformational Polymorphism

SSD: Sum of Squared Deviation

SSR: Simple Sequence Repeat

TAE: Tris-acetate EDTA

TBE: Tris-borate-EDTA

TE: Tris-EDTA

Tm: melting temperature

UBC: University of British Columbia

UNEP: United Nations Environment Programme

UPGMA: unweighted pair group method, arithmetic mean

WG: Western Ghats

Chapter 1

Review of literature

Review of literature

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1.0 What is Biodiversity ?

The term biodiversity was coined by an entomologist, Edward O. Wilson in 1986, in a report for the first American Forum on Biological Diversity, organized by the National Research Council. Biodiversity can be defined as the array of all interacting, genetically distinct biological populations and species in a region, the communities that they comprise and the variety of ecosystems of which they are functioning parts or in short, biodiversity is the totality of genes, species and ecosystem of a region (Chaudhuri and Sarkar, 2002). Fig 1.1 shows a representation of biodiversity including the flora, fauna, other life forms, abiotic factors and their habitat. The three levels of biodiversity

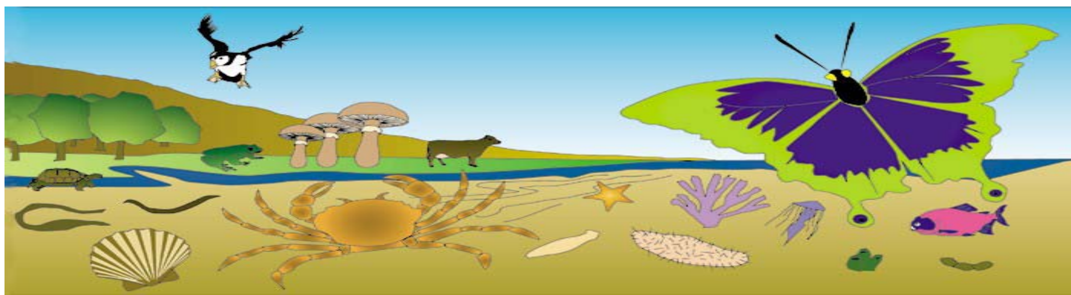

Fig 1.1: Balanced ecosystem

are 1) diversity of genes within a species (genetic diversity), 2) diversity between species (species diversity) and 3) variability in the habitats occurring within a region (ecosystem diversity) (Chaudhuri and Sarkar, 2002). Biodiversity is the prime wealth of the earth and it has contributed in many ways to the development of man. A healthy biodiversity provides a number of natural services to mankind and that to free of charge! Such services include **ecosystem services** (protection of water resources, nutrient storage and recycling, pollution absorption, etc), **biological services** (food, medicines, wood products, ornamental plants, breeding stocks, etc) and **social benefits** (research, education, recreation, tourism, cultural values, etc). A rapid increase in human population and other activities like habitat destruction, extension of agriculture, filling up of wetlands, conversion of rich biodiversity areas for human settlements and industrial development, destruction of coastal areas and uncontrolled commercial expansion are causing destruction of the ecosystem and loss of biodiversity. A majority of biologists believe that a “mass extinction” of biodiversity is underway. Loss of biodiversity has serious environmental, economic and social consequences and costs. Every country has a responsibility to conserve, restore and sustainably use the natural resources within its

jurisdiction. For such sustainable use, there is a need to analyze and survey biodiversity and the first step in doing so is to identify areas of high species diversity and which are experiencing loss of habitat. These areas are also called as ‘Hotspots’. Myers *et al* (2000) have thus identified 25 hotspots all over the globe (Fig 1.2) where 44% of all vascular plant species and 35% of all species in four vertebrate groups are confined to only 1.4% of the land surface of the earth. The details of these 25 hotspots are given in Table 1.1

Fig 1.2: 25 Biodiversity hotspots

and they contribute to several habitat types at the global scale; predominant are the tropical forests, Mediterranean type and islands. Sixteen hotspots are in the tropics which largely represent developing countries where threats are the greatest and conservation strategies are the scarcest. Protection of these hotspots is a prime need where only 38% of them are being protected as parks and reserves, remaining 62% are still to be protected (Myers *et al*, 2000). There are approximately 170 countries in the world, and just 12 of them (Australia, Brazil, China, Colombia, Ecuador, India, Indonesia, Madagascar, Malaysia, Mexico, Peru and Zaire) contain 70% of our planets biodiversity. For this reason, these countries are known as ‘Megadiverse’ (Chaudhuri and Sarkar, 2003). These identified megadiverse countries need to take more efforts to protect their biodiversity and also its sustainable use. For decades, there have been efforts, to bring people together from all over the globe in an attempt to fight against the destruction and loss of species and ecosystem. In 1972, the United Nations Conference on Human Environment (Stockholm) resolved to establish the United Nations Environment Programme (UNEP).

Their main aim was to tackle specific issues, such as protecting wetlands and regulating the international trade in case of endangered species. Later, in 1987, the World

Table 1.1: Details of the 25 hotspots (Myers *et al*, 2000)

Hotspots	Original extent of primary vegetation (km^2)	Remaining primary vegetation (% of original extent)	Area protected (km^2) (% of hotspot)	Plant species	Endemic plants (% of global plants, 300,000)
Tropical Andes	1,258,000	314,500(25.0)	79,687 (25.3)	45,000	20,000 (6.7%)
Mesoamerica	1,155,000	231,000 (20.0)	138,437 (59.9)	24,000	5,000 (1.7%)
Caribbean	263,500	29,840 (11.3)	29,840 (100.0)	12,000	7,000 (2.3%)
Brazil's Atlantic forest	1,227,600	91,930 (7.5)	33,084 (35.9)	20,000	8,000 (2.7%)
Choc\Darien \Western Ecuador	260,600	63,000 (24.2)	16,471 (26.1)	9,000	2,250 (0.8%)
Brazil's Cerrado	1,783,200	356,630 (20.0)	22,000 (6.2)	10,000	4,400 (1.5%)
Centra Chile	300,000	90,000 (30.0)	9,167 (10.2)	3,429	1,605 (0.5%)
California Floristic Province	324,000	80,000 (24.7)	31,443 (39.3)	4,426	2,125 (0.7%)
Madagascar*	594,150	59,038 (9.9)	11,548 (19.6)	12,000	9,704 (3.2%)
Eastern Arc and Coastal Forests of Tanzania/ Kenya	30,000	2,000 (6.7)	2,000 (100.0)	4,000	1,500 (0.5%)
Western African Forest	1,265,000	126, 500 (10.0)	20,324 (16.1)	9,000	2,250 (0.8%)
Cape Floristic Province	74,000	18,000 (24.3)	14,060 (78.1)	8,200	5,682 (1.9%)
Succulent Karoo	112,000	30,000 (26.8)	2,352 (7.8)	4,849	1,940 (0.6%)
Mediterranean Basin	2,362,000	110,000 (4.7)	42,123 (38.3)	25,000	13,000 (4.3%)
Caucasus	500,000	50,000 (10.0)	14,050 (28.1)	6,300	1,600 (0.5%)
Sundaland	1,600,000	125,000 (7.8)	90,000 (72.0)	25,000	15,000 (5.0%)
Wallacea	347,000	52,020 (15.0)	20,415 (39.2)	10,000	1,500 (0.5%)
Philippines	300,800	9,023 (3.0)	3,910 (43.3)	7,620	5,832 (1.9%)
Indo- Burma	**2,060,000**	**100,000 (4.9)**	**100,000 (100.0)**	**13,500**	**7,000 (2.3%)**
South- Central China	800,000	64,000 (8.0)	16,562 (25.9)	12,000	3,500 (1.2%)
Western Ghats/ Sri Lanka	**182,500**	**12,450 (6.8)**	**12,450 (100.0)**	**4,780**	**2,180 (0.7%)**
SW Australia	309,850	33,336 (10.8)	33,336 (100.0)	5,469	4,331 (1.4%)
New Caledonia	18,600	5,200 (28.0)	526.7 (10.1)	3,332	2,551 (0.9%)
New Zealand	270,500	59,400 (22.0)	52,068 (87.7)	2,300	1,865 (0.6%)
Polynesia/ Micronesia	46,000	10,024 (21.8)	4,913 (49.0)	6,557	3,334 (1.1%)
Totals	17,444,300	2,122,891 (12.2)	800,767 (37.7)	↑	133,149 (44%)

*Madagascar includes the nearby islands of Mauritius, Reunion, Seychelles and Comores
↑ This total cannot be summed owing to overlapping between hotspots

Commission on Environment and Development (the Brundland Commission) concluded that economic development must become ecologically less destructive. In 1992, the largest- ever meeting of the world leaders took place at the United Nations Conference on Environment and Development in Rio de Janeiro, Brazil. This was the "Earth Summit" which signed two historic agreements, one was the Convention on Climate Change and the other was the Convention on Biological Diversity (CBD). The three main goals of CBD are:

1) The conservation of biodiversity

2) Sustainable use of the components of biodiversity, and

3) Sharing the benefits arising from the commercial and other utilization of genetic resources in a fair and equitable way.

Thus CBD has created a global forum where governments, non- governmental organizations, academicians, the private sectors, and other interested groups meet and share ideas and compare strategies. The megadiversity countries of the developing world are also trying to develop a common plank for their rights, develop a frame work which will enable them to share experiences, information and maybe even personnel. These 12 megadiversity countries, except Australia, met in February 2003 at Cancun, Mexico to develop a consortium, a sort of negotiating platform, on environmental issues like protecting valued biodiversity and traditional knowledge or further even raising funds to help each other.

1.1 Biodiversity in India

India is one of the 12 megadiversity centers of the world. Western Ghats (WG) and Northeastern Himalayas (NE) constitute 2 hotspots (Fig 1.3) of the 25 biodiversity hotspots in the world (Myers *et al* 2000). India has a rich and varied heritage of biodiversity covering ten biogeographical zones, namely, the trans- Himalayan, the Himalayan, the Indian desert, the semi- arid zone(s), the Western Ghats, the Deccan Penninsula, the Gangetic Plain, North- East India and the islands and coasts (Rodgers *et al*, 2000). India lies at the junction of the 3 major biogeographic realms, namely, the Indo- Malayan, the Eurasian and the Afro- tropical. The forests cover an actual area of 63.73 million ha (19.39%) and consists of 37.74 million ha of dense forests, 25.51 million ha of open forest and 0.487 million ha of mangroves, apart from 5.19 million ha of scrub. It comprises 16 major forest groups accounting for a species diversity of about 1,256 belonging to 245 genera (MoEF, 1999). The endemism of Indian biodiversity is about 33% of the country's recorded flora and is concentrated mainly in the Northeast, Western Ghats, Northwest Himalaya and the Andaman and Nicobar islands. As many as 14 ecoregions lying completely or in part within India figure amongst the Global 200, which are outstanding examples of the world's diverse ecosystems based on criteria such as **species richness, species endemism, unique higher taxa, unusual ecological or evolutionary phenomena and global rarity of major habitat types.** This immense diversity has resulted in the inclusion of the two Indian regions namely, Western Ghats/

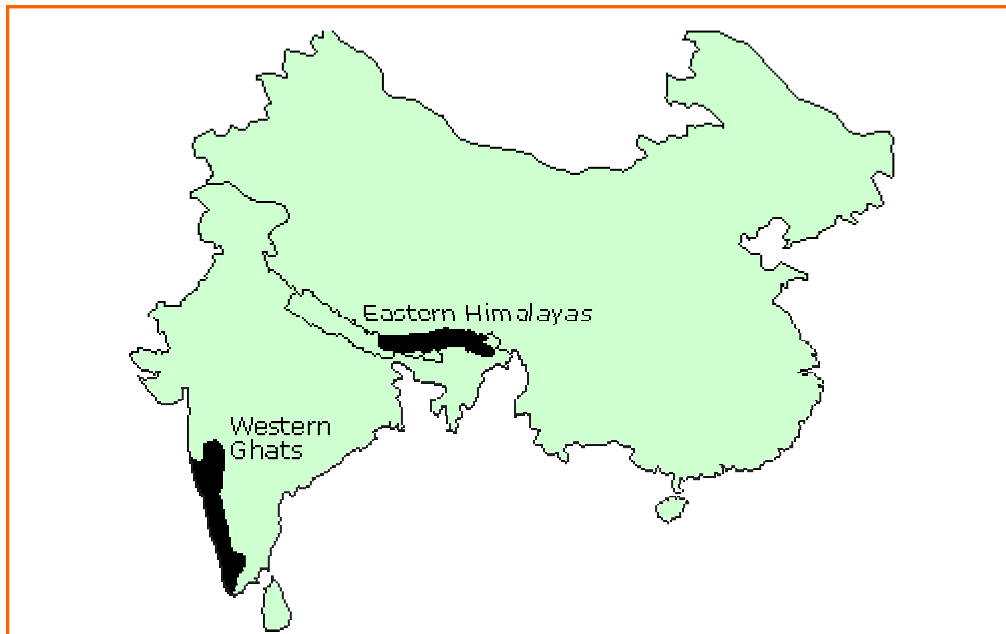

Fig 1.3: Two hotspots in India

Sri Lanka and the Indo- Burma region (covering the Northeastern Himalayas) in the 25 global biodiversity hotspots.

WG is also known as the Malabar rain forest province and is one of the major tropical evergreen forested regions in India which exhibit enormous plant and animal diversity. This mountain range is approximately 1,600km long and runs parallel to the west coast of India from 8°20'N to 21°40'N and 73°E to 77°E (Fig 1.4). The hills rise to 2,800m with an average width of 100km covering an area of approximately 170,000km^2, which includes 12,000km^2 of primary vegetation. The WG are estimated to harbor approximately 5,500 species of flowering plants (Nair and Daniel 1986). According to Myers *et al* (2000) they are also home to 2,180 endemic plants, constituting 0.7% of global plant species. Shola forests (Fig 1.5) occurring above 1500m in the Nilgiri ranges of the WG represent a unique system of evergreen montane forests, which are almost invariably confined to sheltered sites, such as valleys, hollows and depressions.

Fig 1.4: Western Ghats: Hotspot area marked in yellow (www.nationalgeographic.com)

Fig 1.5: Shola forest : Nilgiri hills

These 'Sholas' are the best examples of tropical evergreen forests or tropical rain forests (Malabar rain forest) and are some of the non- renewable natural resources (Hajra and Mudgal, 1997). Palynological studies have revealed that these shola forest communities had been wide spread in the past. They originated through gradual invasion of shrubs and under trees into grassland, about 35,000 years BP, corresponding in time to the last

glaciation in the north and were established about 24,000 years BP. The progressive recession of sholas had started around 7,000 years BP. Both biotic and climatic factors not only reduced the sholas but have also created conditions under which the community has almost completely stopped regeneration outside the sholas (Gupta, 1989).

The Northeastern region of India is a unique transitional zone between the Indian, Indo- Malayan and Indo- Chinese biogeographical zones as well as the confluence of the Himalayan region with peninsular India (Rao, 1993). The Northeastern region comprises the eight states of India (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Tripura and Sikkim). Fig 1.6 shows the representation of temperate forest from Meghalaya. There are 14 peaks in this region above 8000m and hundreds above 7,000m

Fig 1.6: Forest cover in Shillong

in altitude (www.commonwealthknowledge.net). The region contains about 8,000 species of flowering plants including several representatives of primitive and ancient angiosperms (Takhtajan, 1969; Rao, 1994) of which 3,500 are endemic. The high rainfall, moist and cold climate coupled with factors like variable altitude, latitude and longitude have added to the multiplicity of habitats and thus provide varied microclimates and ecological niches both for plants and animals. The region is also recognized as a centre of origin of several cultivated plants. The rise of the Himalayas not only opened several routes for inflow and outflow of flora from central or west Asia, or even Europe, to East Asia but also has dynamically influenced the evolutionary pattern of vegetation in terms of emergence of new taxa and extinction of others.

1.2 History of the tectonic movement of the Indian plate and birth of the two hotspots

1.2.1 History of the tectonic movement of the Indian plate

The supercontinent called "**Pangea**" (Fig 1.7) existed during the Permian period (270 million years ago). The rest of the globe was all covered by water and this ancestral ocean was called "**Panthalassa**". But this single continent was short- lived and by the end of Triassic period (180 million years ago) it started splitting into northern and southern landmasses. The southern landmass was called the **Gondwana land** and the northern landmass was called **Laurasia**. Gondwana land included the present South America, Africa, Indian and Antarctic continents, while Eurasia and North America were derived

Fig 1.7: Supercontinent pangea

from Laurasia. Both these landmasses i.e. Gondwana land and Lauraisa started fragmenting about 120 million years ago. The movement of fragments from Gondwana land led to the placement of South America, Africa, Australia and Antarctica to their present position. Laurasia rotated clockwise, Africa drifted north to meet Laurasia resulting in numerous collisions to form the present Alps of Europe and another collision created a pinching closure to the east that left the Mediterranean Sea. A north Atlantic Rift broke Laurasia into two, to form Portugal and Canada and continued along, what is now the Artic Ocean. The Indian subcontinent broke away from Antarctica and drifted to north towards Laurasia at the end of the Cretaceous Period (80 million years ago) (Bourliere, 1980).

1.2.2 Birth of the Great Himalayas and Western Ghats

Himalaya is amongst the youngest of mountain chains in the world (www.gisdevelopement.net). The Himalayan mountain system developed in a series of stages 30- 50 million years ago. The Himalayan range was created from powerful earth movements that occurred as the Indian plate collided with the Eurasian continental plate. As the two plates collided, the sinking ocean floor generated volcanoes in southern Tibet. By 25 million years ago the fast moving Indian continent (moving at the rate of 15cm/ year) had almost entirely closed over the intervening ocean, squeezing the sediments on the ocean floor. This ocean, '**Tethys**' thus completely disappeared. The sediments on the ocean floor were light weight and instead of sinking along with the plate crumpled into mountain ranges and Himalaya was born. Fig 1.8 shows the step by step formation of Himalayas (www.pbs.org).

The Deccan Plateau itself was once part of Gondwana land, evident in relicts of the ancient southern flora and fauna. After becoming detached from this southern continent during the Cretaceous period (80 million years ago), it drifted northward to finally crash into the northern Laurasian continent. After this initial collision, a series of geological uplifts created the Western Ghats mountain range, with several peaks higher than 2,000m (www.worldwildlife.org).

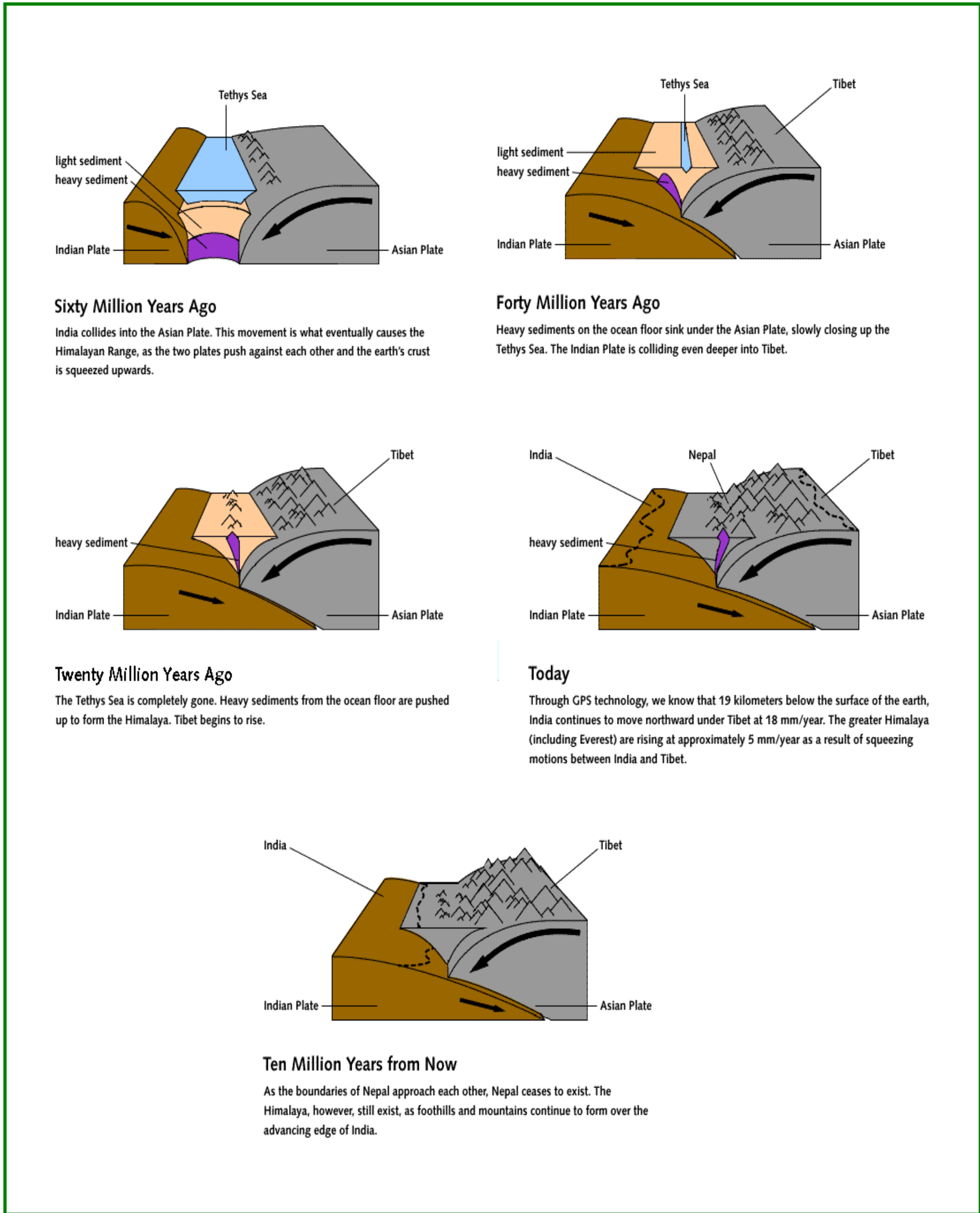

Fig 1.8: Birth of Himalayas (www.pbs.org)

1.3 Flora in WG and NE region, the two hotspots

It has been reported that 325 families represent 17,000 flowering plants in India out of which 15% are believed to be under threat. The most dominant families are Acanthaceae, Asteraceae, Cyperaceae, Euphorbiaceae, Fabaceae, Lamiaceae, Orchidaceae, Poaceae, Rubiaceae and Urticaceae. Apart from the flowering plants there are 64 gymnosperms, 1,200 pteridophytes, 2,850 bryophytes, 13,000 fungi and 12,500 algae (Hajra and Mudgal, 1987). Together the two hotspots have 5,150 endemic plant species, or 20% of the worlds recorded plant species, in only 746,400 km^2 or 0.5% of the earths land surface.

In the Himalayan ranges vegetation can be divided into three broad zones: tropical- subtropical (base to 1,200m), temperate (1,200- 3,600m) and alpine (upto 4,500m) (Singh and Sarkar, 1990). Northeastern Himalayas represent about 8,000 species of flowering plants out of which 3,500 are endemic. In NE region there is high degree of diversity in orchids, rhododendrons, hedychiums, oaks and bamboos alongwith large number of plants of medicinal and ethanobotanical values. It is estimated that out of a total of 800 edible plants in India, more than 300 are found in NE region. Thus, this region is also called as the 'centre of origin' of several cultivated plants including about 200 wild relatives of cultivated plant species. However, presently it is recorded that atleast 55 flowering plants endemic to this region are rare. The pitcher plant (*Nepenthes khasiana*) has become rare while the Taxol plant (*Taxus wallichiana*) has come in the Red Data category due to over exploitation for drug extraction used in cancer treatment (MoEF, 1999).

The types of forests in WG include thorn forests (300- 800m), deciduous forests (800- 1,300m) and tropical evergreen montane forests (above 1,500m) (Meher- Homji, 1975). WG or the Malabar province harbours 4,000 species of flowering plants of which 1,600 are endemic. WG has high degree of diversity in valuable timber species of families such as Bombacaceae, Clusiaceae, Dipterocarpaceae, Fabaceae and Lauraceae, alongwith large variety of bamboos, legumes, medicinal plants, spices and condiments. It is also a rich germplasm centre for a number of wild relatives of cultivated plants. Nearly 235 species of endemic flowering plants are considered to be endangered (MoEF, 1999). Overexploitation of *Mappia foetida* for the anticancer drug component camptothesin and *Renanthera imschortians* commonly known as Red Vanda, a highly threatened species of orchid has been added to the Red Data book

(MoEF, 1999). Apart from plants endemic to these two regions there are plant species common to the Himalayas and the South Indian hilltops. Especially plant species only of the shrub savanna or the shola forest are reported to be common (Meher- Homji, 1972). Fig 1.9 shows the representative forests types in the two hotspots.

Fig 1.9: Various forest types in the two hotspots: a) Subtropical, b) tropical, c) temperate, d) alpine, e) thorn and f) montane

India's ever increasing population has put tremendous pressure on these ecosystems. In order to increase food production, there is an increase in agricultural areas leading to reduction in the forests and grasslands (Upadhyay, 1999). In the NE region a 2,778 km^2 area was used for shifting cultivation from 1993 to 1997 and has caused extensive deforestation. In Meghalaya alone, 295 km^2 area has been used for similar purpose (Upadhyay, 1999). In WG, smaller ranges and deforestation contribute to isolation of the shola forests. A loss of 25.6% of the total forest area has been noted in the WG region during the last two to three decades, whereas the dense forest cover and open forests have decreased by 19.5 and 33.2% of their total occupation, respectively, due to deforestation and land use (Jha *et al*, 2000).

1.4 Distribution and migration of plant species between WG and NE region

Various theories have been put forth by a number of researchers to explain the geographical distribution and migration of plant species between the NE and WG mountain ranges. The most commonly held view for the occurrence of these common species is that the *Pleistocene glaciation is responsible for pushing the Himalayan plants southwards and these species are known as the relicts or vestiges of the last ice age (Medlicot and Blanford, 1879; Burkill, 1924; Hora, 1949; Auden, 1949; Dey, 1949). Hora (1949) believes that the Vindhya- Satpura trend of hills was the connecting link that existed in the past between the east and the west. This hypothesis known as the **Satpura Hypothesis** is based on: 1) Continuity of the Vindhya- Satpura ranges with the Assam Himalayas in the east and the Western Ghats in the west. 2) Five to six thousand feet elevation of the Vindhya- Satpura ranges and of the northern section of the Western Ghats. 3) Continuity of an ecological belt of mountains with rainfall of about 100 inches or above and consequently of tropical evergreen forests between Assam Himalayas and the mountains of Ceylon via the Vindhya- Satpura trend and the Western Ghats and
4) Dispersal of the fauna/ flora from east to the west and the consequent change in topography necessary therefore (Dilger, 1952).

***Pleistocene glaciation and its impact on the Globe:**

Various types of paleoclimatic evidence suggest that the climate of Earth has varied over time. Ice ages occur at regular intervals of 100,000 years with warm interglacial periods lasting 15- 20,000 years as a result of instabilities in the earth's climate caused by the Milankovitch cycles (Bennett, 1990). Glaciation is a geological phenomenon in which massive ice sheets form in the Arctic and Antarctic and advance towards the equator. In the last billion years of Earth history, glacial periods have started at roughly 925, 800, 680, 450, 330 and 2 million years before present (B.P.). The last major glacial period began about 2,000,000 years B.P. and is commonly known as the Pleistocene glaciation or the last Ice Age (www.geog.ouc.bc.ca). Climatic oscillations in the Quaternary have played a major role in changing the geographical distribution of plant species. The change of geographical distribution has also provided opportunities for speciation through isolation and hybridization after secondary contact. Quaternary palaeoecology in Europe and North America has revealed a series of southward range contractions of both plant and animal species during the last glacial period followed by rapid northward range expansions in the wake of deglaciation.There is no direct information on the prevalence of the Pleistocene glaciation in Penninsular India but it is a generally accepted fact that increased glaciation in the Himalaya could have lowered the mean annual temperature of Pennninsula by 5°C to 7°C (Rajguru, 1969) making it favourable to permit some of the animals and plants from the Himalaya to wander south (Pascoe, 1963).

Randhwa (1945) has earlier suggested that a part of India to the north of the Satpura Trend was, in historical times, very much wetter than it is at present time and supported a typical wet tropical fauna and flora. However, the **'**Brij**'** country which was covered with luxuriant evergreen tropical forests about 2,000 years ago has now completely changed. Briggs (1989) further believes the existence of a former wet tropical belt across India between the eastern Himalayas and the west coast north of Vindhya-Satpura Trend and this is probably the area that once supplied the connecting link between the Western Ghats and the Northeastern region of India.

However, Blasco (1970, 1971) is not in favor of the commonly held theory that Pleistocene glaciation is responsible for pushing the Himalayan flora southwards. He has followed the views putforth by Hedbergs (1969) for the mountains of East Africa, wherein direct contact between the various high peaks due to the Pleistocene climatic changes is ruled out and the intermountain migration through independent long distance dispersal is suggested. However, Dilger (1952) has pointed out that the distance involved between the mountains of East Africa is considerably shorter than that between the Himalayas and the South Indian hills. Furthermore, Meher- Homji (1972) has also revealed problems associated with long distance dispersal hypothesis based on the presence of different species of *Rhododendron* and *Mahonia* on WG and NE region eg: *Rhododendron arboreum* in the Himalaya and *R. nilagiricum* on the Ceylon highlands; *Berberis*- syn. *Mahonia nepalensis* in the Himalayas and *M. leschnaultii* in south India.

This suggests that the transport has taken place sufficiently earlier to give enough time to these genera to evolve independently. Apart from such distinct species in WG and NE region, it is also seen that in certain cases there exists one link species between these two regions, namely, *Symplocos laurina*, *Pittosporum floribundum*, *Ardisia solanacea*, etc. (Meher- Homji, 1975). Analysis of such plant species at the genetic level can provide insight into their migration and distribution.

1.5 Detection of genetic variation

A variety of methods exist for the assessment of genetic variation in closely or distantly related plant species. Traditionally, genetic variation is inferred by morphological/ phenotypic variation or the growth response of the organism. Many researchers have also used cytological tools for such studies. Janaki Ammal *et al* (1950) studied the genus Rhododendron in detail using chromosome counts and studied its ploidy. Middleton and Wilcock (1990) carried out chromosomal analysis of 32 taxa in the Gaultheria group for subgeneric classification. He further studied the leaf and stem anatomy of many species in the Gaultheria group of genera of tribe Andromedeae and suggested that these characters would be useful for subgeneric classification (Middleton, 1993). However, it is difficult to tell whether the phenotypic variations are due to environmental effects or the underlying genetic differences within the plant species.

The use of allozyme technique became widespread in the late 1960's and produced a large volume of data on genetic variation in plant species (Loveless and Hamrick, 1984; Hamrick and Godt, 1989). The primary contribution of allozymes to plant population biology has come from their utilization as neutral (or nearly neutral) genetic markers. Allozymes have been employed to characterize patterns of genetic variation, within and among populations and to examine the process of dispersal and the patterns of mating that influence levels of genetic differentiation (Brown 1979, Loveless and Hamrick 1984; Hamrick and Godt 1989; Barrett and Kohn 1991). Hamrick and Godt (1989) in their review of 322 isozyme studies also concluded that woody plants maintain more intraspecific variations than other life forms but generally display less variation between populations. Here are a few examples of the use of allozymes in the study of plant population genetics: Batista and Sosa (2002) have used allozymes to study diversity in natural populations of *Viola palmensis.* They detected no relationship between genetic differentiation and geographical distances between populations and suggested an outcrossing insect mediated breeding system. Matolweni *et al* (2000)

studied genetic diversity and gene flow in the morphologically variable rare endemics *Begonia dregei* and *Begonia homonyma.* They suggested a restricted gene flow and the ancient separation of isolated forest patches. However, the main drawback of allozymes is their low abundance and low level of polymorphism.

1.5.1 DNA markers for assessment of genetic variation

DNA markers play a very important role and help in studying the genetic variations in natural populations. They represent nuclear as well as organellar genome (chloroplast and mitochondrial) and reveal sequence polymorphisms that can be used as highly informative markers for the structure and dynamics of genomes at the level of populations and individuals. They measure the genetic diversity at DNA level, can account for the effects of selection, are environment- independent and are available in an enormous number. Further, the invention of PCR technology by Saiki *et al* (1988) and its technical simplicity made it a very popular technique among researchers. Table 2 summarizes popular marker types and their suitability for various applications in plant population (within and between) studies in comparison with allozyme markers.

Table 2: Comparison of various molecular marker systems

	Allozymes	RAPD	AFLP	Nuclear RFLP	Cytoplasmic RFLP
Polymorphism	+	++	+++	++	+
Dominance	Codominant	Dominant	Dominant	Codominant	(Haploid)
Mode of inheritance	Bi- parental	Bi- parental	Bi- parental	Bi- parental	Uniparental
Recombination	Yes	Yes	Yes	Yes	No
Costs (development and application)	Low	Low	High	Intermediate	Intermediate
Applications:					
Indirect, within populations	++	-/+	-/+	++	-
Indirect, between populations	+++	-/+	-/+	++	++
Direct	+	++	+++	++	++
Phylogeography	+	-	-	-/+	+++

* Suitability ranking for applications: -, not suitable; -\+, to be avoided; +, suitable; ++, well suited; +++, very well suited. (Ouborg *et al* 1999)

1.5.2 Use of various marker systems for population analysis

The most commonly used marker systems for population analysis alone have been discussed below, although a number of them have been made available so far (Bachmann, 1994).

1) Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) (Nuclear random markers)

Randomly Amplified Poplymorphic DNA markers (RAPD), introduced by Williams *et al* (1990) and Inter Simple Sequence Repeat markers (ISSR) as reported by Zietkiewicz *et al* (1994) are both PCR derived dominant markers. RAPD is the most commonly used marker system and is mainly used in applied research, eg plant breeding (review in Weising *et al*, 1995) and also in studies of natural plant populations (reviews in Bartish *et al*, 1999; Bussell, 1999). Muller and Fischer (2001) used RAPD in the study of genetic structure of animal weed *Seneco vulgaris* in relation to habitat type and population size while Lacerda *et al*, (2001) in their study of genetic diversity and structure of natural populations of *Plathymenia reticulate* (Mimosoideae) concluded that their study would provide a genetic database to monitor populations of *P. reticulate.* Esselmann *et al*, (2000) studied within- and among species diversity in *Dendroseris*

(Asteracea: Lactuceae) and suggested that RAPD might provide a useful alternative to allozymes for assessing diversity in rare species.

Culley *et al* (2001) used both allozymes and ISSR to study population genetic structure of the Cleistogamous plant species *Viola pubescens* Aiton (Violaceae) and inferred high genetic variation, population structuring and mixed mating system. Camacho and Liston (2001) analysed population structure, genetic diversity and significance of asexual reproduction in *Botrychium pumicola* (Ophloglossaceae) suggesting that the long distance dispersal of gemmmae is a rare event while Wolfe *et al* (1998) studied hybridization in natural populations of Penstemon (Scrophulariaceae) and did not support hybrid origin of *P. spectabilis* using ISSR markers.

2) Amplified fragment length polymorphism (AFLP)

AFLP is an ingenious combination of RFLP (Restriction Fragment Length Polymorphism) and PCR (Polymerase Chain Reaction) techniques (Vos *et al* 1995). During last few years, there have been a few reports on the use of AFLP in the studies of natural populations. Gaudeul *et al* (2000) analyzed genetic diversity in an endangered alpine plant *Eryngium alpinum* L. (Apiaceae). Their study showed that although the endangered species occurred in small isolated populations, contained high genetic diversity indicating chances of recovery of the species. Mariette *et al*, (2001) studied genetic diversity within and among *Pinus pinaster* populations and suggested very weak cross species amplification in Pinus genus. Ribeiro *et al* (2002) studied within and among population genetic diversity of maritime pine using AFLP and cpSSR and concluded that gene flow was the predominant force shaping nuclear and chloroplast genetic variation.

3) Cytoplasmic DNA markers

Chloroplast and mitochondrial genomes are the extranuclear or organellar genomes which are also exploited for the studies on population structure, phylogeography and genetic diversity. These organellar genomes are uniparenatally inherited, are homoplasmic and non recombinant. Availability of intergenic chloroplast (cp) and mitochondrial (mt)DNA markers (Demesure 1995, Taberlet 1991 and Dumolin-Lapague, 1997) and use of PCR-RFLP or CAPS technique (Cleaved Amplified Polymorphic Sequence) have helped researchers to study genetic diversity in natural populations. Mohanty *et al*, (2002) analysed chloroplast DNA polymorphism in wild populations of *Prunus spinosa* L. and showed incongruency between the phylogeny of haplotypes and their geographic locations due to intensive seed movements. Palme *et al*

(2003) analysed postglacial recolonization and cpDNA variation of Silver birch. Johansen and Latta (2003) studied mitochondrial haplotype distribution, seed dispersal and patterns of postglacial expansion of ponderosa pine where results suggested a small number of long distance colonists with diffusive dispersal.

1.5.3 Molecular markers in plant population biology for planning conservation strategies

In order to prevent depletion of biodiversity, it is necessary to understand how the diversity of life, particularly at the genetic level, is maintained under natural conditions. Charles Darwin's theory of evolution by natural selection is predicted upon the existence of genetic diversity –a major component of biodiversity. With a loss of genetic variability within a species, the pool of genetic resources for further evolution is gradually drained (Narain, 2000). The goal of a conservation strategy should therefore, be to ensure that evolution continues and gene pools are maintained.

In the wake of the Convention on Biological Diversity (Rio de Janeiro, Brazil, 1992), intraspecific variation has increasingly been accepted as a focus for conservation, an approach consistent with the general aim of maintaining the evolutionary potential of species. Studies on intraspecific variations can contribute to the development of conservation strategies, by identifying appropriate units for conservation called as Evolutionary Significant Units (ESU). DNA based markers can provide wealth of information in this regard since they provide an unbiased comparison of the adaptations of plants to their environment, its genetic basis and its effect on evolution. For example, different cpDNA lineages detected in a variety of European species, such as Oak, Beech and Alder, could represent ESUs (Newton *et al*, 2000). Similarly cpDNA haplotypes of silver birch detected by Palme *et al* (2003) and Japanese beech identified by Okaura and Harada (2002) could serve as ESU. Assessments of genetic variation are, therefore, of key importance to the development of effective conservation strategies.

DNA based markers can provide a wealth of information for the study of plant evolutionary biology. Molecular markers that can be determined without regard to the phenotype permit an unbiased comparison of the adaptation of organisms to their environment, its genetic basis and its effect on evolution. Conservation strategies need the support of a thorough knowledge of population genetics and population ecology which can be achieved with the use of molecular markers.

1.6 *Gaultheria fragrantissima*: A plant under present study

G. fragrantissima Wall. is found from Nepal to Bhutan at altitudes of 1,800m to 2,500m and also in Burma, Ceylon, Khasia hills, Western Ghats, the Nilgiri hills, the Palni hills and hills of Travancore at altitudes of over 1,500m (Anonymous, 1956). The classification of *G. fragrantissima* is given in the box below.

Classification of *G. fragrantissima* Wall. (Bentham and Hooker)

Division: Phanerogams

Subdivision: Dicotyledons

Class: Angiosperms

Subclass: Gamopetalae

Series: Heteromerae

Cohort: Ericales

Family: Ericaceae

Subfamily: Ericaceae

Tribe: Andromeda

Genus: *Gaultheria*

Species: *fragrantissima*

The genus ***Gaultheria*** is named by Kalm after **Dr Gaulthier,** a physician in Quebec and the species ***fragrantissima*** is described by Wallich, 1820.

Vernacular names of *G. fragrantissima* are as follows: in Lepcha- Kalomba, in Nepali- Machino, in Tamil- Kolakkaai, in Kannada- Moolai; Gandhapooram and English/ popular name is Wintergreen.

G. fragrantissima is a stout shrub with lanceolate to ovate leaves of 3 × 1-1.5 inch in size, placed alternately. They are shortly acute or subobtuse at tip and rhomboid at base with crenate or serrate margins and are gland dotted. Flowers are white or pinkish and are closely placed in dense, more or less pubescent racemes which are 1-3 inch long. Anther cells are each with 2 terminal bristles. Fruits are 1/5 inch in diameter, brown, hairy, surrounded by deep blue succulent calyx (Fig 1.10 and 1.11) (Clarke, 1882).

Fig 1.10: Line diagram of *G. fragrantissima*

Fig 1.11: a) Habitat, b) shrub, c) flowers and d) fruits of *G. fragrantissima*

Uses and properties: The fruit of this plant is known to be edible. It is a medicinally important plant where oil extracted from its leaves is popularly known as "Oil of Indian

Wintergreen". The oil contains methyl salycilate as the chief constituent which is used as a prescription for rheumatic arthritis, sciatica, neuralgia and is also used in most of the proprietary balms, liniments or ointments and is also used as a flovouring agent in tooth pastes (Chopra, 1932). A similar oil is obtained in large quantities from *G. procumbens*. Linn., a plant indigenous to the United States of America and from *Betula lenta* Linn. (sweet birch). It has also been discovered in many plants of the families Cupuliferae, Rosaceae, Polygalaceae, Ericaceae and Leguminosae.

1.7 Genesis of thesis and its organization

Although *G. fragrantissima* is a medicinally important plant, there is hardly any information available about the genetic variation in this plant species using molecular markers. I, therefore undertook studies towards molecular analysis of *G. fragrantissima* with the following objectives:

1) To study genetic diversity in *G. fragrantissima* from the two hotspots, using various molecular marker systems. This would help in understanding the nature of gene flow, the presence of haplotypes and to assess different theories on migration and evolution.

3) To study the variation in the yield of oil of wintergreen.

Organization of Thesis

I have organized my thesis into five chapters and the contents of each chapter are as follows:

Chapter 1: Review of literature.

In this chapter, I have discussed the importance of the two hotspots in respect to their birth, flora and theories of plant migration. The potential of DNA markers in natural plant population analysis has also been discussed.

Chapter 2: Genetic diversity analysis of *G. fragrantissima* from the two hotspots in India using ISSR markers.

This chapter deals with the study of genetic diversity in *Gaultheria fragrantissima* using ISSR markers. This study has given an insight into the genetic structure, gene flow and geographic distribution of *G. fragrantissima* in the two hotspots in India.

Chapter 3: Chloroplast and mitochondrial DNA diversity in *G. fragrantissima* from the two hotspots in India.

Chloroplast and mitochondrial DNA diversity of *Gaultheria fragrantissima* is detailed in this chapter. This study has identified different chloroplast and mitochondrial

haplotypes in *G. fragrantissima* which help in understanding the phylogeography (geographic distribution of genetic variation) and evolutionary history of the plant.

Chapter 4: Comparative analysis of quantity and quality of oil of Indian Wintergreen from the two hotspots in India.

Variations in the yield of oil and methyl salicylate content are studied in relation to the season of collection of leaves and the geographic location of the plants (Western Ghats and Northeastern Himalayas). Oil yield from individual plants of NE region has also been studied in this chapter.

Chapter 5: Thesis summary and future prospects

Here, I have very briefly outlined the summary of the results obtained in the earlier chapters and discussed the usefulness of both the types of markers viz. nuclear as well as cytoplasmic.

The bibliographic details and my curriculum vitae are given at the end of the thesis chapters.

Chapter 2

Genetic diversity analysis of *G. fragrantissima* from the two hotspots in India using ISSR markers

Part of the contents of this chapter has been submitted as a full length paper to Taxon

Genetic diversity analysis of *G. fragrantissima* from the two hotspots in India using ISSR markers

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- **2.5.1 Genetic diversity clustering and gene flow in the populations in *G. fragrantissima***
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2.1 Abstract

The present study involves use of Inter Simple Sequence Repeat (ISSR) markers to analyze genetic diversity of an undershrub, *Gaultheria fragrantissima*, Wall. collected from seven populations separated by a minimum aerial distance of 14km, from two plant diversity hotspots in India, namely Western Ghats (WG) and Northeastern Himalayas (NE). Sixtyfive ISSR genotypes were identified from sixtysix individuals. The percentage of polymorphic bands (PPB) ranged from 6.98- 37.98 in seven populations. The total heterozygosity, H_T was 0.505 and was higher than the average heterozygosity that ranged from 0.147- 0.380. AMOVA was performed to partition the variance between the two hotspot regions, among populations and among individuals within populations. Within population variance was higher (15.56%) than among population variance (8.31%) as seen in outcrossing plant species. Mantel's Test showed a strong positive correlation between the genetic and geographic distances. This study has thrown light on the genetic structure, gene flow and geographic distribution of *G. fragrantissima* in the two hotspots in India.

2.2 Introduction

Gaultheria fragrantissima,Wall. is an outcrossing, insect pollinated plant (chromosome no. n= 22), that grows at the fringes of shola forest or slopes above 1500 m altitude (Meher-Homji 1975). It occurs only in the two biodiversity hotspots in India viz. Western Ghats (WG) also known as the Malabar rainforest and the Northeastern Himalayas (NE). *G. fragrantissima* forms a group of plant species along with *Rhododendron arboreum*, *Eurya nitida*, *Symplocos laurina*, *Mahonia leschnaultii*, etc. that are common to both WG and NE regions and belongs to the shrub savanna or shola forest (Meher- Homji, 1972). Various theories as detailed in Chapter 1 (Review of literature) have been put forth by different researchers to explain the geographical distribution and migration of plant species between the NE and WG mountain regions. To verify these theories on migration, it is necessary to study the plant species common to both the regions at genetic level.

Availability of a variety of DNA markers during the last few decades has enabled researchers to use them in population genetic studies of forest plants. Most of such studies have been carried out using allozymes [in *Bromus tectorum* (Poaceae), Bartlett *et al* (2002); in *Calystegia collina* (Convolvulaceae), Wolf *et al* (2000); in *Pueraria lobata* (Fabaceae), Pappert *et al* (2000)] and randomly amplified polymorphic DNA (RAPD) markers [in *Senecio vulgaris* (Asteraceae), Muller and Fischer (2001); in *Digitalis minor* (Scrophulariaceae), Sales *et al* (2001); in *Plathymenia reticulata* (Mimosoideae), Lacerda *et al* (2001)]. Inter simple sequence repeat (ISSR) markers is one more class of random DNA markers exhibiting great potential in such studies of natural populations. These markers amplify the unique sequence between two inversely oriented, closely spaced simple sequence repeats (SSRs) of the same type, at several loci simultaneously yielding a multilocus marker system useful for diversity analysis (Zeitkiewicz *et al*, 1994). They rely on the ubiquity of SSRs in eukaryotic genome (Langercrantz *et al*, 1993) and thus can be targeted towards particular sequences which are reported to be abundant in that genome and can overcome the technical difficulties of RFLP and RAPD. Additionally, no prior sequence information for primer synthesis is required. Their utility in analysis of a number of cultivated species has already been demonstrated and exploited. For example, in rice, citrus and oil seed rape by Blair *et al* (1999), Fang and Rose (1997) and Charters *et al* (1996), respectively. Our laboratory has also shown earlier the utilily of these markers in the analysis of various crop plants like rice, wheat and chickpea. For example

Joshi *et al* (2000) studied genetic diversity and phylogenetic relationship in genus Oryza, Dholakia *et al* (2001) analyzed the influence of varied agroclimatic conditions on an important agronomic character like grain protein content in wheat and Rajesh *et al* (2001) used them to study genetic relationship among annual and perennial species of *Cicer*. These markers can be used in population genetic studies as they can detect very low levels of genetic variation (Zietkiewcz *et al*, 1994). According to Wolfe *et al*, (1998), they might have the potential for analyzing biogeographic patterns among populations of a single plant species. They have been used to study genetic diversity in natural populations of *Primula obconica* (Nan *et al*, 2003), *Botrychium pumicola* (Camacho and Liston, 2001) and to examine patterns of hybridization and hybrid speciation in *Penstermons* (Wolfe *et al*, 1998). In our laboratory, we have demonstrated use of these markers in studying genetic diversity in natural populations of *Eurya nitida* (Bahulikar *et al*, 2004) and genetic variations in three montane plant species that are common to the two hotspots, namely WG and NE region (Deshpande *et al*, 2001). In the present work I have analyzed 66 plants of *G. fragrantissima* from WG and NE region using the ISSR markers. Such efforts are expected to give an insight in assessing different theories on migration and evolution and are also useful in designing conservation strategies.

2.3 Materials and methods

2.3.1 Study sites and sampling

G. fragrantissima has disjunct distribution in the areas understudy and the frequency of its occurrence varies from 8- 30% per km^2 (BSI record). Depending on its density more than 50- 90% of the available plants were collected from each population. The details of sampling are given in Table 2.1. A total of 66 plants from seven populations representing the WG and NE region were collected. Number of plants per population ranged from 5 to 16. Among population distance was not less than 10km as suggested by Nybom and Bartish (2000). In fact, the minimum distance observed among populations from Naduvattum and Avalanche was about 14km aerial distance while the maximum distance was 2325km aerial distance in the populations from Munnar and Shillong peak road. All the seven populations were collected from over an altitude of 1400m (Table 2.1).

Table 2.1: Details of sampling of populations of *G. fragrantissima*

Region	Area of collection	No. of plants	Latitude Longitude	Altitude (m)
WG	Naduvattum	5	11°28'60N 76°52'60E	1952
	Avalanche	16	11°22'0N 76°31'00E	2147
	Kotagiri road	10	11°25'60N 76°52'60E	1792
	Kodaikanal	10	10°13'0N 77°28'60E	1966
	Munnar	6	10°5'60N 77°04'00E	1603
NE	Cherapunji road	6	25°26'928N 91°49'101E	1485
	Shillong peak road	13	25°34'0N 91°52'60E	1525

Three populations were collected from the Nilgiri hills, namely Naduvattum, Avalanche and Kotagiri road whereas Kodaikanal and Munnar were represented by one population each. In the NE hotspot, Cherapunji road and Shillong peak road were the two populations representing Meghalaya state. The collection tours were planned and executed with the help of the Botanical Survey of India (BSI). BSI is a government organization with regional centers all over the country and is engaged in surveying and documenting the floral wealth in the country. Prior to collection, the herbarium records were checked for the identification of collection sites. During the actual sampling, it was noticed that some of the sites identified earlier were cleared of its vegetation for human settlements or other human activities. As a result even after extensive exploration only limited number of plants could be sampled.

2.3.2 DNA isolation

On collection, leaf tissue was packed in polythene bags along with wet cotton swabs to retain moisture and carried to the laboratory at room temperature and then stored at –80°C until further use. DNA was extracted using the Sarkosyl method as described by

Doyle & Doyle (1987), in which 1% β-mercaptoethanol was additionally used in extraction buffer. As a first step, leaf tissue was ground in liquid nitrogen to a fine powder, which was quickly transferred to a tube containing 7.5ml of ice–cold extraction buffer (0.35M sorbitol, 0.1M Tris-HCl, 5mM EDTA, pH 7.5) per gram of ground tissue. The tube was briefly shaken and 7.5ml nuclei lysis buffer (2M NaCl, 0.2M Tris-HCl, 50mM EDTA, 2% CTAB, pH 7.5) per gram was quickly added, followed by 3ml of 5% sarkosyl solution per gram. Sample sets were incubated in 65°C water bath for 20min and were allowed to cool. 18ml of chloroform: isoamyl alcohol (24:1) was added to each tube. The tubes were centrifuged at 5000*g* for 15min. Further, the aqueous layer was removed and given another chloroform:isoamyl alcohol treatment. The aqueous layer was transferred to a fresh tube and DNA was precipitated with double volume of chilled ethanol. The DNA pellet was dried, suspended in 500ml TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8.0). DNA was further purified by adding hexadecyl-trimethyl-ammonium bromide (CTAB) to a final concentration of 1% followed by chloroform treatment and precipitation. The DNA was quantified spectrophotometrically at 260/ 280 nm and visually by ethidium bromide staining on 0.8% agarose gel and by comparing them with commercial DNA samples with known concentrations (Sambrook *et al*, 1989).

2.3.3 Oligonucleotide primers, PCR amplification and electrophoresis

2.3.3a ISSR primers

A set of 100 ISSR primers was procured from University of British Columbia (UBC), Vancouver, Canada. These primers were 15- to 22-mer and most of them comprised di-, tri-, tetra- and pentanucleotide repeat motifs of which dinucleotide repeats were with 3' or 5' anchors. They were used for initial screening with a few individuals. Primers that generated good patterns with the representative sample group were further used with all the individuals.

2.3.3b Polymerase chain reaction (PCR) amplification and electrophoresis

Amplification of 20 ng DNA was performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM spermidine, 2% formamide, 0.1 mM dNTPs, 0.3 μM primer and 0.8 U *Taq* DNA polymerase (Ampli*Taq*, Perkin- Elmer, USA) in 20 μl reaction for 44 cycles, using Gene Amp PCR System 9700 from PE Applied Biosystems (Perkin- Elmer, USA). After initial denaturation at 94 °C for 5 min, each cycle comprised 1 min denaturation at 94°C, 45 s annealing at 50°C and 1 min extension at 72°C along with 7 min extension at 72°C at the end of 44 cycles. Amplification products were

electrophoresed on 2% agarose gel using 0.5X TAE (0.02M Tris acetate, 5mM EDTA, pH 8.0) buffer and visualized with ethidium bromide (0.5μg/ml) staining.

2.3.4 Statistical analysis

To avoid bias in parameter estimation, analysis was restricted to those ISSR loci that fulfilled the 3/N criterion (observed frequencies were less than 1-[3/N]) where N is the number of plants (Lynch and Milligan, 1994). The percentage of polymorphic bands (PPB) and the ISSR genotypes were calculated manually. Polymorphism information content (PIC) scores were calculated on the basis of the formula, PIC $=2\times p_i q_i$ (Botstein *et al*, 1980), where p_i is the frequency of the present allele and q_i is the frequency of the null allele. Other parameters that were computed included Nei's (1973 and 1977) measures of heterozygosity which includes: a) total heterozygosity, H_T, (calculated with Lynch and Milligan (1994) correction for biallelic dominant markers), $H_T = 2q_i(1-q_i)+Var(q_i)$ where q_i is the frequency of the null allele at i^{th} locus in a population; $Var(q_i) = (1-x)/ 4N$, where $x = q_i^2$ and N is the total number of plants, b) mean heterozygosity within a population (H_s), c) diversity among populations ($D_{ST} = H_T-H_S$) and d) coefficient of population differentiation ($G_{ST} = D_{ST} / H_T$). Gene flow (N_m) or the number of migrants entering a population in each generation was estimated indirectly using Wright (1931) formula, $Nm = (1-F_{ST}/4F_{ST})$, where F_{ST} is the standardized variance among populations and is considered equivalent to G_{ST} (Nei, 1977).

To illustrate relatedness among different individuals and among populations, the presence-absence matrix of ISSR bands was analyzed using cluster analyses based on the unweighted pair group method with arithmetic mean (UPGMA). Dendrogram was constructed with the help of the commercial software package WINBOOT (Yap and Nelson, 1996) using Jaccard coefficient as this coefficient does not incorporate shared band absence. Similarity index within population was calculated using WINDIST programme (Yap and Nelson, 1996).

AMOVA (Analyses of Molecular Variance) (Excoffier *et al*, 1992) was used to partition the variance between main geographic regions, among populations and among individuals within populations using program WIN AMOVA 1.55 provided by Excoffier *et al* (1992) (www.anthropologie.unige.ch/ftp/ comp).

Distance matrix was employed for PCO (Principal Co-ordinate) analysis, which was performed with the help of NTSYS-PC programme version 1.8 (Rohlf, 1989). The

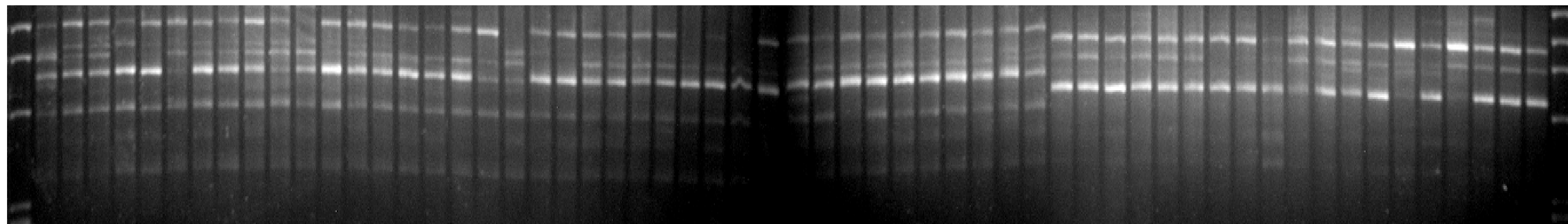
Mantel's test (Mantel, 1967) was applied for correlations between the matrix of genetic diversity and spatial distance.

2.4 Results

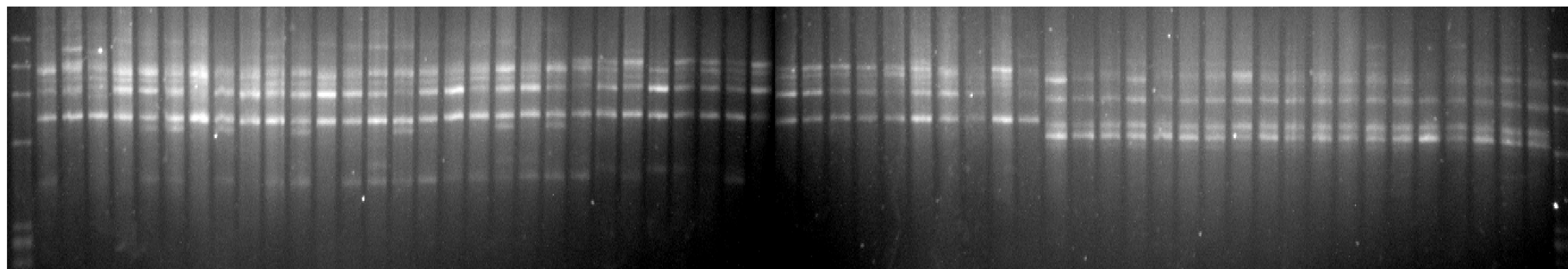
2.4.1: Information potential of different ISSR primers in diversity analysis in *G. fragrantissima*

A set of 100 ISSR primers (UBC, Canada) was initially used to analyze DNA samples of *G. fragrantissima*. From these, 17 ISSR primers including various dinucleotide motifs with single or double nucleotide anchors on 3' end that were polymorphic and gave good banding pattern were selected for the complete set of plants. Three repetitions were carried out with each primer. As seen from Table 2.1, 47 plants of *G. fragrantissima* represented WG and 19 represented the NE Himalayas. Figure 1A, 1B, 1C and 1D show the representative amplification profile of various individuals of *G. fragrantissima* using ISSR primers $(CT)_8RC$, $(AC)_8YT$, $(TC)_8G$ and $(AG)_8YT$. The selected 17 ISSR primers amplified 129 loci in total 66 individuals of *G. fragrantissima* of which 112 loci were polymorphic (86.82%) and 17 loci representing 13.17% were common to all the plants under study (Table 2.2). Each primer amplified 3 to 12 bands with a mean of 7.5 in the size range of 200 bp to 1500 bp, while 43 unique bands (22 from WG and 21 from NE) were obtained. $(AG)_8YA$ repeat amplified maximum number of polymorphic bands (12) and a minimum of 2 were amplified by $(TC)_8RT$ (Table 2.2). The polymorphism information content (PIC) score for all the 17 primers was calculated which ranged from 0.41- 0.49, with an average of 0.47 (Table 2.2). A total of 65 ISSR genotypes were identified from 66 individual plants where only two plants from Shillong peak road shared a genotype.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

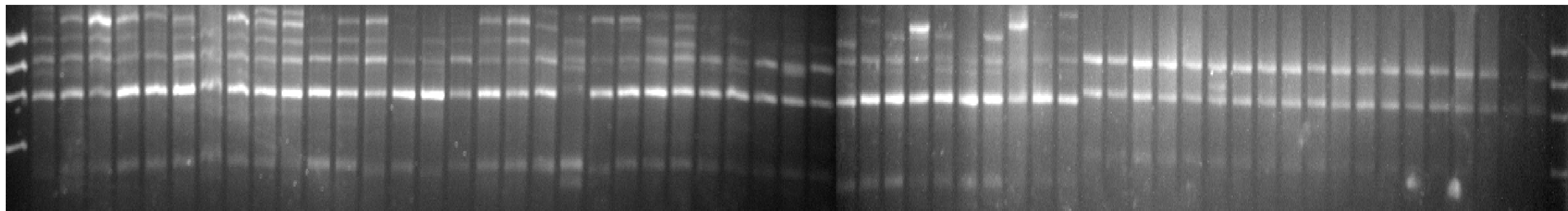


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60



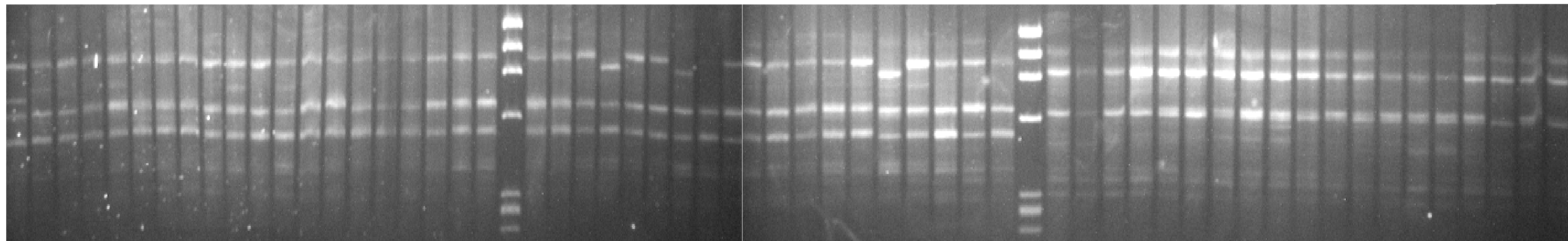
(B) UBC 855 $(AC)_8$YT

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60



(C) UBC 824 $(TC)_8G$

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60



(D) UBC 834 $(AG)_8YT$

Fig 2.1: Amplification profile of *Gaultharia fragrantissima* using ISSR markers

Lanes 1 and 60 represent molecular weight marker (ØX174 *HaeIII*), lanes 2- 22 Nilgiri hills, Lanes 23- 32 Kodaikanal, Lanes 33- 38 Munnar, Lanes 39- 40 Nilgiri hills (repeated randomly) and 41-59 Meghalaya. For Fig 2.1D alone lane no. 21 and 41 represent molecular weight marker while the order of loading other samples is same as in others.

Table 2.2: Genetic diversity revealed by various repeat motifs in *G. fragrantissima*

Repeat	**Anchor**	**PIC scores**	**Monomorphic loci**	**Polymorphic loci**	**Percentage polymorphism**
$(CT)_8$	A	0.49	2	6	75.0
$(CT)_8$	G	0.47	0	6	100.0
$(CT)_8$	RC	0.49	1	10	90.9
$(CT)_8$	RG	0.49	1	7	87.5
$(CT)_8$	T	0.48	0	8	100.0
Mean					**90.68**
$(TC)_8$	G	0.49	1	6	85.7
$(TC)_8$	RA	0.48	1	6	85.7
$(TC)_8$	RG	0.49	0	3	100.0
$(TC)_8$	RT	0.41	2	2	50.0
Mean					**80.35**
$(AG)_8$	YT	0.48	0	8	100.0
$(AG)_8$	YC	0.47	2	8	80.0
$(AG)_8$	YA	0.44	0	12	100.0
Mean					**93.33**
$(AC)_8$	YT	0.49	2	5	71.4
$(AC)_8$	YA	0.48	1	7	87.5
Mean					**79.45**
$(GA)_8$	C	0.44	3	6	66.6
$(GA)_8$	T	0.49	0	9	100.0
Mean					**83.3**
$(CA)_8$	G	0.47	1	3	**75.0**
Total			17	112	**86.82**

R- A/G

Y- C/T

2.4.2 Genetic differentiation in *G. fragrantissima* populations and gene flow

Similarity index within population ranged from a maximum of 0.990 (G f 9A1 and G f 9A3) to a minimum of 0.33 (G f 4J11 and G f 9B6; G f 9B1; G f 9A5) (Table 2.3).The diversity within populations of *G. fragrantissima* was assessed using various parameters (Table 2.4). The percentage of polymorphic bands (PPB) for each population ranged between 6.98- 37.98. Total heterozygosity (H_T) was 0.505 while average heterozygosity (H_S) was in the range of 0.147-0.380. The other parameters like average gene diversity within subpopulations (D_{ST}), proportion of genetic diversity (G_{ST}) and gene flow (Nm) were 0.212, 0.419 and 0.346, respectively. Gene flow was also calculated considering two populations at a time (Table 2.5). It ranged between 0.339 to 0.692 in the five populations from WG and it was 0.143 between the two populations of NE region. The average gene flow was 0.472 when the aerial distance in km ranged from 14 to 172 whereas it was 0.272 when the aerial distance in km increased from 173 upto 2325.

Table 2.3: Similarity index within different populations *G.fragrantissima*

Population	Maximum	Minimum
Naduvattum	0.952 (Gf4H4and Gf4C7)	0.882(Gf4C6; Gf4G8 and Gf4A1)
Avalanche	0.947 (Gf4J27 and Gf4J25)	0.794 (Gf4J30 and Gf4J11)
Kotagiri road	0.970 (Gf4M14 and Gf4M13)	0.873 (Gf4M5 and Gf4M15)
Kodaikanal	0.966 (Gf7E18 and Gf7E17)	0.83 (Gf7E21and Gf7E13)
Munnar	0.927 (Gf12A7 and Gf 12A3)	0.793 (Gf12A3 and Gf12A1)
Cherapunji road	0.990 (Gf9A1 and Gf9A3)	0.930 (Gf9A2 and Gf9A6)
Shillong peak	0.989 (Gf9B9and Gf9B11)	0.877(Gf9B7 and Gf9B1)
WG- NE	0.458 (Gf4M11 and Gf9B5)	0.33 (Gf4J11 and Gf9B6;Gf9B1; Gf9A5)

Gf : *Gaultheria fragrantissima*, the number after Gf indicates the number given to the location, namely 4 : Nilgiri hills (Naduvattam, Avalanche and Kotagiri road); 7 : Kodaikanal; 9 : Shillong (Cherapunji and Shillong peak road) and 12 :Munnar, the alphabet after the number indicates the name of the respective site followed by the number of the plant.

Table 2.4: Various parameters of diversity within populations of *G. fragrantissima*

	Naduvattum	Avalanche	Kotagiri road	Kodaikanal	Munnar	Cherapunji road	Shillong peak road	Total
PP	15.50	37.98	24.03	27.91	29.46	6.98	11.63	86.82
H_T	-	-	-	-	-	-	-	0.505
H_S	0.277	0.362	0.305	0.380	0.359	0.147	0.222	-
D_{ST}	-	-	-	-	-	-	-	0.212
G_{ST}	-	-	-	-	-	-	-	0.419
N_m	-	-	-	-	-	-	-	0.346

PPB (percentage of polymorphic bands), H_T (Total heterozygosity),H_S (Average heterozygosity), D_{ST} (Average gene diversity), G_{ST} (Proportion of genetic diversity), Nm (Gene flow) were calculated as given in Materials and Methods

Table 2.5: Gene flow calculated among populations considering pairwise combination

Naduvattam	-	-	-	-	-	-	-
Avalanche	0.430	-	-	-	-	-	-
Kotagiri road	0.339	0.486	-	-	-	-	-
Kodaikanal	0.440	0.692	0.526	-	-	-	-
Munnar	0.425	0.623	0.479	0.681	-	-	-
Cherapunji road	0.180	0.253	0.202	0.272	0.250	-	-
Shillong peak road	0.244	0.342	0.272	0.368	0.338	0.143	-
	Naduvattum	Avalanche	Kotagiri road	Kodaikanal	Munnar	Cherapunji road	Shillong peak road

2.4.3 AMOVA and cluster analysis

The details of AMOVA have been depicted in Table 2.6. A substantial variation of the order of 76.13% was found between the 2 main distant regions (WG and NE) by AMOVA. It revealed 15.56 % variance within the individuals when all the 66

individuals were considered, which was higher than 8.31 % variance among the seven populations. Cluster analysis was performed using UPGMA algorithms as well as PCO approach. Dendrogram drawn using the software WINBOOT (Yap and Nelson, 1996), showed 2 distinct groups representing NE and WG populations separately with a similarity of 0.33 between them (Table 2.3). The values at the fork are the bootstrap values which indicate the robustness of the groups. Within WG the three populations from Nilgiri hills (Naduvattum, Avalanche and Kotagiri road) formed one group and those from Kodaikanal and Munnar formed the second group though individuals from Kodaikanal formed a closer group than the Munnar group. There were only a few exceptions where Gf12A1 and Gf12A3 from Munnar was placed in Kodaikanal group and Gf4J30 from Avalanche grouped more closely with Kodaikanal and Munnar group (Fig 2.2). PCO analysis revealed the same distribution pattern of the population shown by the dendrogram and confirmed the groups (Fig 2.3). The Mantel's correlogram calculated for genetic distance and geographic distance for *G. fragrantissima* revealed a strong positive correlation between them (r = 0.938, P<0.001) when all the individuals were considered together while within WG it was r = 0.6, P <0.001 and within NE it was r = 0.4, P <0.001.

Table 2.6: Analysis of molecular variance (AMOVA) for the 66 individuals sampled from 7 populations of *G. fragrantissima*

Source of variation	**d.f**	**SSD**	**MSD**	**VC**	**%total variance**	**P-value**
Among regions (WG and NE)	1	810.24	810.24	28.47	76.13	< 0.001
Among populations	5	166.60	33.32	3.11	8.31	< 0.001
Within population	59	343.38	5.82	5.82	15.56	< 0.001

d.f. (degree of freedom), SSD (sum of squared deviation), MSD (mean squared deviation), VC (variance component), % Total variance (percentage of total variance contributed by each component), P- value (probability of obtaining a more extreme component estimate by chance alone) as calculated using program WIN AMOVA 1.55 provided by Excoffier *et al* (1992) (http://anthropologie.unige.ch/ftp/ comp).

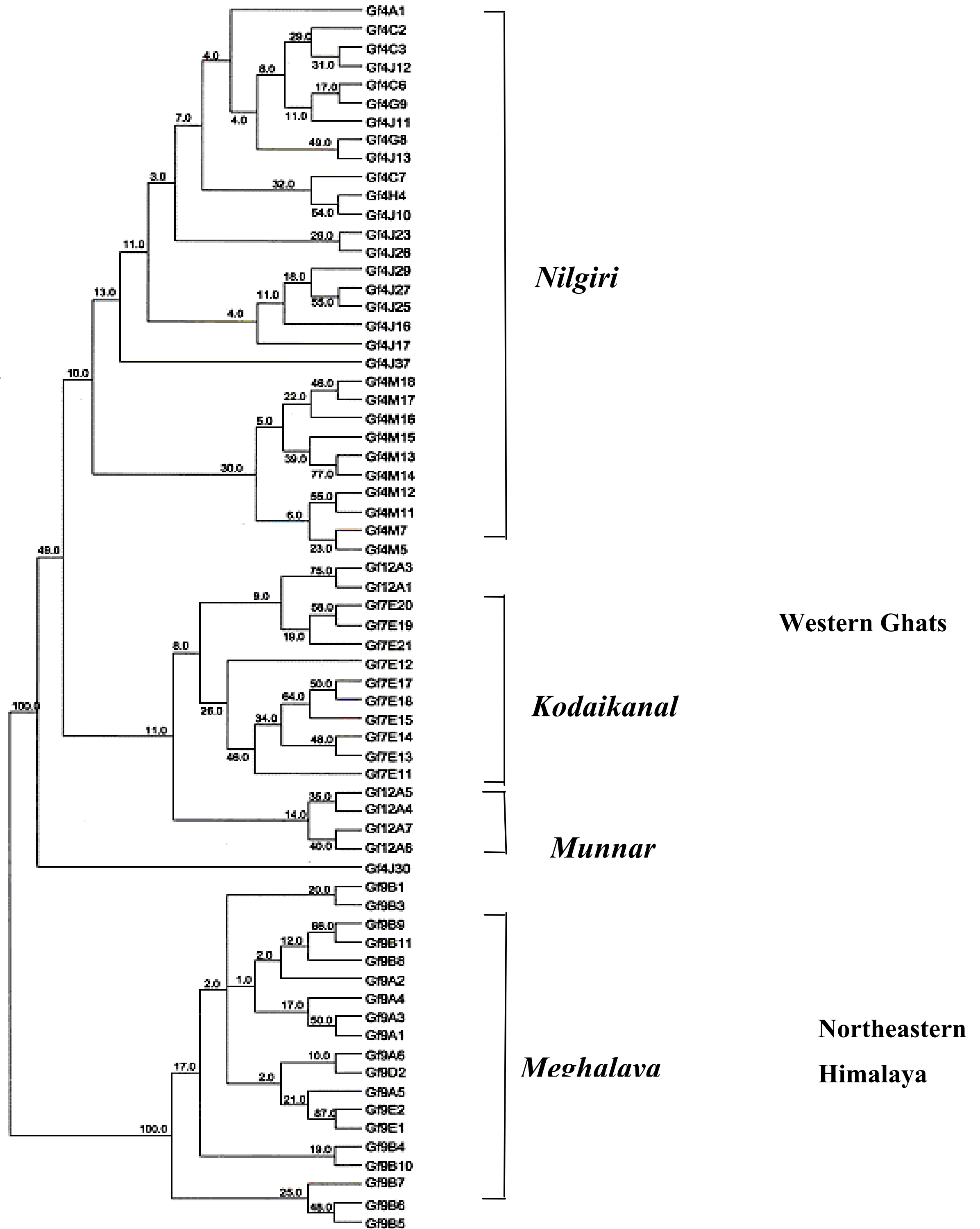


Figure 2.2: UPGMA phenogram of *G. fragrantissima* population from WG and NE regions in India

Gf : *Gaultheria fragrantissima*, the number after Gf indicates the number given to the location, namely 4: Nilgiri hills(Naduvattum, Avalanche, Kotagiri road); 7 : Kodaikanal; 12 : Munnar: (populations from Western Ghats) and 9: Meghalaya (Shillong peak, Cherapunji road): (populations from Northeastern Himalaya), the alphabet after the number indicates the name of the site in the respective location followed by the number of the plant.

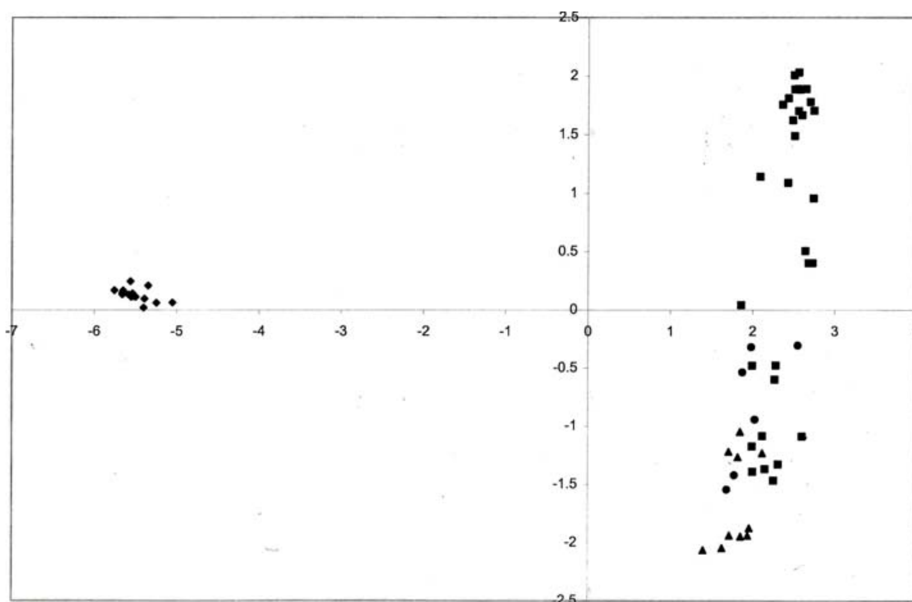


Fig 2.3 Representation of PCO analysis for populations of *G. fragrantissima*

■- Nilgiri hills, ▲– Kodaikanal, •- Munnar, ♦- Meghalaya

2.5 Discussion

2.5.1 Genetic diversity, clustering and gene flow in populations of *G. fragrantissima*

The 17 ISSR primers used in the present study gave an average PIC score of 0.47 which is higher as compared to the previously reported PIC scores for dominant markers where an average value of 0.32 and 0.31 for RAPD markers was reported by Li and Nelson (2001) and Rana and Bhat (2004), respectively. According to Li and Nelson (2001) ISSR markers being dominant in nature the highest PIC score of 0.5 can be obtained. Thus the markers used in the present study are useful for surveying the genetic diversity in *G. fragrantissima* populations as higher is the PIC score of a marker, higher is the probability that it will obtain polymorphism (Rana and Bhat, 2004). Furthermore, the maximum number of genotypes obtained within each population was equal to the number of individuals in the same population. Thus, the genotypic diversity (number of genotypes/ number of individuals) (Wolfe and Randle, 2001) for each population was 1.0, except for Shillong peak road population where it was 0.92 as two individuals shared a genotype. The total heterozygosity was observed to be 0.505 in *G.*

fragrantissima which was higher as compared to those reported earlier (0.238 in *S. laurina* , Deshpande *et al*, 2001 and 0.290 in *Pueraria lobata* , Pappert *et al*, 2000).This suggests that there is high genetic variation within *G. fragrantissima* though the plants from WG and NE regions do not show any distinct morphological variation (personal observation). However, in case of individual populations, the average heterozygosity (H_S) values for NE region populations were low as compared to H_S of WG (Table 2.4). According to Frankel *et al* (1995), higher the heterozygosity better is the fitness of populations, whereas lower the heterozygosity less is the population viability. Thus the populations from NE region are more homogenous as compared to the populations from WG.

This was also revealed by cluster analysis (Fig 2.2). The dendrogram clustered the individuals of *G. fragrantissima* in distinct groups according to their geographical regions. Two major clusters were formed, each grouping individuals from WG and NE regions, respectively. Plants from WG showed genetic similarity in the range of 0.794 to 0.970 while those from NE showed a narrow range between 0.877-0.990 (Table 2.3). The narrow range of similarity index values seen in NE as compared to WG was in accordance with the low average heterozygosity values (Table 2.4) in NE plants. Furthermore, plants from WG grouped according to their specific geographic location with only a few exceptions as detailed in the results. The PCO analysis (Fig 2.3) showed similar clustering as that in the dendrogram supporting the grouping of individuals. Significant positive correlations between genetic and geographic distances using Mantel's Test ($r = 0.938$, $P < 0.001$) also supported the dendrogram and PCO analysis.

The G_{ST} value (Table 2.4) of *G. fragrantissima* was higher as compared with studies by Loveless and Hamrick (1984), Hamrick and Godt (1989) and Heywood (1991). High G_{ST} value indicates differentiation among the populations of *G. fragrantissima*. This was supported by the low gene flow ($Nm = 0.346$) among the populations indicating effective migration of only one migrant in more than two generations. Theoretically, gene flow of more than four migrants per generation ($Nm > 4$) among populations is sufficient to prevent genetic drift causing local genetic differentiation and therefore, population divergence. Thus the low gene flow might lead to genetic drift in *G. fragrantissima.* Furthermore, the gene flow calculated by considering two populations at a time (Table 2.5) revealed higher gene flow with less aerial distance and lower gene flow with high aerial distance. The only exception was

for the two populations from NE region where the gene flow was 0.143 although the aerial distance in km was 51. This might suggest that the mechanism of seed dispersal and pollination is better in WG as compared to that in NE region. Thus the WG populations have a better chance to avoid local adaptations than NE populations because of better gene flow in WG.

AMOVA revealed high variation among individuals within populations (15.56 %, $P < 0.001$) whereas the variation among populations within the two regions was 8.31 %, $P < 0.001$ (Table 2.6). According to Hamrick and Godt (1989), outcrossing species tend to have 10-20% of the genetic variation among populations while selfing species present on an average 50% of this variation among populations. Here, among population variance was low as compared to that with other reported values (13% in *Grevillea scapigera,* Rossetto *et al*, 1995 and 12% in *Cordia alliodora* a highly outcrossing species, Chase *et al*, 1995).

The present study has revealed the status of genetic diversity present in *G. fragrantissima* especially from the two hotspots in India which was previously not studied. Low gene flow, high among population differentiation and clustering of individuals according to their geographic location indicate the need to plan aggressive conservation strategies which should aim at preservation of genotypes from maximum number of populations from the two hotspots covering the entire genetic diversity available in *G. fragrantissima*.

Chapter 3

Chloroplast and mitochondrial DNA diversity in *G. fragrantissima* from the two hotspots in India

Part of the contents of this chapter has been submitted as a full length paper to Annals of Botany

Chloroplast and mitochondrial DNA diversity in *G.fragrantissima* from the two hotspots in India

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3.1 Abstract

Universal primers reported from chloroplast (cp) and mitochondrial (mt) DNA for intergenic amplification followed by restriction enzyme digestion is a marker system being used to study diversity in natural populations. The cp and mtDNA diversity in *G. fragrantissima* in the present study was also analyzed using PCR- RFLP technique. Nine cpDNA and seven mtDNA haplotypes were detected in eight populations. None of the cpDNA haplotypes was shared by all the eight populations. Unlike cpDNA, one mtDNA haplotype was shared by all the populations and was the most common haplotype with a frequency of 0.618. Other three mtDNA haplotypes were common to both the hotspots while three were unique to WG and one to NE region. CpDNA diversity (h_T= 0.639) was higher as compared to mtDNA diversity (h_T= 0.582) among the populations. However, within population diversity was higher in mtDNA (h_S= 0.467) than in cpDNA (h_S= 0.224). The level of population subdivision for unordered and ordered alleles was much higher in cpDNA (G_{ST}= 0.615; N_{ST}= 0.866) as compared to mtDNA (G_{ST}= 0.197; N_{ST}= 0.252). Also higher value of N_{ST} than G_{ST} in cpDNA was observed which suggests phylogeographical organisation of the populations.

3.2 Introduction

Chloroplast (cp) and mitochondrial (mt) DNA are nonrecombinant (no recombination occurs and is essentially inherited as a single gene.), homoplasmic (copies of their DNA within an individual are identical) and highly conserved molecules (Ouborg *et al*, 1999). However, mtDNA shows intramolecular recombinations occasionally, due to the long hairpin like secondary structure (Dumolin- Lapegue, 1998). In angiosperms both chloroplast (cp) and mitochondrial (mt) DNA show maternal inheritance in most species (Rebound and Zeyl, 1994). As a consequence, the two organellar genomes are expected to remain associated, that is, they should behave as if they are completely linked (Schnabel and Asmussen, 1989) and to give similar information on dispersal and gene flow by seeds. However, there are reports where cpDNA is biparentally inherited or shows 'paternal leakage' in plants (Rebound and Zeyl 1994). There are a few reports where some mitochondria from the male parent seem to reach the egg (Chapmann, 1986) and therefore show paternal leakage. Petit *et al* (1993) have shown that paternal transmission rate as low as 1% in cytoplasmic genomes can have a significant effect on their genetic structures, especially when there is an asymmetry between pollen and seed flow. According to Birky (1978) if there are very low levels of paternal gene transmission and recombination, these must be measured, for they may become important over evolutionary time scale even though they are negligible when one looks at the results of a single mating. Furthermore, chloroplast DNA evolves four times slowly than nuclear genome and mitochondrial DNA evolves three times slowly than cpDNA (Wolfe *et al*, 1987).

Availability of universal intergenic cp and mtDNA markers (Taberlet *et al* 1991, Demesure *et al* 1995 and Dumolin- Lapague *et al* 1997) and use of the PCR-RFLP (or CAPS- Cleaved Amplified Polymorphic Sequence) technique have helped researchers to study the intraspecific variation in various plant species. It is a sensitive technique where, a noncoding region between two genes in cp/ mt DNA is amplified with available universal primers followed by restriction digestion of the resultant amplicon. King and Ferris (1998) used this technique to study cpDNA pylogeography of *Alnus glutinosa*. Fineschi *et al* (2000) studied cpDNA polymorphism in *Castanea sativa* and revealed that there was little geographical structure throughout European countries. Mohanty *et al* (2003) studied cp and mtDNA haplotypes in *Prunus spinosa* and showed association

between the two genomes. Bakker *et al* (2000) studied phylogenies in *Pelargonium* using cp and mtDNA, in an attempt to test for linkage of organellar genome inheritance.

In the present work, I have exploited cytoplasmic DNA markers (cpDNA and mtDNA) using PCR- RFLP technique to study the genetic variation within and among populations of *G. fragrantissima* from two hotspot regions in India. This has further extrapolated to explain presence of *G. fragrantissima* in WG and NE regions and to verify hypotheses of its migration from NE to WG. This is the first report about study of intraspecific cp and mtDNA variation in *G. fragrantissima* from India.

3.3 Materials and methods

3.3.1 Plant material

The present study included all the seven populations analysed in Chapter 1 in addition to one population from Elephanta falls and six more plants in Avalanche population and one less from Kotagiri population (Table 3.1).

Table 3.1 Details of sampling of *G. fragrantissima*

Region	Population	Latitude and Longitude	Number of plants
WG	Naduvattum	11°28'60N and 76°34'00E	5
	Avalanche	11°22'00N and 76°31'00E	22
	Kotagiri road	11°25'60N and 76°52'60E	9
	Kodaikanal	10°13'00N and 77°28'60E	10
	Munnar	10°05'60N and 77°04'00E	6
NE	Cherapunji road	25°26'92N and 91°49'10E	6
	Shillong peak road	25°34'00N and 91°52'60E	13
	Elephanta falls	25°32'240N and 91°49'338E	3
Total			74

3.3.2 DNA isolation, PCR amplification and restriction digestion

Isolation of DNA was carried out using the method as given in the previous chapter (Chapter 2). Twenty pairs of universal primers representing various intergenic regions in cp and mtDNA as described by Demesure *et al* (1995), Dumolin- Lapague *et al* (1997) and Tzen- Yuh Chiang *et al* (1998) were used for initial screening to check the

degree of amplification and size of the amplified fragment (Table 3.2). Amplification of 40 ng DNA was performed in 25 μl of reaction mixture consisting of 0.2 μM of each primer, 100 μM of dNTPs, 1.5 mM $MgCl_2$ and 1.0 U of DNA polymerase in the buffer provided by the manufacturer of the enzyme (Perkin- Elmer, USA). The PCR amplifications were carried out in Gene Amp PCR system 9700 (PE Applied Biosystems, USA Perkin- Elmer) with heated lid, using an initial denaturation at 94° C for 4 min, followed by 34 cycles of 1 min at 94°C, 45s at 55°C to 65°C (based on the GC composition of the primer pair) and 2min at 72°C with a final extension of 5min at 72°C.

Ten primer pairs which gave good amplification were selected for the present investigation (Table 3.2). Initially 10 individuals representing all the populations were used for screening with various restriction enzymes namely, *Alu I, EcoRI, Hae III, Hha I, Hind III, Hinf I, Hpa II, Mbo I, Msp I, Pst I, Pvu II, Rsa I, Sal I, Sau3AI,*and *Taq I* (Promega, USA). Those enzymes which gave amplicon digestion and showed polymorphism in atleast one individual were further used with all the samples (Table 3.3). 5μl of PCR product was digested overnight with 2.4 U of respective restriction enzyme in a 10μl reaction at 37°C (in case of *Taq I* at 65°C). The digests were resolved on 2.5 to 3.0 % metaphor agarose gels (Bio Whittaker, Molecular Applications, USA) along with ϕX *Hae III* molecular weight marker in 0.5X Tris Borate EDTA buffer, stained with ethidium bromide and visualised in uv light. The sizes of the digested products were analyzed using the software SEQAID II version 3.81 (Rhoads and Roufa 1991).

3.3.3 Data analysis

Various parameters of diversity (h_T - total diversity, h_S - average intrapopulation diversity, G_{ST} - level of population subdivision of diversity using unordered alleles and frequency of haplotypes) were calculated using the HAPLODIV software (Pons and Petit, 1995). HAPLONST software (Pons and Petit, 1996) was used to calculate N_{ST} -the level of population subdivision for ordered alleles and v_T and v_S (the analogues of h_T and h_S).

A matrix of frequencies of haplotypes in each population was used for generating a matrix of distances between populations, using the coefficient of Euclidean distances squared. This later matrix was used to construct a dendrogram by UPGMA algorithm (Everitt, 1986). The ratio of the pollen flow to seed flow was calculated using the formula: $[(1/G_{STb}- 1)-2(1/G_{STc}- 1)]/ (1/G_{STc}- 1)$, where G_{STb} is the level of population subdivision based on biparentally (nuclear) inherited genome (refer to Chapter 2) and

G_{STc} is the level of population subdivision for cytoplasmically inherited genome (cp/ mtDNA) using unordered alleles (King and Ferris, 1998).

3.4 Results

3.4.1 Screening of cpDNA and mtDNA markers for their utility

Twenty pairs of universal primers of cpDNA and mtDNA were initially screened to assess the degree of amplification and suitability of amplicons for restriction enzyme digestion (Table 3.2). Based on the quality of amplification, ten pairs of primers, five from cpDNA and five from mtDNA were selected for the present study. The amplified fragments were digested with various tetra- and hexa- cutting restriction enzymes as detailed in materials and methods and only those combinations which gave polymorphic restriction patterns were used for the analysis. Thus, finally for the complete study seven (four from cpDNA and three from mtDNA) polymorphic patterns representing five pairs of primers and eight populations from the 2 hotspots in India including 74 individuals were considered (Table 3.3). The size range of polymorphic fragments was between 88 bp to 1431bp. Figure 3.1 shows the size range of various amplicons amplified using the specific cp and mtDNA primer pairs, namely: trnC- trnD, psbC- trnS, trnS-trnT, nad1B-nad1C and nad4/1- nad4/2 and used for further restriction enzyme digestion in the present study. Figures 3.2, 3.3 and 3.4 show the restriction patterns in *G. fragrantissima* using the primer pairs trnS- trnT with *Hinf I* and nad1B- nad1C with *Taq I* and *Alu I*, respectively.

Table 3.2: Details of primers used for initial screening

Primer pair	Genome type	Degree of amplification	Polymorphic status
trnC- trnD **5'CCAGTTCAAATCTGGGTGTC3'** **3'GGGATTGTAGTTCAATTGGT5'**	chloroplast	++	polymorphic
psbC- trnS **5'GGTCGTGACCAAGAAACCAC3'** **3'GGTTCGAATCCCTCTCTCTC5'**	chloroplast	++	polymorphic
trnD- trnT **5'ACCAATTGAACTACAATCCC3'** **3'CTACCACTGAGTTAAAAGGG5'**	chloroplast	NA	-
trnS- trnfM **5'GAGAGAGAGGGATTCGAACC3'** **3'CATAACCTTGAGGTCACGGG5'**	chloroplast	++	monomorphic
psaA- trnS **5'ACTTCTGGTTCCGGCGAACGAA3'** **3'AACCACTCGGCCATCTCTCCTA5'**	chloroplast	More than one band	-
trnS- trnT **5'CGAGGGTTCGAATCCCTCTC3'** **3'AGAGCATCGCATTTGTAATG5'**	chloroplast	++	polymorphic
trnM- rbcL **5'TGCTTTCATACGGCGGGAGT3'** **3'GCTTTAGTCTCTGTTTGTGG5'**	chloroplast	+	-
trnQ- trnCr **5'GGGACGGAAGGATTCGAACC3'** **3'CGACACCCRGATTTGAACTGG5'**	chloroplast	More than one band	-
trnRr- rpoC1 **5'ATTGCGTCCAATAGGATTTGAA3'** **3'GCACAAATTCCRCTTTTTATRGG5'**	chloroplast	NA	-
atpB- rbcL **5'ACATCKARTACKGGACCAATAA3'** **3'AACACCAGCTTTRAATCCAA5'**	chloroplast	++	monomorphic
nad 1B- nad 1C **5'GCATTACGATCTGCAGCTCA3'** **3'GGAGCTCGATTAGTTTCTGC5'**	mitochondria	++	polymorphic

Contd.

Primer pair	Genome type	Degree of amplification	Polymorphic status
nad 4/1- nad 4/2 **5'CAGTGGGTTGGTCTGGTATG3'** **3'TCATATGGGCTACTGAGGAG5'**	mitochondria	++	polymorphic
nad 4/2- nad 4/4 **5'TGTTTCCCGAAGCGACACTT3'** **3'GGAACACTTTGGGGTGAACA5'**	mitochondria	More than one band	-
rps 14- cob **5'CACGGGTCGCCCTCGTTCCG3'** **3'GTGTGGAGGATATAGGTTGT5'**	mitochondria	More than one band	-
cox 2/1- cox 2/2r **5'TTTTCTTCCTCATTCTKATTT3'** **3'CCACTCTATTGTCCACTTCTA5'**	mitochondria	++	monomorphic
nad 1/4 - nad 1/5r **5'GCCAATATGATCTTAATGAG3'** **3'TCACCTTGATACTAAACCAG5'**	mitochondria	+	-
nad 4/2- nad 4/3r **5'CTCCTCAGTAGCCCATATGA3'** **3'AACCAGTCCATGACTTAACA5'**	mitochondria	++	monomorphic
nad 4/3- nad 4/4r **5'GGAGCTTTCCAAAGAAATAG3'** **3'GCCATGTTGCACTAAGTTAC5'**	mitochondria	NA	-
nad 7/1- nad 7/2r **5'ACCTCAACATCCTGCTGCTC3'** **3'CGATCAGAATAAGGTAAAGC5'**	mitochondria	++	monomorphic
nad 7/2- nad 7/3r **5'GCTTTACCTTATTCTGATCG3'** **3'TGTTCTTGGGCCATCATAGA5'**	mitochondria	NA	-

+ & ++ : degree of amplification (++ amplicons were clear and prominent and used for further digestion with restriction enzymes), NA : Not amplified and -: not used for digestion

Table 3.3: Details of primers, amplification conditions, size of amplified product and restriction enzymes used in the present study

Genome	Primer pair	Annealing temperature	Size (kb)	Restriction enzyme
cpDNA	trnC- trnD	63° C	3.8	*Sau3A I and EcoR I*
	psbC- trnS	64° C	1.6	*TaqI*
	trnS- trnT	65° C	1.4	*Hinf I*
mtDNA	nad 1B- nad 1C	55° C	1.5	*Alu I and Taq I*
	nad 4/1- nad 4/2	65° C	2.2	*EcoR I*

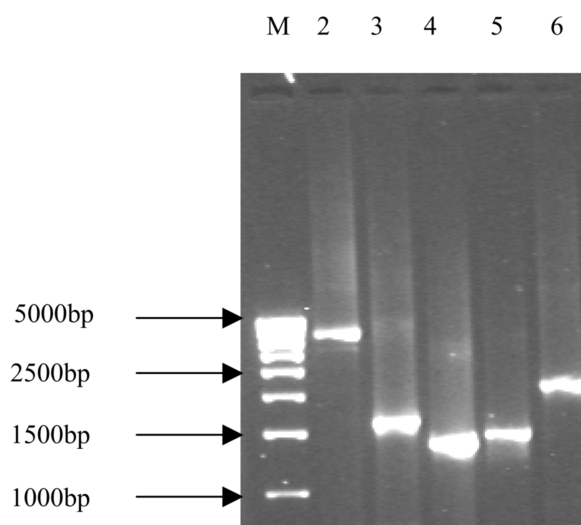

Fig 3.1: Size range of various amplicons

Lane 1(M): marker (500bp ladder, Biogenei, Bangalore), lane 2- 6: primer pairs used trnC- trnD (3.8kb), psbC- trnS (1.6kb), trnS-trnT (1.4kb), nad1B- nad1C (1.5kb) and nad4/1- nad4/2 (2.2kb), respectively.

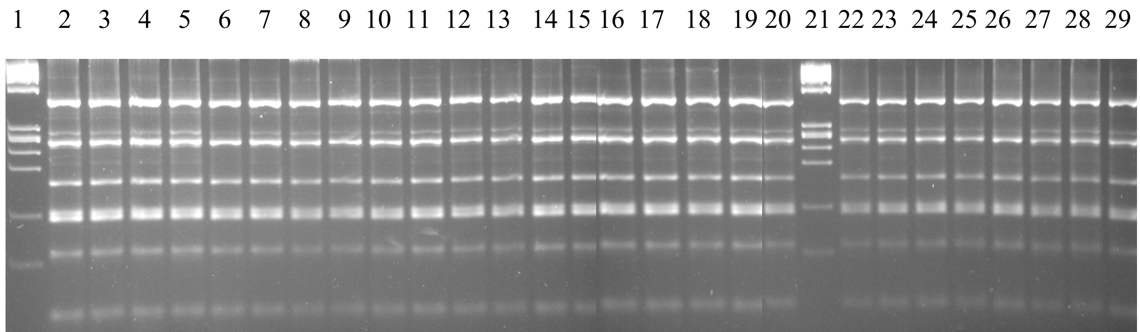

Fig3.2a

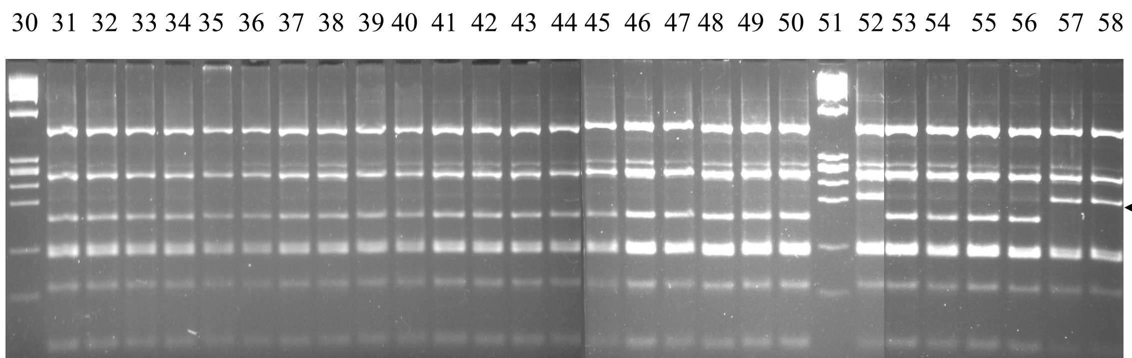

Fig 3.2b

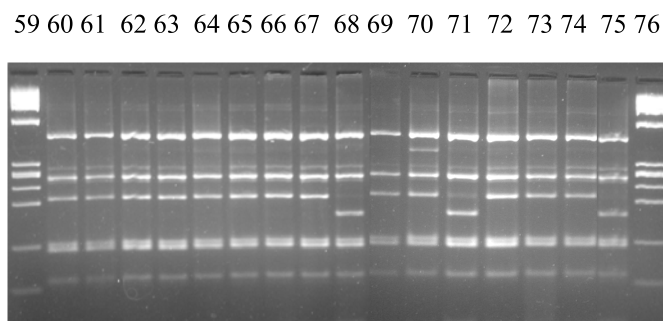

Fig 3.2 c

Fig 3.2: Representative restriction pattern in *G. fragrantissima* using primer pair trnS- trnT and *Hinf I restriction enzyme*. Arrow shows the polymorphic band.

Lanes 1, 21, 30, 51, 59 and 76 represent molecular marker (ØX174/ HaeIII). Further order of loading is as follows: Nilgiri hills (36 samples), Kodaikanal (10 samples), Munnar (6 samples), Megahalaya (16 samples) and lanes 20 and 75 loaded randomly with samples from Kodaikanal.

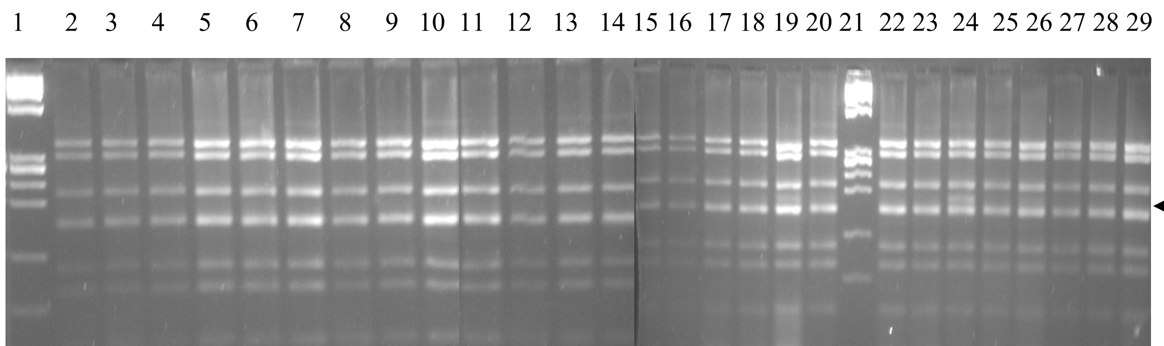

Fig 3.3a

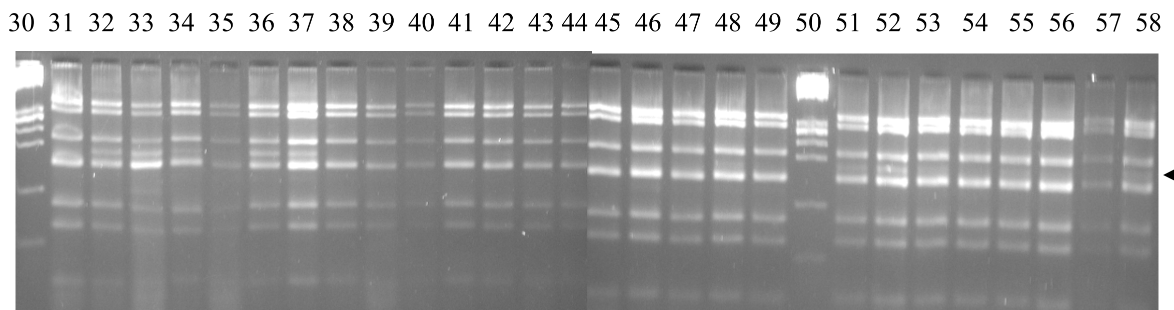

Fig 3.3b

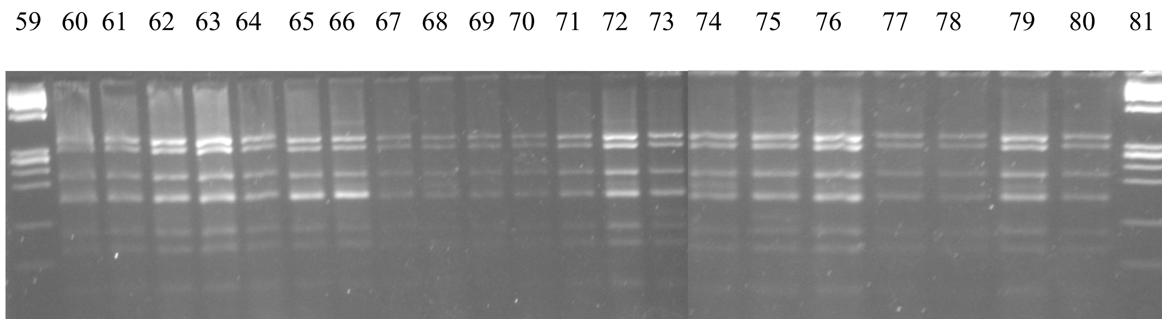

Fig 3.3c

Fig 3.3: Representative restriction pattern in *G. fragrantissima* using primer pair nad1B- nad1C and *Alu I restriction enzyme*. Arrow shows the polymorphic band.

Lanes 1, 21, 30, 51, 59 and 82 represent molecular marker (ØX174/ HaeIII). Further order of loading is as follows: Nilgiri hills (36 samples), Kodaikanal (10 samples), Munnar (6 samples), Megahalaya (21 samples) and lanes 20 and 82 loaded randomly with samples from Kodaikanal.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

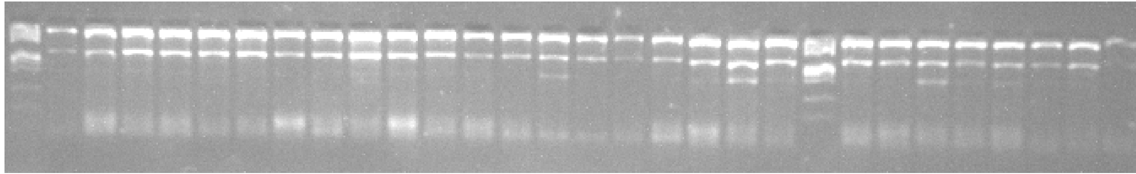

Fig 3.4a

31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

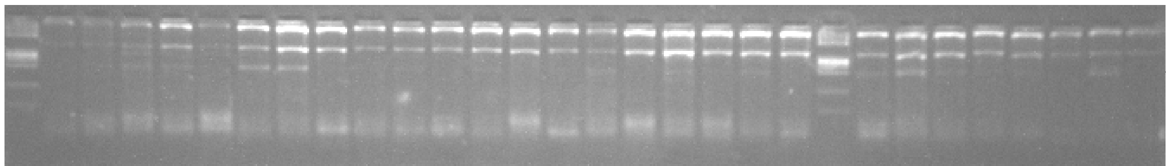

Fig 3.4b

61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84

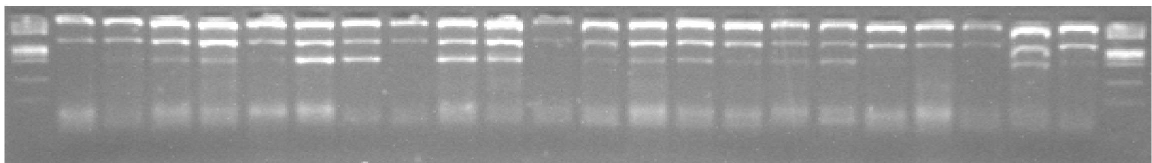

Fig 3.4c

Fig 3.4.: Representative restriction pattern in *G. fragrantissima* using primer pair nad1B- nad1C and *Taq I restriction enzyme*. Arrow shows the polymorphic band.

Lanes 1, 22, 31, 52, 60 and 84 represent molecular marker (ØX174/ HaeIII). Further order of loading is as follows: Nilgiri hills (36 samples), Kodaikanal (10 samples), Munnar (6 samples), Megahalaya (22 samples) and lanes 20, 21 and 82 and 83 loaded randomly with samples from Kodaikanal.

3.4.2 Haplotypes based on cpDNA analysis

Nine haplotypes (CH1 through CH9) based on cpDNA were represented by the eight populations of *G. fragrantissima* under present study. Among these, four populations (Avalanche, Munnar, Cherapunji road and Shillong peak road) revealed more than one haplotype, four populations (Naduvattum, Kotagiri road, Kodaikanal and Elephanta falls) depicted only one haplotype while four populations (Avalanche, Munnar, Cherapunji road and Shillong peak road) showed unique haplotypes within cpDNA (Table 3.4). No haplotype was shared by all the populations. Both the regions represented unique haplotypes with maximum of three unique haplotypes within one population of Shillong peak road from NE region. CH1 showed maximum frequency of 0.566 while CH5 showed minimum frequency of 0.009 (Table 3.4). Haplotype CH4 with a frequency of 0.285 was common to the NE region and CH1 was common to WG. CH2 was unique to Avalanche population while CH3 and CH9 were unique to Munnar population (both the populations represent WG). CH5, CH6 and CH7 were unique to Shillong peak road population and CH8 was unique to Cherapunji road population (both the populations represent NE) (Table 3.4). The genetic diversity parameters based on cpDNA profiles were determined using HAPLODIV and HAPLONST software programme (Pons and Petit 1995 and 1996). Total diversity (h_T) was observed to be 0.648, average diversity (h_S) was 0.249, and their analogues v_T and v_S were 0.668 and 0.089, respectively. The level of population subdivision of diversity using unordered alleles (G_{ST}) and using ordered alleles (N_{ST}) was 0.615 and 0.866, respectively (Table 3.6).

In case of cpDNA, the dendrogram drawn using UPGMA algorithm considering frequencies of individual haplotypes in each population is represented in Fig. 3.5. Interestingly it shows clustering according to their geographic locations, WG populations form one group while the NE populations form the other group. In WG cluster Naduvattum, Kotagiri road and Kodaikanal populations form a close cluster with no dissimilarity. Population from Avalanche joins this cluster at 0.19 dissimilarity while Munnar population joins this cluster at 0.41 dissimilarity. In NE region, the Cherapunji road and Elephanta falls populations join at 0.44 dissimilarity which in turn is joined by Shillong peak road population at 0.73 dissimilarity.

Table 3.4: cpDNA haplotype frequencies and composition of eight populations of *G. fragrantissima*

Populations → / Haplotype ↓	Naduvattam	Avalanche	Kotagiri road	Kodaikanal	Munnar	Cherapunji road	Shillong peak road	Elephanta falls	Total	Frequency
CH1	5	19	9	10	4	0	0	0	47	0.566
CH2	0	3	0	0	0	0	0	0	3	0.017
CH3	0	0	0	0	1	0	0	0	1	0.020
CH4	0	0	0	0	0	4	8	3	15	0.285
CH5	0	0	0	0	0	0	1	0	1	0.009
CH6	0	0	0	0	0	0	2	0	2	0.019
CH7	0	0	0	0	0	0	2	0	2	0.019
CH8	0	0	0	0	0	2	0	0	2	0.041
CH9	0	0	0	0	1	0	0	0	1	0.020
Total	5	22	9	10	6	6	13	3	74	

3.4.3 Haplotypes based on mtDNA analysis

Based on mtDNA profiles, seven haplotypes (MH1 through MH7) were observed in all the eight populations together. Six populations from Avalanche, Kotagiri road, Kodaikanal, Munnar, Cherapunji road and Shillong peak road had two or more than two haplotypes, two populations Naduvattum and Elephanta falls had only one haplotype while two populations from Avalanche and Kotagiri road showed unique haplotypes. As against cpDNA haplotypes, in case of mtDNA, unique haplotypes were distributed only in the populations from WG. MH1 showed maximum frequency of 0.618 and was the only haplotype shared by all the populations (Table 3.5). Minimum frequency (0.005) was shown by MH3 represented uniquely in only Avalanche population. MH2 with a frequency of 0.22 was common to five populations and MH4 to four populations from both the regions. MH5 and MH6 with frequencies of 0.027 and 0.013, respectively were unique to Kotagiri road population. MH7 was common to Cherapunji road and Shillong peak road populations from NE region (Table 3.5). Based on these patterns the diversity parameters were calculated. Total heterozygosity h_T was 0.582, average heterozygosity h_S was 0.467 and v_T and v_S were 0.586 and 0.438, respectively. The level of population subdivision of diversity using unordered alleles (G_{ST}) and using ordered alleles (N_{ST}) was 0.197 and 0.252, respectively (Table 3.6).

The dendrogram constructed by using mtDNA haplotype frequencies in each population is represented in Fig 3.6 which reveals some interesting observations. The Elephanta falls population from NE region groups at 0.21 dissimilarity with other two populations, namely, Naduvattum and Avalanche from WG. This cluster shows dissimilarity of 0.45 with populations from Kodaikanal and Munnar of WG, while Kotagiri road population from WG joins this group at 0.49 dissimilarity. Remaining two populations from NE, namely Cherapunji road and Shillong peak road form a cluster with 0.23 dissimilarity.

3.4.4 Ratio of pollen to seed flow

Based on the diversity studies carried out using nuclear DNA markers in case of *G. fragrantissima* in Chapter 2, G_{STb} was found to be 0.386. The genetic diversity studies using cpDNA and mtDNA, G_{STc} for cpDNA was 0.615, while G_{STc} for mtDNA was 0.197. These values were used to calculate the ratio of pollen/seed flow which indirectly gives information on the relative contribution of pollen and seed flow to the total gene flow (formula as per given in Data analysis). The ratio for cpDNA was 0.56 and for mtDNA was -1.60, respectively.

Table 3.5: mtDNA haplotype frequencies and composition of eight populations of *G. fragrantissima*

Populations → / Haplotype ↓	Naduvattam	Avalanche	Kotagiri road	Kodaikanal	Munnar	Cherapunji road	Shillong peak road	Elephanta falls	Total	Frequency
MH1	5	18	4	7	3	2	2	3	44	0.618
MH2	0	2	0	3	2	3	7	0	17	0.220
MH3	0	1	0	0	0	0	0	0	1	0.005
MH4	0	1	2	0	1	0	2	0	6	0.073
MH5	0	0	2	0	0	0	0	0	2	0.027
MH6	0	0	1	0	0	0	0	0	1	0.013
MH7	0	0	0	0	0	1	2	0	3	0.040
Total	5	22	9	10	6	6	13	3	74	

Table 3.6: Analysis of diversity using HAPLODIV and HAPLONST software

Genome	h_S	h_T	G_{ST}	v_S	v_T	N_{ST}
CpDNA	0.249 (0.102)	0.648 (0.116)	0.615 (0.120)	0.089 (0.05)	0.668 (0.098)	0.866 (0.078)
MtDNA	0.467 (0.115)	0.582 (0.105)	0.197 (0.087)	0.438 (0.134)	0.586 (0.134)	0.252 (0.068)

Values in parentheses indicate the standard error of the estimates

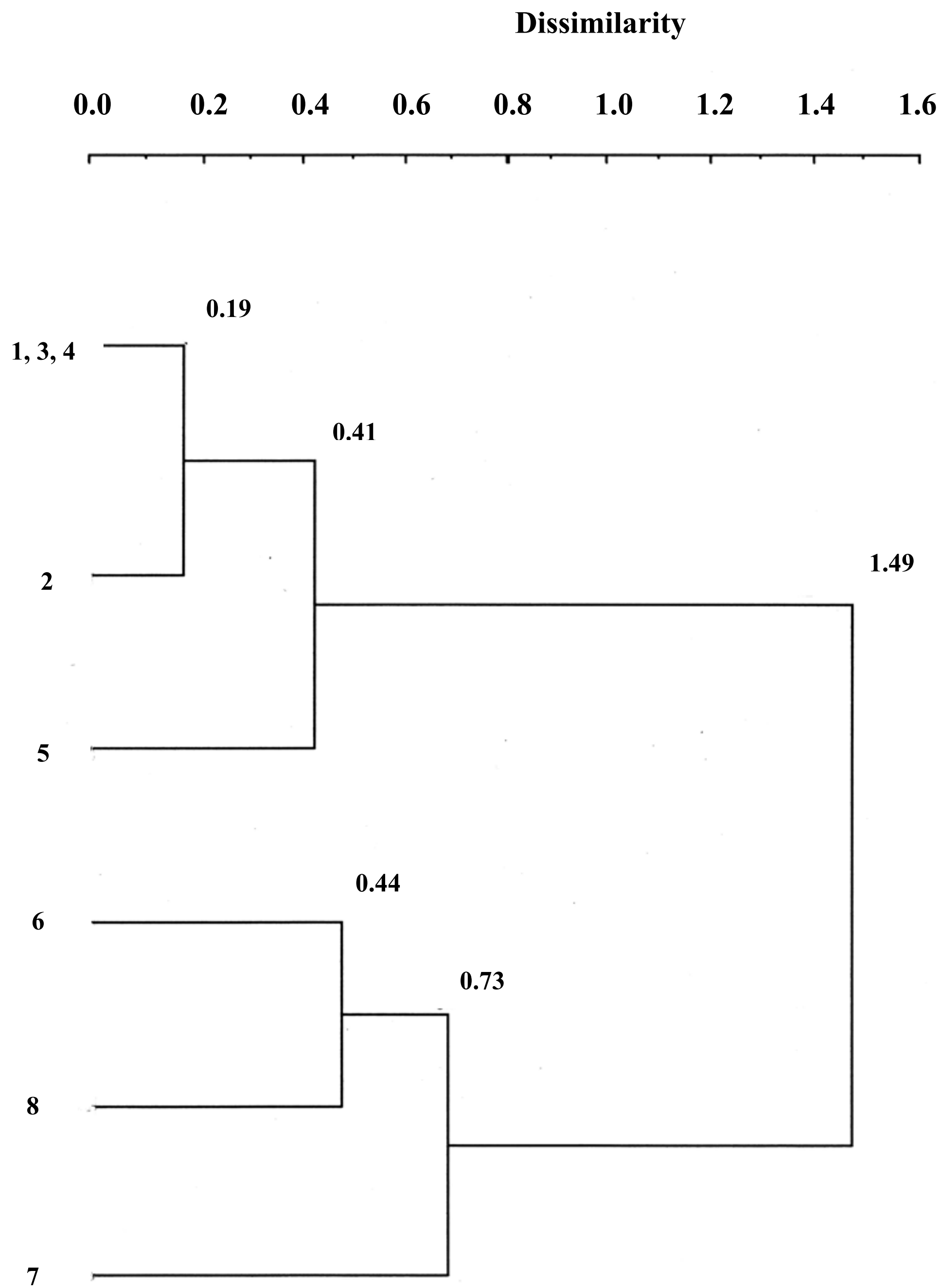

Fig 3.5: Dendrogram drawn based on frequencies of cpDNA haplotypes

1- Naduvattum, 2- Avalanche, 3- Kotagiri road, 4- Kodaikanal, 5- Munnar,
6- Cherapunji road, 7- Shillong peak road, 8- Elephanta falls

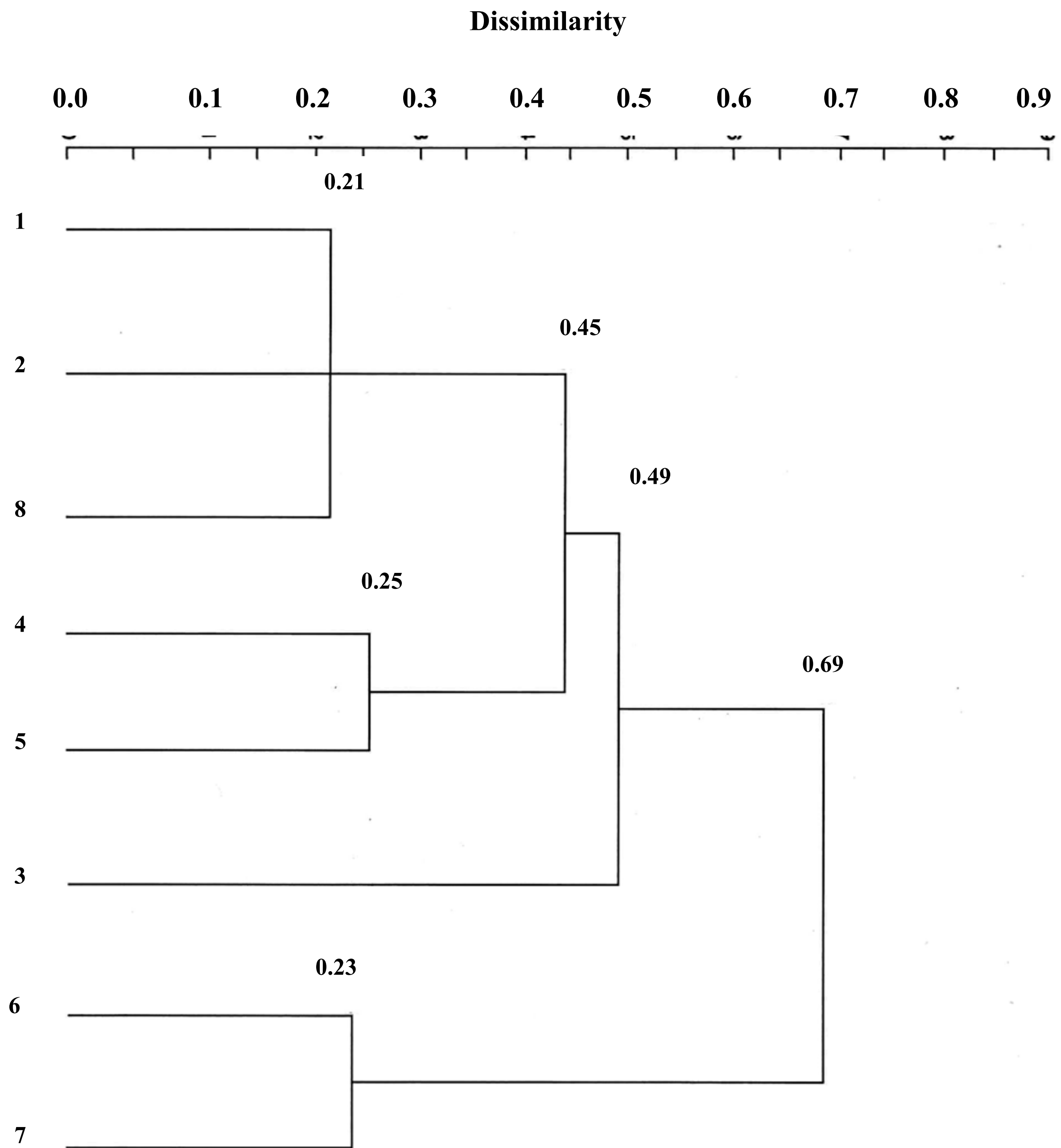

Fig 3.6: Dendrogram drawn based on frequencies of mtDNA haplotypes

1- Naduvattum, 2- Avalanche, 3- Kotagiri road, 4- Kodaikanal, 5- Munnar,

6- Cherapunji road, 7- Shillong peak road, 8- Elephanta falls

3.5 Discussion

The slow rate of evolution of chloroplast and mitochondrial DNA compared to nuclear DNA in plants earlier limited its use in population studies at intraspecific level (Palmer 1987). Both chloroplast and mitochondrial genomes being conserved molecules, the probability of detecting intraspecific variation was considered to be low. However, a sensitive technique like PCR-RFLP allows use of sequence specific restriction enzymes (which are now available in high number) for the digestion of a specific noncoding amplicon from cp/ mtDNA to detect large number of variations, since atleast 50% of cpDNA variations are due to small insertions and deletions (indels) (Gielly and Taberlet, 1994). Hence, there are many reports in the last decade on the use of cp- and mtDNA to study intraspecific variations, population genetics and phylogeographic studies in plants (Soltis *et al* 1992, Demesure *et al* 1996, El Mousadik and Petit 1996, King and Ferris 1998, Newton *et al* 2000, Bakker *et al* 2000, Sperisen *et al* 2001, Mohanty *et al* 2001 a,b, 2002, 2003 and Palme *et al* 2003). Use of genetic markers from these organellar genomes is also a powerful tool for detecting population differentiation because they are more sensitive to the effects of genetic drift than the nuclear genome (Gugerli *et al,* 2001). In the present study, PCR-RFLP technique was used to reveal chloroplast and mitochondrial DNA diversity in the natural populations of *G. fragrantissima* from the two hotspots in India.

3.5.1 Distribution of cpDNA haplotypes

In the present study none of the cpDNA haplotypes was shared by all the eight populations. However, haplotype CH1 was shared by all the populations in WG, while CH4 was shared by all the populations of NE region. Seven cpDNA haplotypes except CH1 and CH4 were unique with low frequencies (Table 3.4). They suggest recent changes in cpDNA of *G. fragrantissima*, as low frequencies of the unique haplotypes were reported to reflect their recent origin in case of *Prunus* also (Mohanty *et al* 2001). Total heterozygosity h_T (for unordered alleles) and v_T (for ordered alleles) were higher than the corresponding average heterozygosities, h_S and v_S (Table 3.6). Furthermore the values for G_{ST} (unordered alleles) and N_{ST} (ordered alleles) were high (0.615 and 0.866, respectively) (Values of G_{ST} for other plants species are: *Prunus avium*, 0.29; *Prunus spinosa*, 0.19; *Argania spinosa*, 0.60 and *Alnus glutinosa*, 0.87; Mohanty *et al*, 2001 and 2000; El Mousadik and Petit, 1996 and King and Ferris, 1998, respectively and values of N_{ST} are: *Prunus spinosa*, 0.136 and *Prunus avium*, 0.33; Mohanty *et al*, 2000 and 2001,

respectively) and N_{ST} value was higher than G_{ST} value in *G. fragrantissima* in the present study. The high values of G_{ST} and N_{ST} as well as N_{ST} value higher than G_{ST} are usually observed for phylogeographically organized populations (Pons and Petit, 1996). The dendrogram based on cpDNA haplotype frequencies (Fig. 3.4) shows grouping of populations according to their geographic distribution. This observation is in accordance with several other plant population studies where cpDNA variation is geographically structured (Soltis *et al,* 1992 and Sewell *et al,* 1996).

3.5.2 Distribution of mtDNA haplotypes

Contrary to cpDNA, one mtDNA haplotype (MH1) was shared by all the eight populations and had a high frequency and MH2 and MH4 were shared by five and four populations, respectively (Table 3.5). These mtDNA haplotypes may correspond to the ancestral type. Three mtDNA haplotypes (MH3, MH5 and MH6) were unique with low frequency to population from Avalanche and Kotagiri road indicating recent changes in mtDNA of *G. fragrantissima* from WG alone since no such unique haplotypes were observed in NE region. The total mtDNA diversity (h_T) was high and a major portion of it was contributed by within population diversity (h_S) (Table 3.6). Similarly, values of v_S and v_T were also high. In case of mtDNA the values of population subdivision (G_{ST} and N_{ST}) were very low as compared to those in case of cpDNA and there was not much difference between these values (Table 3.6). This indicates that at the mtDNA level the populations do not show much differentiation. They are more or less phylogenetically equivalent and are not yet phylogeographically well structured although some unique haplotypes at low frequency were observed (Pons and Petit, 1996). Figure 3.5 also shows that in terms of haplotype relationship, Elephanta falls population from NE region was closer to Naduvattum and Avalanche populations from WG although more plants need to be obtained and analyzed from this population. This supported the theory that *G. fragrantissima* probably migrated during glaciation from NE to WG and showed common haplotypes between the two hotspots due to the slow rate of evolution of mtDNA (12 times slower than nuclear and 3 times slower than cpDNA, Wolfe *et al*, 1987). On the other hand cpDNA did not show even a single haplotype common between the two hotspots.

Nine cpDNA haplotypes and seven mtDNA haplotypes were detected from a total of eight populations indicating relatively higher level of chloroplast DNA variation than mitochondrial DNA variation in case of *G. fragrantissima*. This has also reflected higher h_T value for cpDNA than that for mtDNA (Table 3.6). This could be explained on

the basis of the three times slower rate of nucleotide substitution in mt DNA than in cp DNA (Wolfe *et al* 1987, Palmer 1992). Similar observation was made in case of *Prunus spinosa* (Mohanty *et al* 2003), however, there are also some examples where mt DNA variation is higher than cpDNA (Laurent *et al* 1993, Caha *et al* 1998).

3.5.3 Correlation between cp and mtDNA haplotypes

In *G. fragrantissima* there were nine cp and seven mtDNA haplotypes observed as detailed in the results. The most frequent mtDNA haplotypes MH1 and MH2 were always associated with all the nine cpDNA haplotypes and MH4 was associated with eight cpDNA haplotypes. However, four mtDNA haplotypes (MH3, MH4, MH5 and MH6) common to WG and MH7 common to NE were associated with the cpDNA haplotypes from their respective regions. Therefore, no strict association was seen between the two genomes. This is in contrast to the observation made in case of *Prunus spinosa* (Mohanty *et al* 2003) where the two mtDNA haplotypes (M1 and M2) were specifically associated with cpDNA haplotypes with only two cases of dissociation and in case of Oaks (Dumolin- Lapegue *et al*, 1998) a congruence of groupings based on cp and mtDNA was observed with only three cases of uncoupling. This suggests two possibilities in case of *G. fragrantissima* that probably there might not be similar pattern of inheritance for cp and mtDNA. Inheritance patterns of cp and mtDNA in angiosperms can vary among species (Dumolin- Lapegue *et al*, 1998) or in cpDNA paternal leakage may have occurred causing dissociation. However, the second possibility appears to be rare as there are more cases of dissociation than association.

3.5.4 Contribution of pollen and seed flow to the total gene flow

In the present study, the ratio of pollen/seed flow is less (0.56 for cpDNA and -1.60 for mtDNA, respectively) as compared to earlier reports where the values are: 1 for *Castanea sativa* (Fineschi *et al*, 2000), 3.4 for *Silene alba*, 8.9 for *Silene vulgaris* (*Ouborg et al*, 1999), 24 for *Pinus contorta*, 84 for *Fagus sylvatica*, 286 for *Quercus robur* and 500 for *Quercus petrataea* which are wind pollinated plants and 23 for *Alnus glutinosa* which shows aquatic seed dispersal (King and Ferris, 1998). It is reported that the ratio is low for tree species characterized by insect pollination or by very efficient seed dispersal mechanisms (Petit, 1999). *G. fragrantissima* is an insect pollinated plant and the seeds are edible thereby facilitating efficient seed dispersal. These together may lead to low pollen/seed flow ratio. Furthermore the negative ratio for mtDNA suggests better seed flow than pollen flow compared to that in cpDNA. It is also reported that ratio of pollen/seed flow decreases with increase in the distance i.e. within population

gene flow is mainly pollen mediated while between populations the contribution of seed and pollen flow to dispersal is more or less the same (Ouborg *et al*, 1999). In the present study the populations of *G. fragrantissima* are from two spatially separated regions which might also contribute to the low ratio of pollen/seed flow.

Chapter 4

Comparative analysis of quantity and quality of oil of Indian Wintergreen from the two hotspots in India

Comparative analysis of quantity and quality of oil of Indian Wintergreen from the two hotspots in India

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4.1 Abstract

Oil extracted from the leaves of *Gaultheria fragrantissima* Wall. is similar in its physical and chemical properties to the oil of Wintergreen obtained from *Gaultheria procumbens*, Linn. and *Betula lenta*, both natives of North America. Comparative analysis of quantity and quality of Oil of Indian Wintergreen was carried out in the present study. Significant variation in the yield of oil as well as content of methyl salicylate was observed in the plants from the two hotspots. The average yield of oil was 1.36% in Northeastern Himalayas (NE) and it was 0.074% in Western Ghats (WG). Content of methyl salicylate ranged from 98.2- 99.4% and 89.9- 91.3% for plants from NE region and WG, respectively. Furthermore, within population variation in oil yield was also observed for plants collected from Cherapunji road and Shillong peak road populations (populations from NE region) where the yield ranged from 1.10- 1.67% and 1.23- 1.79%, respectively.

4.2 Introduction

Gaultheria fragrantissima Wall. belongs to family Ericaceae also known as the Heath family and is a medicinally and economically important plant because of the presence of an essential oil in its leaves. This volatile oil obtained by steam/water distillation of its leaves is identical to the oil of Wintergreen, obtained from *Gaultheria procumbens* Linn. and *Betula lenta*, both natives of North America (Anonymous, 1956). The oil is colourless and possesses an agreeable odour and flavor. Following are the reported physical properties of the oil from Assam leaves (Anonymous, 1956).

Specific gravity at 16°C	1.185
Optical rotation	0°
Refrative index at 25°C	1.4000
Ester number	362.9
Ester content, calculated as methyl salicylate	96- 99 %
Solubility at 20°C	Clearly soluble in 6- 8 volume of 70% alcohol

Methyl salicylate is the major component (96- 99 %) of the oil and the other compounds reported are triacontane, an aldehyde or ketone, an alcohol and an ester (Anonymous, 1956). The presence of methyl salicylate as the chief component of oil of Wintergreen was recognized more than a centuary ago by Procter and Cahours (1844). Methyl salicylate does not occur in *Gaultheria* in free form, but as a glycoside. This glycoside know as gaultherin is a monotropitin which is a conjugate of methyl salicylate with a disaccharide of glucose and xylose. Methyl salicylate is released from gaultherin after hydrolysis of the disaccharide also known as primeverose, by an hydrolase known as gaultherase in plants (Ribnicky *et al*, 2003) (Fig 4.1). Other compounds reported to be isolated from the leaves of *G. fragrantissima* are β- sitosterol, quercetin- 3- galactoside and ursolic acid (Murthy and Babu, 1972).

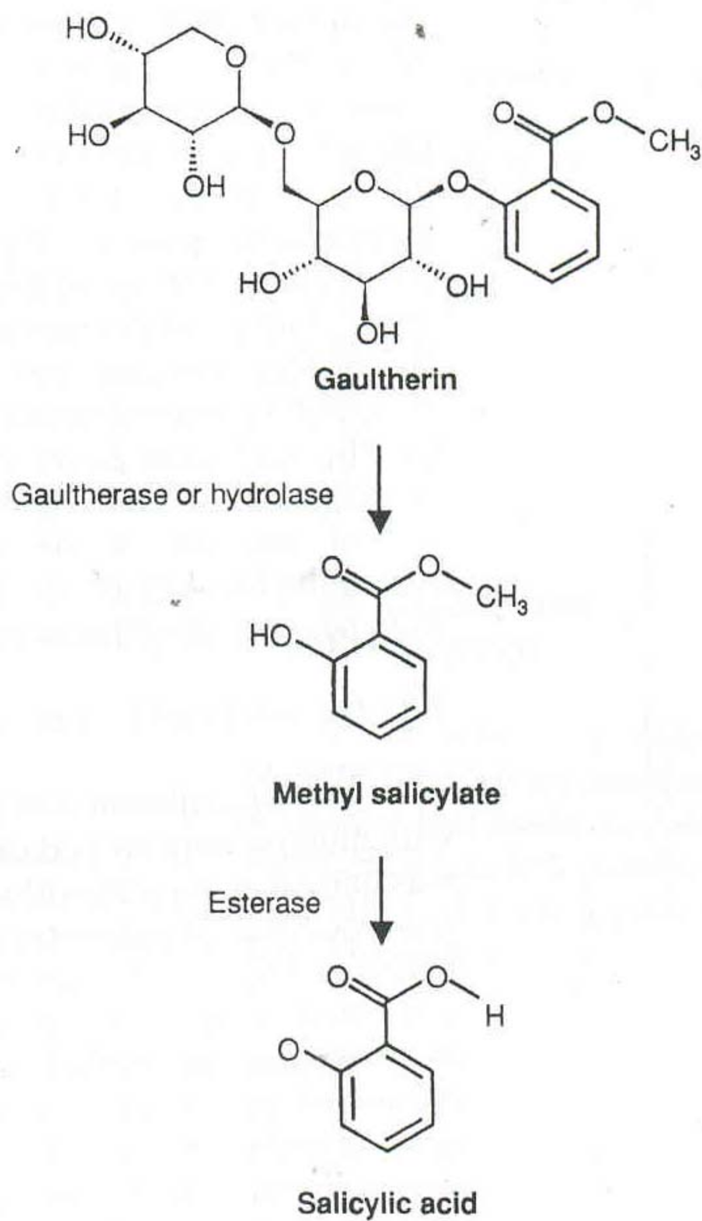


Fig 4.1: Conversion of gaultherin into methyl salicylate by gaultherase and further to salicylic acid

Oil of Gaultheria is a stimulant, carminative and an antiseptic due to the presence of methyl salicylate. It is applied externally in the form of a liniment or an ointment in rheumatism, sciatica and neuralgia. External application may cause eruptions. The oil is also given internally and is best administered in the form of an emulsion. It has vermicidal action against hookworm. It is a constituent of several insecticidal and insect – repellent preparations and is used as a flavoring agent in confectionary, soft drinks and dentifrices. Experiments on tumor- susceptible mice showed that the onset of cancer is delayed when small amounts of oil of gaultheria are administered (Anonymous, 1956). It is reported that the synthetic methyl salicylate has largely replaced the natural product due to its lower cost (Chopra, 1932). However, most of the pharmaceutical companies manufacturing ayurvedic (ancient alternative medicine in India) pain balms even today, claim to use the natural product rather than the synthetic methyl salicylate (www.nutripharm.net, www.sportsbalm.conz/amrutanjan_liquid_balm.htm,). Naturally occurring gaultherin (conjugate of methyl salicylate and a dissacharide of glucose and xylose) can be used as a natural substitute for aspirin. Aspirin is used as a painkiller and in the treatment for heart attacks and strokes, the leading cause of death. It is proposed that the natural gaultherin may have therapeutic value as a safer and alternative to aspirin with minimal negative gastric side effects (Ribnicky *et al*, 2003). β- sitosterol alone and in combination with similar plant sterols reduce blood levels of cholesterol and is effective in reducing symptoms of benign prostatic hyperplasia (Lee *et al*, 1977 and Berges *et al*, 1995). Quercetin an antioxidant, has anti cancer properties, help prevent heart diseases and act as an antihistamine (www.berkleywellness.com). Ursolic acid is also medicinally active both topically and internally with anti inflammatory, anti tumor (skin cancer) and anti microbial properties (Reico *et al*, 1991; Ishida *et al*, 1990 and Zaletova *et al*, 1987). Hence, there is a need to make efforts towards efficient extraction of oil. In this chapter, I have studied the yield of oil in relation to its season of collection and also the site of collection (WG and NE region).

4.3 Materials and methods

4.3.1 Field sampling

Leaf tissue was collected from two populations, namely, Naduvattum and Avalanche representing Western Ghats and one population, namely, Cherapunji road representing Northeastern Himalayas (leaf tissue from a number of plants for each population was pooled together). 500g to 1kg of leaf tissue was collected from each of

the above mentioned regions during months of February, July and October (Table 4.1). Later, individual plant tissue was also collected from two populations, namely Cherapunji road and Shillong peak road from NE region in the month of December.

4.3.2 Extraction of oil from *G. fragrantissima* leaves

100g of fresh leaves were cut into fine pieces and macerated in 1L round bottom flask in 200ml water at 49°C (120°F) for 18 hrs in water bath (Anonymous, 1956) followed by distillation of oil using Dean- Stark assembly (Fig 4.2) for 4- 5 hours till the milky distillate became clear. Initially the distilled water was milky due to its emulsion with oil (Fig 4.2a), but as the distillation proceeded it became clear (Fig 4.2b) and the oil which is heavier than water settled down (Fig 4.2c). Oil was extracted from the distillate by extracting it with diethyl ether as follows: the distillate was collected in a flask and equal amount of diethyl ether was added to it. Oil dissolved in diethyl ether and water formed a separate layer. Water layer was removed using a separating funnel and the lighter layer of solvent (diethyl ether) was evaporated at 40°C, 100mm of Hg using Rota vapor R- 114 (Buchi, Switzerland). The yield obtained was dried and weighed. Each extraction was carried out three times and the yields given in Table 4.1 represent the average values.

4.3.3 Conditions used for GC analysis

GC- analyses were accomplished using an Agilent 6890N instrument equipped with HP-5 capillary column (30m × 0.32 mm, 0.25 μm film thickness), working with the following temperature programme: 50°C to 250°C at the rate of 20°C per min, kept at 250°C for 5min, injector and detector temperatures 250°C, carrier gas nitrogen (2ml/ min), detector FID, split ratio 1: 100, injection of 0.5μl. Methyl salicylate was estimated quantitatively by comparing its retention time with that of authentic methyl salicylate (procured from s.d. fine chem., Ltd, Mumbai) which gave a peak of area 99.30% at 7.767 retention time. All the oil samples were analyzed under similar conditions.

Fig 4.2a **Fig 4.2b** **Fig 4.2c**

Fig 4.2: Dean- Stark assembly used for oil extraction by water distillation.

1: Dean- Stark assembly, 2: Round bottom flsak with leaves macerated in water, 3: Heating mantel, 4: Oil heavier than water settles down.

4.4 Results

Leaf tissue was collected from WG (Nilgiri hills) and NE region (Shillong) during different months (February, July and October) in the year 2002 and 2003. Leaf tissue thus collected was pooled together from various plants in the respective regions. The aim of such a collection was to compare the overall percentage yield of oil from the plants of the two main hotspots. Percentage oil yield on fresh weight basis (water content is 53.5% in the leaves) was calculated for all the samples and the results are given in Table 4.1. Plants from NE region gave higher yield in the range of 1.4 to 1.49% (average is 1.44%), as compared to the plants from WG where the yield of oil ranged from 0.056- 0.086% (average is 0.072%), irrespective of the month of collection. GC- analysis was carried out to measure the methyl salicylate content of the oil. Fig 4.3 gives the representative GC profiles of the standard (synthetic methyl salicylate), wintergreen oil from NE region and WG collected during July and October. It was also observed that the content of methyl salicylate varied in the two hotspots. Percentage of methyl salicylate ranged from 98.2- 99.4% for plants from NE region whereas it ranged from 89.9- 92.3% for plants from WG (Table 4.1). As shown in Fig 4.3b (i & ii) oil from Shillong shows a major peak of methyl salicylate at 7.6 retention time. Other peaks detected here are very

few with very less area percentage (less than 1%) hence were not identified. Fig 4.3c (i & ii) represents the GC profile of oil from Nilgiri hills which shows that methyl salicylate gives the major peak at 7.6 retention time. The other 28 peaks are with area percentage less than 2% and hence were not identified.

Table 4.1: Oil yield and methyl salicylate content in leaves of *G. fragrantissima* from various populations and from different months

Population	Month of collection	% yield based on fresh weight	∗Methyl salicylate content (GC)
Cherapunji road	February	1.4	98.2
Naduvattam	February	0.082	91.3
Cherapunji road	July	1.49	99.4
Naduvattam	July	0.064	89.9
Cherapunji road	October	1.43	98.5
Naduvattam	October	0.086	91.7
Avalanche	October	0.056	92.3

GC: Gas chromatography, ∗Percentages obtained as area percentage

Furthermore, as the plants from NE region yielded more quantity of oil, leaf tissue from 13 individuals from Cherapunji road population and 4 individuals from Shillong peak road population were collected during the month of December, 2003 to study the variation in oil yield within a population. In these samples percentage oil yield, within Cherapunji road population ranged from 1.10- 1.67% and within Shillong peak road population it ranged between 1.23- 1.79% (Table 4.2).

Table 4.2: Percentage oil yield within population in *G. fragrantissima* from Northeastern region

Location	Oil yield (%) on fresh wt. basis
Cherapunji road	
Gf 1	1.12
Gf 2	1.10
Gf 3	1.23
Gf 4	1.18
Gf 5	1.36
Gf 6	1.67
Gf 7	1.43
Gf 8	1.22
Gf 9	1.48
Gf 10	1.21
Gf 11	1.52
Gf 12	1.26
Gf 13	1.41
Shillong peak road	
Gf 1	1.79
Gf 2	1.68
Gf 3	1.23
Gf 4	1.37

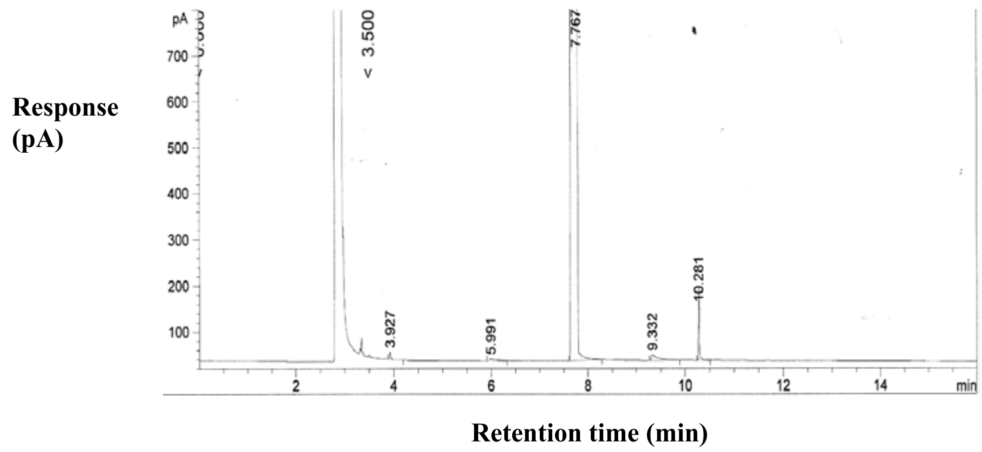
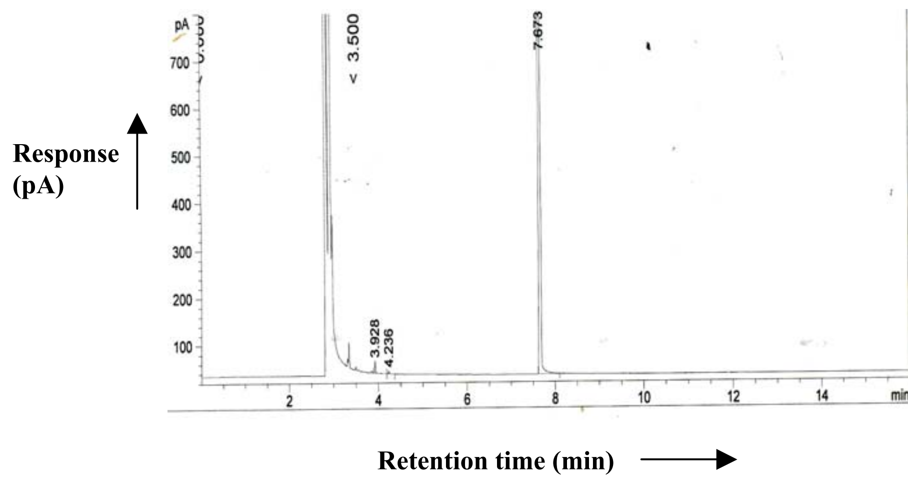
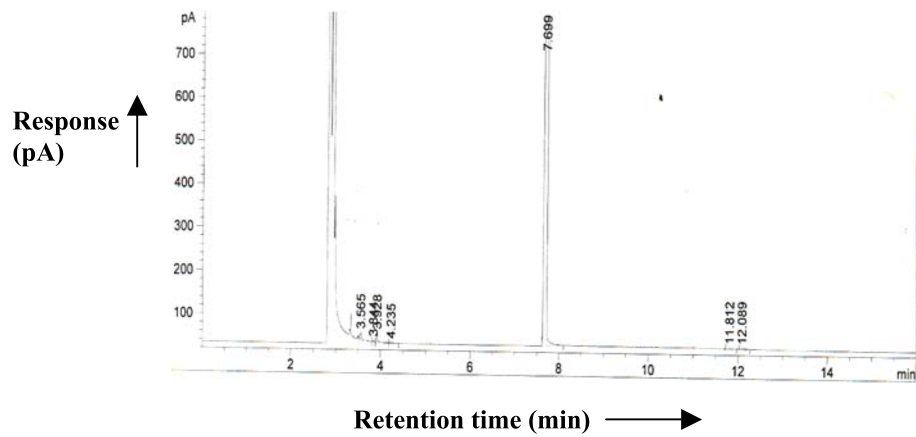


Fig 4.3a: GC profile of standard (synthetic methyl salicylate)

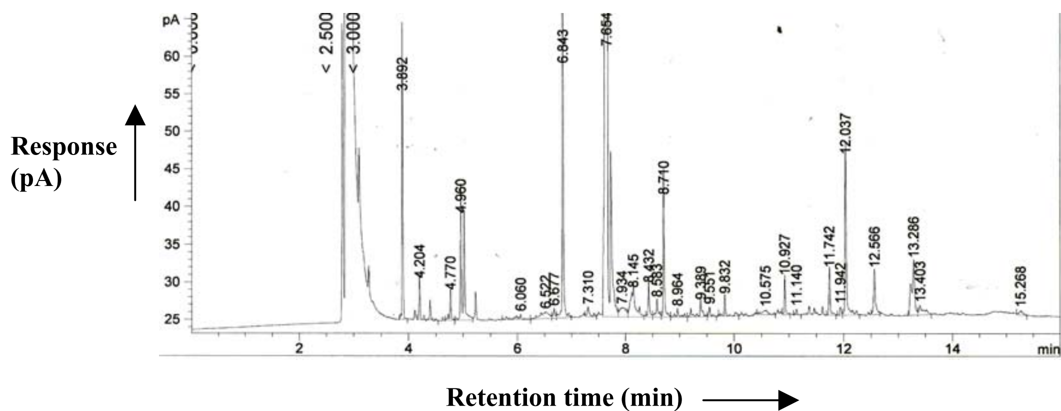


[i]

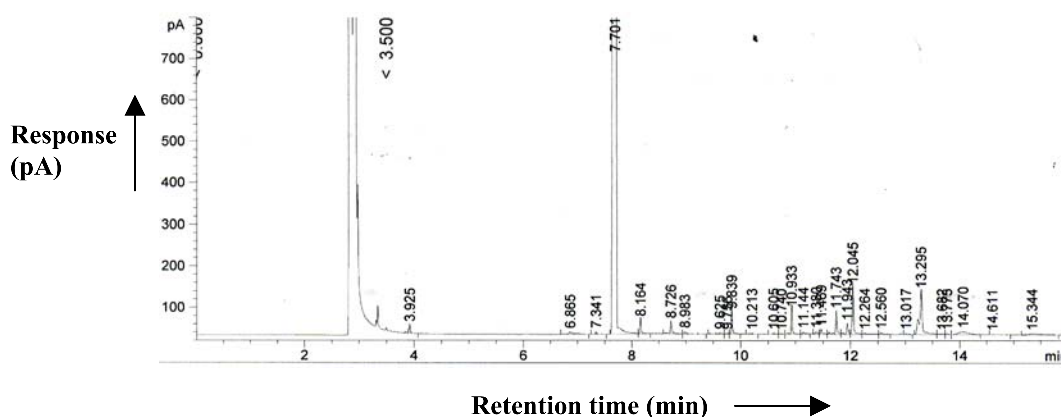


[ii]

Fig 4.3b: GC profile of wintergreen oil from NE region for months of:i) July and ii) October



[i]



[ii]

Fig 4.3c: GC profile if wintergreen oil from WG for months of: i) July and ii) October

4 5 Discussion

4.5.1 Comparison of oil yield from *G. fragrantissima* and *G. procumbens*

Oil of Indian Wintergreen extracted by water distillation from the leaves of *G. fragrantissima* is identical with 'Oil of Gaultheria', also know as 'Wintergreen Oil', obtained from *G. procumbens* Linn., a native of North America and oil distilled from the wood and bark of Betula lenta (sweet birch), also a native of America. The physical properties of oil of Wintergreen from *G. procumbens* Linn (Anonymous, 1956) are as follows:

Specific gravity at 15°C	1.180- 1.193
Optical rotation	-0° 25' to -1° 30'
Refrative index at 20°C	1.535- 1.536
Ester number	354- 365
Ester content, calculated as methyl salicylate	96- 99 %
Solubility at 20°C	Clearly soluble in 6- 8 volume of 70% alcohol

The average yield of oil from *G. procumbens* Linn. is 0.66% during summer which gives the highest yield (Anonymous, 1966) and that from sweet birch is 0.2% (Chopra, 1932) where the distillation process does not include prior maceration of the leaves. However, if the leaves are macerated prior to distillation the yield increases from 0.66% to 1.30% for *G. procumbens* Linn. and from 0.2% to 0.41% for Betula lenta. The yield of oil as earlier reported for *G. fragrantissima* for the winter season is 0.036% for plants from Nilgiri hills and 0.65% for plants from Assam ie NE region where the distillation process does not include prior maceration of the leaves (Chopra, 1932). In the present study, I have reported an average yield of 1.44% for plants from NE region during winter and 0.072% for plants from WG during winter where the leaves were macerated before distillation. Thus the maceration of leaves of *G. fragrantissima* before distillation increased the oil yield by two times as compared to the oil yield where maceration was not done. This suggests that oil yield in *G. fragrantissima* is as good as that from *G. procumbens* Linn. and can be used for commercial exploitation.

4.5.2 Effect of genetic and environmental factors on oil yield

Due to the importance of *G. fragrantissima* as a medicinal plant there have been a few reports earlier on oil exraction. It was reported by Adhikary and Bashyal (1985) that the oil yield showed seasonal variation for plants collected from Nepal. In the present study no such variation was obtained in the yield of oil for plants collected during different months (Table 4.1). However, variation in the yield of oil was recorded for plants from WG and NE region. Plants from NE region yielded more oil than plants from WG. The two regions are spatially separated by an aerial distance of more than 2000km with difference in their annual rainfall, climate, soil type, etc (Meher- Homji, 1972). Thus, the environment might be playing a crucial role in oil yield. It is reported that the production of secondary metabolites is affected by the environment as well as the genotype of the plant. Chatwachirawong *et al* (1999) studied G-E interactions on starch content from five Cassava genotypes. Their results indicated that there was less seasonal

effect on genotypic performance. Doran *et al* (1995) studied the effect of environment and genotype on oil yield from the leaves of *Eucalyptus camaldulensis* and obtained significant variation in oil yield in the plants collected from their natural habitat in Zimbabwe. Katerina *et al* (2001) studied composition of essential oil from Yarrow (*Achillea millefolium*) growing in natural habitats of Czech Republic and inferred that the content and composition of the active substances depended on both, climatic conditions and individual subspecies. In the present study, it was noted that the content of methyl salicylate also varied in the two hotspots in India.

Based on the genetic diversity studies using random nuclear markers presented in Chapter 2, it was observed that the plants of *G. fragrantissima* clustered according to their geographic regions (Fig 2.2, Chapter 2). Using cytoplasmic markers (Chapter 3) it was suggested that plants of *G. fragrantissima* from NE region probably migrated towards WG during Pleistocene glaciation. Thus the plants had a common genetic background before glaciation but after glaciation they started adapting to their local environment and evolving independently in different climatic conditions. These findings, together suggest that there might be genetic basis for the difference in the yield of oil and methyl salicylate content from the two spatially separated hotspots. However, the possibility of attributing the oil yield difference also to climatic variations in the two hotspots cannot be ruled out unless the same plants are grown in the two regions and are used for oil extraction. Similarly, Buben *et al* (1992) studied the contents and composition of essential oil in various Thuja species collected from four genomically different localities in Czechoslovakia during different seasons. They obtained variability of the main component of the essential oil in these areas. Skoula *et al* (1999) studied the genetic diversity in *Salvia fruticosa* from Crete and its relation with the essential oil profiles and suggested a genetic basis for the chemical profiles observed. They reported quantitative changes in the essential oil content and suggested that the microclimate played an important role in affecting the essential oil content. Thus, both the genetic and environmental factors might be responsible for oil yield and methyl salicylate content in *G. fragrantissima* from the two hotspots in India.

In order to determine the effect of environment/ genetic background on oil content at a specific location, within population variation in oil yield for plants collected from Cherapunji road and Shillong peak road populations was also analyzed (Table 4.2) where the yield of oil ranged from 1.10- 1.67% and 1.23- 1.79%, respectively. Considerable variation in the oil yield was thus observed in the plants collected from the

same populations. This within population variation could be attributed to their genetic background alone and not to the environment as the individual plants belong to the same climatic zone. Study using cytoplasmic markers (Chapter 3) inferred that Cherapunji road and Shillong peak road populations showed more than one cp and mtDNA haplotypes and also represented unique haplotypes. Furthermore, Shillong peak population had maximum number of unique cpDNA haplotypes (Table 3.4, Chapter 3).It would, therefore be interesting to analyze haplotypes of these plants as well as more plants of the same haplotypes for the correlation of haplotype with oil content if such a correlation exists. The collections for individual plants from the two populations in NE region were made recently based on the previous pooled sample data of oil yield and methyl salicylate content, therefore the genotype analysis of these plants has been initiated as the next step based on the outcome of the present study.

Chapter 5

Thesis summary and future directions

Thesis summary

Contents

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5.2 Genetic diversity in *G. fragrantissima* based on cytoplasmic markers

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5.4 Assessment of theories on postglacial migration

5.5 Application of genetic diversity driven conservation strategies in *G. fragrantissima*

5.6 Future directions

In my thesis, I have used both nuclear (ISSR) and cytoplasmic (cp and mtDNA) markers to study the genetic diversity in an undershrub *Gaultheria fragrantissima* Wall. from the two biodiversity hotspots in India, namely Western Ghats (WG) and Northeastern Himalayas (NE). *G. fragrantissima* is an insect pollinated, shrub savanna type of plant growing above 1500m altitude. The two hotspots mentioned above are among the 25 hotspots recognized all over the globe and are also among the 8 hottest hotspots (Myers *et al*, 2000). For the past 4-5 decades researchers have studied the plant species common to the two hotspots with respect to their morphological characters, habitat type, endemism, phytogeography, etc (Meher- Homji, 1972 and 1975; MoEF, 1999). However, there are very few reports on the study of the genetic diversity of these plant species (Jain *et al*, 2000, Deshpande *et al,* 2001 and Bahulikar *et al*, 2004) Furthermore, there are theories proposed by various researchers regarding the migration of flora and fauna from NE region towards WG during Pleistocene glaciation (as detailed in Chapter 1, Review of literature). In the light of this, I used molecular tools to determine the genetic diversity of one such plant, *G. fragrantissima* which is, not only common to the two hotspots, but is also medicinally and economically important plant due to the presence of methyl salicylate as a major constituent in the oil from its leaves.

5.1 Geographic clustering of *G. fragrantissima* from the two hotspots based on ISSR markers

ISSR markers are random nuclear (biparentally inherited genome and hence transmitted through both seeds and pollen) dominant markers which do not require any previous sequence information for their synthesis. Such markers are particularly useful for an initial examination of the genetic diversity in plants. Here, I analyzed seven populations of *G. fragrantissima* from the two hotspots in India. ISSR primers with good PIC score were used in the analysis which obtained percentage polymorphism of 86.82 with a total diversity of 0.505. UPGMA phenogram was developed which clustered the plants according to their geographic regions. Furthermore, low gene flow among populations and high among population differentiation were recorded. This indicated that *G. fragrantissima* populations might be progressing towards genetic drift.

5.2 Genetic diversity in *G. fragrantissima* based on cytoplasmic markers

In order to study the phylogeography (geographic distribution of genetic variation) and evolutionary history of the plant, I used cytoplasmic markers (cp and mtDNA markers). Cytoplasmic DNA is uniparentally inherited, transmitted exclusively

through seeds, is homoplasmic and non recombinant. Here I detected nine cpDNA and seven mtDNA haplotypes in the eight populations of *G fragrantissima* from the two hotspots in India. Interestingly, none of the cpDNA haplotypes was common between the two hotspots which could be due to the faster rate of evolution in cpDNA as compared to mtDNA (Wolfe *et al*, 1987). However, one mtDNA haplotype was shared by all the eight populations that could be considered as the ancestral haplotype for *G fragrantissima.* It was revealed that the cpDNA diversity was higher than mtDNA diversity, however, within population diversity was higher in mtDNA. The level of population subdivision for unordered and ordered alleles was higher in cpDNA and N_{ST} value was also higher than G_{ST} in cpDNA together suggesting phylogeographic structuring in *G. fragrantissima*.

Among the seven haplotypes of mtDNA, two most frequent (MH1 and MH2) were always associated with all the nine cpDNA haplotypes whileone mtDNA haplotype (MH4) was associated with eight cpDNA haplotypes. On the other hand, four mtDNA haplotypes (MH3, MH4, MH5 and MH6) common to WG and MH7 common to NE were associated with the cpDNA haplotypes from their respective regions. Thus, no strict association was observed between the two genomes. More cases of dissociation than association between the cp and mtDNA haplotypes probably suggest different patterns of inheritance for cp and mtDNA in *G. fragrantissima*. Low ratio of pollen/seed flow is a characteristic of plants with insect pollination or efficient seed dispersal and the ratio decreases with increase in distance (Petit, 1999 and Ouborg *et al*, 1999). This supports the result in case of *G. fragrantissima* where low ratio of pollen to seed flow was observed.

5.3 Towards biomolecule prospecting

G. fragrantissima Wall. is know for its oil with methyl salicylate as its chief constituent. This oil is similar in its physical and chemical properties to the Oil of Wintergreen obtained from *G. procumbens*, Linn. and *Betula lenta*, both natives of North America and hence is known as Oil of Indian Wintergreen. I carried out comparative analysis of oil yield and methyl salicylate content in *G. fragrantissima* plants from the two hotspots in India. Here, I detected variation in oil yield and methyl salicylate content in the plants from the two hotspots, although no variation in oil yield was observed for plants collected during different seasons. NE region gave high yield of oil in the range of 1.40- 1.49% and high methyl salicylate content in the range of 98.2- 99.4% as compared to the plants from WG where oil yield ranged from 0.056- 0.086% and methyl salicylate

content ranged from 89.9- 92.3%. Furthermore, within population variation in oil yield was also observed for plants from Cherapunji road and Shillong peak road populations where the yield ranged from 1.10- 1.67% and 1.23- 1.79%, respectively. These results can be further used to study the correlation between oil yield and the individual plant haplotype. This will help in identifying elite haplotypes giving better oil yield which need to be conserved.

5.4 Assessment of theories on postglacial migration

It has been well recorded that the pleistocene glaciation was probably responsible for pushing the Himalayan flora southwards (Burkill 1924; Hora 1949; Auden 1949; Dey 1949). During glaciation the plant species from NE region must have advanced towards the southern Indian peninsula by stepwise migration and during post glaciation, only the hardy species might have survived in WG. Plants of temperate shrub savanna type such as *Gaultheria, Mahonia, Rhododendron*, etc could survive only at the high altitude (above 1500m) in WG while only tree species of tropical forest type such as *Symplocos, Pittosporum, Ardisia, Elaeocarpus*, etc could survive at lower altitudes (Meher- Homji 1975). Interestingly, in case of *Gaultheria* only one species, *G. fragrantissima* is observed to be common to the two regions although there are 7-8 species of *Gaultheria* reported in the NE region (Meher- Homji 1975). There could be two explanations for this, one that *G. fragrantissima* was the only species of genus *Gaultheria* hardy enough to survive after glaciation in WG at high altitudes. Secondly, the other species might have migrated very slowly, hence could not reach the high altitudes of WG and therefore, could not survive after glaciation in WG.

This can be strongly supported by fossil records, however unfortunately, no fossil record could be obtained in particular for *Gaultheria*. Gupta (1971) traced the sequence of vegetation and climatic changes in the Nilgiri hills based on the analysis of fossil pollen. According to him, montane forests gained dominance about 14000 years ago as evident from the preponderance of pollen of *Gordonia, Elaeocarpus* and *Euonymus*. After this phase, pollen record showed progressive decline of forest in favour of grassland. Also a key to the past distribution of the present disjunct taxa of the montane shrub- savanna *Rhododendron, Gaultheria, Mahonia* etc was provided by the present distribution of the montane forest genera like *Pittosporum, Symplocos, Elaeocarpus*, etc on lower altitudes (Meher- Homji, 1975). These genera have representation from Sikkim to Garhwal in the sub- tropical Himalayas; in western India in WG; in Eastern India in Chota Nagpur and Mahendragiri hills and in Sri Lanka in Southern hilly region (Meher-

Homji, 1975). Intensive sampling of *G. fragrantissima* within India as well as neighboring countries like Nepal, Burma, Bhutan and Srilanka and their genetic analysis may lead to a better understanding of the migration pathways and the history of distribution of *G. fragrantissima* after the last ice age.

5.5 Application of genetic diversity driven conservation strategies in G. fragrantissima

The rich biodiversity in the two hotspots is threatened largely due to various anthropogenic activities due to which there is an ever increasing loss of the forests in these two hotspots (Upadhyay, 1999 and Jha *et al*, 2000), hence there is a need to protect and conserve the threatened forests community. Therefore, the two regions under study, namely, WG and NE region, have been marked for the overall national strategy of protecting the ecosystems and safe guarding the genetic diversity. India is a developing country where threats are the greatest and conservation resources are the scarcest. Therefore, efforts are needed especially in India, to conserve and maintain genes, species and ecosystems for the sustainable use and management of biological resources. In the light of above scenario, genetic diversity directed conservation would be a better proposition to be adopted in a country like India. Thus an assessment of genetic variation is a primary step towards this. Precise knowledge of the subdivision of genetic variation within and among populations will help in identifying the genetically heterogeneous species which require different conservation strategies as compared to genetically uniform species (Pons and Petit 1995). Based on my studies, it is possible to select the populations with common as well as unique genetic backgrounds which need to be conserved in order to maintain the genetic diversity in *G. fragrantissima* in India.

5.6 Future directions

I have made a preliminary effort to analyze the diversity in *G. fragrantissima* from the two hotspot regions in India based on DNA markers as well as the chemical markers such as methyl salicylate content. However, more in depth analysis need to be done in both the cases. Vigorous attempts need to be made to make extensive collections in the same as well as other areas to have more number of populations in order to have more reliable estimation of the population structure and genetic diversity. Nucleotide sequencing of the chloroplast and mitochondrial DNA fragments as well as Single Strand Conformational Polymorphism (SSCP) analysis if performed, will throw light on the mutational events in these genomes. Furthermore, use of controlled crosses in the population will give an idea about the inheritance patterns of these extranuclear genomes

since my studies have suggested different inheritance patterns of cp and mtDNA in *G. fragrantissima*. Apart from this, the haplotype and oil content correlation needs to be done for commercial exploitation of *G. fragrantissima*. Plantation of such elite genotypes on a very large scale can be attempted for oil extraction in collaboration with the commercial organizations who have been exploiting *G. fragrantissima* for their product preparation. This will save nonselective destruction of natural populations of *G. fragrantissima*. Communities and tribals in these areas can be involved in such efforts which will provide them sustainable occupation and will help in their upliftment. Conservation of various genotypes can also be achieved through their participation in such programmes. The present studies performed by me only form the first step towards such an endeavor.

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Curriculum Vitae

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Education

B.Sc. (Microbiology) from University of Pune, India, with **first class**

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Ph.D. thesis title: "Genetic diversity analysis in *Gaultheria fragrantissima* Wall. from the two biodiversity hotspots in India using molecular markers".

Postgraduation dissertation title "Bacterial deterioration of metal working fluids: Studies on aspects of biocide evaluation."

Awards

Awarded Senior Research Fellowship by Council of Scientific and Industrial Research, Government of India (CSIR).

Presentations and Poster

1) **Apte G S**, Lagu MD, Ranjekar PK and Gupta VS. Study of chloroplast and mitochondrial DNA diversity in the natural populations of *Gaultheria fragrantissima* in the two hotspots in India. 11th New Phytologists Symposium. Plant speciation at Plant Canada, Antagonish. June 26- 28, 2003.

2) **Apte G S**, Lagu MD, Kulkarni BG, Suresh HS, Rao MKV, Ranjekar PK Gupta VS. Chloroplast and mitochondrial DNA diversity studies in *Gaultheria fragrantissima* from the two hotspots in India. IUPAC International Conference on Biodiversity and Natural Products: Chemistry and Medical Applications, New Delhi, January 26- 31, 2004.

3) Attended International conference " NEEM 2002" in Mumbai.

Seminars Presented during Postgraduation

1) Growth of Mosaic virus-free sugarcane plants from Apical meristems. Reference : Indian Phytopathology (June 1975)

2) Decolourisation of Pulp & paper mill effluent by growth of *Aspergillus Niger*. Reference : World Journal of Microbiology & Biotechnology (January 1990)

3) Cytogenetic studies reveal increased genomic damage among 'Pan Masala' consumers.
Reference : Mutagenesis (November 1990)

4) A fibre optic carbon dioxide sensor for fermentation monitoring. Reference : Biotechnology (June 1995)

Publications

1. Deshpande AU, **Apte GS**, Bahulikar RA, Lagu MD, Kulkarni BG, Suresh HS, Singh N, Rao MKV, Gupta VS, Pant A, Ranjekar PK. **(2001)**. Genetic diversity across the natural populations of three montane plant species from the WG, India revealed by ISSR repeats. Molecular Ecology 10: 2397-2408.

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1. **Apte G S**, Bahulikar R A, Lagu MD, Kulkarni BG, Suresh HS, Rao MKV, Ranjekar PK, Gupta VS. Genetic diversity analysis in Gaultheria fragrantissima from the two hotspots in India using ISSR markers.
Communicated to Taxon.

2. **Apte G S**, Lagu MD, Kulkarni BG, Suresh HS, Rao MKV, Ranjekar PK Gupta VS. Chloroplast and mitochondrial DNA diversity studies in Gaultheria fragrantissima from the two hotspots in India.
Communicated to Annals of Botany.