STUDIES ON ECOLOGY, PROPAGATION, MOLECULAR GENETIC DIVERSITY AND CONSERVATION OF CRITICALLY ENDANGERED PLANTS *PHYLLANTHUS TALBOTII* AND *PSEUDOGLOCHIDION ANAMALAYANUM* FROM WESTERN GHATS OF INDIA

A Thesis submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

BOTANY

By

Mr. SIDHESH SHRIKANT NAIK

Research Guide

Prof. S. KRISHNAN

Co-Guide

Prof. M. K. JANARTHANAM

Goa University
Taleigao, Goa

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STATEMENT

As required by the University Ordinance OB 9.9 (iv), I state that the present thesis "**Studies on Ecology, Propagation, Molecular Genetic Diversity and Conservation of Critically Endangered Plants *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum* from Western Ghats of India**", is my original contribution and the same has not been submitted on any occasion for any other degree or diploma of this University or any other University/ Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Place: Goa University **(Mr. Sidhesh S. Naik)**

Date: **Candidate**

CERTIFICATE

This is to certify that the thesis entitled "**Studies on Ecology, Propagation, Molecular Genetic Diversity and Conservation of Critically Endangered Plants *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum* from Western Ghats of India**", submitted by **Mr. Sidhesh S. Naik** for the award of the degree of Doctor of Philosophy in Botany, is based on his original and independent work carried out by him during the period of study, under my supervision.

The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institute.

(Prof. M. K. Janarthanam)
Co-Guide

(Prof. S. Krishnan)
Research Guide

Place: Goa University
Date:

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Dedicated to the Memories of My Beloved

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ABBREVIATIONS

BAP	6- Benzylamino purine
cm	Centimeter
DAF	Day after fertilization
CR	Critically Endangered
°C	Degree centigrade
2, 4-D	2, 4-Dichlorophenoxyacetic acid
EDTA	Ethylenediaminetetraacetic acid
g	Grams
HCl	Hydrochloric acid
h	Hours
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ISSR	Inter simple sequence repeat
Kg	Kilogram
KIN	Kinetin
L	Litre
µl	Microlitre
µM	Micro mole
M	Molar
m	Meter
mg	Milligram
min	Minutes
min	Minutes
ml	Milliliter

mm	Millimeter
mM	Mill molar
MS	Murashige and Skoog medium
ng	Nanogram
nm	Nanometer
N	Normal
NAA	1-naphthalene acetic acid
OD	Optical density
ppm	Part per million
%	Percent
PGRs	Plant growth regulators
±	Plus or minus
psi	Pounds per square inch
rpm	Revolutions per minute
RT	Room temperature
sec	Seconds
NaOH	Sodium hydroxide
UV	Ultra violet
v/v	Volume by volume
w/v	Weight by volume
WPM	Woody plant medium
ZEA	Zeatine
TDZ	Thidiazuron
'CR'	Critically endangered
IUCN	International Union for Conservation of Nature and Natural

2-iP	2-isopentanyl adenine
ITS	Internal transcribed spacers
*mat*K	MaturaseK
*rbc*L	ribulose-bisphosphate carboxylase
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PHYC	Phytochrome C
SCAR	Sequence characterized amplified regions
AFLP	Amplified fragment length polymorphism
RFLP	Restriction fragment length polymorphism
SSR	Simple sequence repeats
RET	Rare, endangered and threatened
UPGMA	un-weighted pair group method
GPS	Global positioning system
EC	Electrical Conductivity
2, 4–D	2, 4-dichlorophenoxyacetic acid
CTAB	Cetyl trimethyl ammonium bromide
RGCB	Rajiv Gandhi Centre for Biotechnology
MEGA	Molecular Evolutionary Genetics Analysis

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INTRODUCTION

1. INTRODUCTION

Western Ghats or Sahyadri is one of the 25 “hotspots” of biological diversity in the world (Myers *et al*., 1988; 2000). It is also declared as ‘world heritage site’ by United Nations Educational, Scientific and Cultural Organization (UNESCO) because of its high number of endemic plant species (Kumar *et al*., 2012; Gaikwad *et al*., 2014). Due to anthropogenic pressures several plant species, especially the endemics have already been appeared in Red Data Book of Indian plants (Nayar and Sastry, 1987, 1988, 1990). The Southern Western Ghats are rich in endemic tree species and genera (Ahmedullah and Nayar, 1986; Nayar, 1996; Ramesh and Pascal, 1997; Myers *et al.,* 2000; Puyravaud *et al*., 2003). Some of these species are very narrow in their distribution and exist in a very few fragmented populations, which lead to genetic depletion due to inbreeding (Charlesworth, 2003; Charlesworth and Willis, 2009).

India is very rich in terms of biodiversity and its endemic plant species having 5,725 angiosperms, 10 gymnosperms, 193 pteridophytes, 678 bryophytes, 260 liverworts, 466 lichens, 3500 fungi and 1924 algae (Sanjappa, 2005). Floristically Western Ghats is one of the richest in the country (Myers *et al*. 2000). Out of nearly 17,000 plant species recorded in India, it is estimated that Western Ghats may have about 4,500 more flowering plants species, of which nearly 1,700 (nearly 38%) of these are endemic (Nair and Daniel, 1986; Subbaiyan *et al*., 2014). It is also estimated that about 63% of India’s woody evergreen taxa are endemic to the Western Ghats (Johnsingh, 2001). The Southern Western Ghats are rich in endemic trees species and genera, while herb species endemism appears to be more in the Northern

Western Ghats (Ahmedullah and Nayar, 1986; Nair, 1991; Nayar, 1996; Ramesh and Pascal, 1997; Daniels, 2001).

Western Ghats consists of various type of forests which includes tropical wet evergreen, semi evergreen, moist deciduous, dry deciduous, mountain sub-tropical and temperate sholas and grasslands (Champion and Seth, 1968; Chauhan, 2014). However, it is reported that flora and wildlife in the Western Ghats region is under threat from various sources. This includes threat from commercial forestry and other competing agricultural land use options. Many studies noted that deforestation is quite high in the forests of Western Ghats (Chattopadhyay, 1985; Gunawardene *et al*., 2007; Kumar *et al*., 2012; Reddy *et al*., 2013).

Biodiversity is categorized into three fundamental levels such as ecological, species and genetic by the International Union for Conservation of Nature and Natural Resources (IUCN) (McNeely *et al*., 1990). The ability of population to adopt environmental changes decreases due to human anthropogenic activities like habitat destruction, mass collection in case of medicinal plants and logging which ultimately decreases the biodiversity on the earth (Chung and Park, 2008). In this scenario, maintenance and conservation of biodiversity is important and worldwide issue (Izawa *et al*., 2007; Sodhi *et al*., 2014).

The understanding of the level of genetic diversity and variation within and among the populations is needed for conservation and sustainable utilization. Population genetic diversity studies are essential for the research, conservation program and restoration of threatened populations of critically endangered and endemic plants. Evaluation of genetic diversity plays

an important role for their conservation management (Hamrick and Godt, 1996; Francisco-Ortega *et al*., 2000; Zonneveld *et al*., 2012).

Comparative population studies using molecular markers are essential to collect information on the level and pattern of genetic diversity of wild plant species, which is the initial step towards their conservation (Geburek, 1997). To obtain consistent information on the existing genetic diversity, a number of reliable and widely used markers have been developed for *Phyllanthus* species (Uma Shaanker and Ganeshaiah, 1997; Chaurasia *et al*., 2009; Rout and Aparajita, 2010).

ISSR and RAPD analysis is a popular method for estimating genetic diversity in plant populations with several advantages such as speed, low cost and the use of small amounts of plant material. ISSR techniques are also best suited for most of the plants since no sequence information is required (Huff *et al*., 1993; Zietkiewicz *et al*., 1994; Ge *et al*., 1999; Nybom and Bartish, 2000; Kingston *et al*., 2004; Parab *et al*., 2008; Parab and Krishnan, 2009; Rout and Aparajita, 2010; Patel *et al*., 2014).

The conservation of biodiversity is a universal issue, which is predicated by governments and communities (Sinclair *et al*., 1995). Biodiversity can also provide financial returns (Daily *et al*., 2000). Hence, it is essential to document and conserve these biological resources to utilize them for the human welfare (Sahu *et al*., 2008; Htun *et al*., 2010; Thapa and Chapman, 2010; Htun *et al*., 2011).

Ongoing development, upcoming industries and greedy nature of human for food and shelter have caused great extinction of species. After the critical evaluation on their status and

threat perception, a compiled Red Data Sheets have made, which are published in five volumes of the Red Data Books of Indian Plants (Nayar and Sastry, 1987, 1988, 1990).

The critically endangered (CR) and endemic plant species are valuable natural resources due to habit specificity, most of the endemic plants are susceptible to habitat deterioration and fragmentation. At present many endemics are considered to be at a high risk of extinction and hence most of these are included in the IUCN conservation lists. It becomes important to conserve these plant species by way of *in-situ* and *ex-situ* conservation measures for their survival (Nevo, 1998; Volis and Blecher, 2010; Reed *et al.*, 2011).

In the recent years, conservation of biodiversity is widely considered as a high priority field and several research projects have been funded worldwide for their conservation. Genetic resources are renewable, if proper care is taken for its conservation and management. For the conservation of the biodiversity, one should be wise enough to use the natural resources and conserve the natural ecosystem for sustainable development (Kessler *et al*., 1992). The tropical and subtropical areas are very rich in terms of floral and faunal biodiversity (Barrett *et al*., 2001; Slik *et al*., 2015). India, being at the tropical region of the Earth, is rich in terms of biodiversity due to varied climatic and physical features. Conservation can be achieved by two means: (i) *in-situ* conservation of species diversity in nature by establishing Biosphere Reserves, National Parks, Sanctuaries, Sacred groves etc. (ii) *Ex-situ* conservation such as *in-vitro* propagation, gene banks, botanical gardens, herbal garden, cryopreservation etc (Pavendan *et al*., 2012; Bastin and Jeyachandran, 2015).

The *in-situ* conservation is more difficult and may not always be feasible due to limitation of resources and accessibility of the area. *Ex-situ* conservation, on the other hand,

involves a higher degree of protection and consequently greater isolation of germplasm than *in-situ* conservation. Even where *in-situ* conservation is carried out like biosphere reserves, wildlife sanctuaries have been set up, the status of RET (rare, endangered and threatened) species have not been systematically investigated.

To strengthen *in-situ* conservation of the 'CR' plant resources, the following three factors are essential and may be incorporated in all conservation oriented programmes: (a) Prevention of destruction of populations and their habitats; (b) Maintenance and enhancement of the population level and variability; (c) Prevention of collection and excessive commercial exploitation of species (Withers, 1990).

OBJECTIVES OF THE PRESENT INVESTIGATION

With the above background, the present study aimed to assess the populations of *Phyllanthus talbotii* Sedgw. and *Pseudoglochidion anamalayanum* Gamble and to locate new population in their geographical distributional range, ecology, phenology and find its taxonomic position using molecular sequences such as ITS, *mat*K, and *rbc*L. The efforts were also made to develop simple and rapid regeneration protocol using *in vitro* propagation techniques and also by vegetative propagation using stem cuttings, air layering and root suckers.

As a part of the study, we have investigated the genetic variation of these two critically endangered plant species using ISSR markers. Such information will contribute to the better understanding of the genetic profile of the plant populations that can be used to develop strategies for their conservation. This will also become a starting point for further research on

population and evolutionary genetics of these species. Hence, the present work was undertaken with the following specific objectives.

1. To survey and estimate the populations, study the ecology (environmental and phenological data) of the two critically endangered plants *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum* from Western Ghats.

2. To develop and standardize the protocol for vegetative propagation with stem cuttings using various plant growth regulators for both *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum***.**

3. To develop and standardize the protocol for *in-vitro* regeneration, mass multiplication, hardening and field transfer of *Phyllanthus talbotii*.

4. *In-situ* conservation of *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum* by reintroduction into their natural habitats to establish new populations, enrich the existing natural populations and monitor their growth and performance.

5. Assessment of genetic diversity and variations within and among the populations of *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum* using inter simple sequence repeats (ISSR) molecular markers and systematic positions using molecular gene sequences.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Genus *Phyllanthus*

Phyllanthus L. is the largest genus in family Phyllanthaceae estimating over 1,270 species worldwide spreading over the Asian, African, Australian and American continents (Unander *et al*., 1995; Govaerts *et al*., 2000; Mabberley, 2008; Kathriarachchi *et al*., 2006). Hooker (1887) in his Flora of British India provided the first comprehensive taxonomic treatment of this genus for India. *Phyllanthus* dominates the tribe *Phyllantheae* Dumort., being the largest genus in the family Phyllanthaceae. It has a wide variety of floral morphologies and chromosome numbers and has one of the widest varieties of pollen types than any other plant genus (Webster and Carpenter, 2002). Despite their variety almost all *Phyllanthus* species express a specific type of growth called "phyllanthoid branching" in which the leaves on the main (vertical) plant axes are reduced to scales called "cataphylls" while leaves on the other axes plagiotropic (horizontal), deciduous and floriferous (flower-bearing) develop normally.

Earlier, Roxburgh (1832) described 25 species of *Phyllanthus*. Webster (1956, 1957, 1958) divided the genus into eight subgenera namely *Phyllanthus* subgen. *Isocladus* G.L. Webster, *Phyllanthus* subgen. *Kirganelia* (Juss.) G.L. Webster, *Phyllanthus* subgen. *Emblica* (Gaertn.) Kurz, *Phyllanthus* subgen. *Phyllanthus* L., *Phyllanthus* subgen. *Cyclanthera* G.L. Webster, *Phyllanthus* subgen. *Eriococcus*, *Phyllanthus* subgen. *Conami* (Aubl.) G.L. Webster, *Phyllanthus* subgen. *Xylophylla* Scheele and over 62 sections based on vegetative architecture and pollen morphology in addition to floral characters (Kathriarachchi *et al*., 2006).

2.4. Geographical Distribution

Genus *Phyllanthus* is mostly confined to humid tropics of the world (Govaerts *et al*., 2000). In India, 53 species of *Phyllanthus* has been reported (Gangopadhyay *et al*., 2007). *Phyllanthus* is distributed in all tropical and subtropical regions of the Earth. Leaf-flower is the common name for all *Phyllanthus* species (Bancilhon, 1971). *Pseudoglochidion* Gamble belonging to family Euphorbiaceae and it is an endemic monotypic tree genus found in evergreen forests of Anamalai hills of Valparai, Coimbatore District, Tamil Nadu, at an altitude of 1400m.

2.5. Habit and Habitat

Phyllanthus species have varying habit to adjust different environmental conditions showing remarkable diversity of growth forms and floral morphology including annual and perennial herbaceous, arborescent, climbing, floating aquatic, pachycaulous and phyllocladous. *Phyllanthus* species are known to occur globally, mainly tropical and subtropical regions of the world. It is found growing in all kinds of habitats including areas like yards, fields, roadsides, river side's and all forest types because of its diverse growth forms. *Pseudoglochidion* is a small tree, growing up to 10–12 m high. Mostly erect tree and sometimes branched especially those found along the road sides. Habitat is very specific and confined to evergreen forest with canopy density of 50 to 80%. The plants are found to be absent where vegetation is very thick and canopy is multi-storied.

2.6. Chemical Constituents

Phyllanthus species have been traditionally used to cure a wide number of diseases. In Indian ayurvedic medicine, various herbaceous *Phyllanthus* species are commonly known as Bhui Amla, a name previously assigned to *Phyllanthus niruri* L. only. Bhui Amla is prescribed for jaundice, gonorrhea and diabetes (internal use) as well as poultices, skin ulcer and other skin problems (external use) (Naik and Juvekar, 2003; Samali *et al*., 2012).

Phyllanthus primarily contains lignans (phyllanthine and hypophyllanthin, urinatetralin, dextrobursehernin, urinaligran), alkaloids (phyllanthoside), flavonoids, terpenes (Quercetin, Quercetol, quercitrin), tannin and polyphenolic compounds (methyl brevifolin carboxylate, trimethyl ester dehydrochebulic acid, n-octadecane, beta-sitosterol, ellagic acid, daucosterol, kaempferol, quercetin, gallic acid, rutin) (Williamson, 2002; Samali *et al*., 2012). The current research has focused on *Phyllanthus* potential for the treatment of Hepatitis B virus. Indeed, studies suggest that it may suppress the growth and replication of the virus (Venkateswaran *et al*., 1987; Lam *et al*., 2005; Sheng *et al*., 2014).

2.7. Conservation of Rare and Endangered Plants

The major threat to 'CR' plant populations are human activities, land clearing for agriculture, mining and urban development, weed invasion, grazing and collection of plants for medicinal, horticulture and ethnobotanical reasons (Coates and Dixon, 2007). The habitat fragmentation, removal of key species from the particular ecosystem, susceptibility to fire, pollinator decline and introduction of undomesticated animals have resulted in drastic losses

in plant diversity and populations (Lienert, 2004; Thomas, 2004; Coates and Dixon, 2007; Saw *et al*., 2010). Smaller population size, isolation of population due to habitat destruction and forest degradation leads to significant losses of unique evolutionary lineages (Coates, 2000; Hopper, 2000; Charlesworth and Willis, 2009).

During the formulation of policies for conservation programmes, existing and future environmental threats such as taxonomic distinctiveness, geographic distribution, habitat specialization, reproductive biology, evolutionary processes influencing population structure and *ex-situ* conservation methods should be taken in to consideration (Havens *et al*., 2006). *Ex-situ* conservation is often taken as the key conservation aspects, but preservation of germplasm off-site should be considered as an 'emergency ward' targeting at extinction of those species under the immediate threat. Despite of off-site conservation such as seed and germplasm banks and *in-vitro* propagation, the *in-situ* conservation and conservation via assisted migration are the premier approaches for global biodiversity conservation (Havens *et al*., 2006; Singh and Tekale, 2009). The effective conservation strategies must be developed to avoid further loss of essential species and ecosystem (Havens *et al*., 2006). The adaptive management approach was one of the useful methods for linking research to operational practicality in management and restoration of species and ecosystems (Bormann *et al*., 2007). Integrated conservation approach mainly depends on the understanding of ecological and genetic studies, *in-situ* research and *ex-situ* propagation (Falk, 1990; Singh and Tekale, 2009).

2.8. Conservation of Plants Through Tissue Culture

The studies have been carried out for the conservation of endangered and rare plant species through biotechnological tools. Plant tissue culture plays an important role in

propagation and mass multiplication of 'CR' plants (Seeni and Latha, 2000; Decruse *et al*., 2003). *In vitro* propagation under controlled laboratory conditions have proven to be a potential technique for mass production of critically endangered plant species (Faisal and Anis, 2003; Martin and Pradeep, 2003; Pacheco *et al*., 2009; Chandore *et al.,* 2010; Sharma *et al*., 2010; Rani and Dantu, 2012; Daniel *et al*., 2015).

2.8.1. Tissue Culture Studies in Genus *Phyllanthus*

The achievements and advances in *in-vitro* regeneration of the genus *Phyllanthus* are reviewed. Bhattacharyya and Bhattacharyya (2001) used Murashige and Skoog's medium (MS) supplemented with kinetin (KIN), 6-benzylamino purine (BAP) singly or in combination with indole-3-acetic acid (IAA) to develop direct regeneration of shoots from shoot tip culture of *Phyllanthus amarus* Schumach. & Thonn.

Banu and Handique (2003) developed protocol for *in-vitro* regeneration of medicinal plants *Phyllanthus fraternus* G. L. Webster using nodal explants by MS medium supplemented with 1 mg/L BAP and 0.2–0.3 mg/L IAA and rooting was achieved with 87% of the micro shoots on MS medium containing 1mg/L IAA and 0.5 mg/L indole-3-butyric acid (IBA). No rooting was seen without plant growth regulators.

Catapan *et al*. (2000) developed efficient micropropagation protocol for the medicinal plant *Phyllanthus caroliniensis* Walter used nodal segments for axillary shoot proliferation. Maximum regeneration (21–23 shoots per ex-plant) was achieved on MS or Anderson Rhododendron (AR) medium supplemented with either 5 µM BA or 1.25–5.0 µM kinetin or 2.5–5 µM 2-isopentanyl adenine (2-iP). Rooting was achieved 80–100% of the micro shoots

on MS medium without growth regulators, although 1.25 µM 1-naphthaleneacetic acid (NAA) and 1.25–5.0µM IAA promoted significant increase in the number of roots per ex-plant.

Shekhawat and Dixit (2007) developed a protocol for large scale cloning and plant production of *Phyllanthus amarus*. Explants such as nodal shoot segments were used. After sterilization the explants such as axillary buds, meristem were inoculated on MS medium + 2.0 mg/L 6-benzylaminopurine (BAP). Multiple shoots of *P. amarus* were obtained on modified MS (MMS) + 0.1 mg/L IAA + 0.5 mg/L each of BAP and kinetin. Rooting of *in vitro* shoots was done on half-strength MS medium containing 2.0 mg/L IBA + 2.0 mg/L IAA with 100 mg/L activated charcoal, the success of rooting of shoots was 78%.

Malayaman *et al*. (2014) standardized tissue culture protocol for the medicinal plant *Phyllanthus debilis* J.G. Klein ex Willd. by using explants such as leaf and inter nodal segments on MS medium with different concentrations and combinations of various growth regulators. Maximum callus was produced on the leaf segments after 45 days (82.5%) when MS medium supplemented with BAP (3.5 mg/L), NAA (2.5 mg/L) and 2, 4-D (0.5 mg/L). The inter-nodal callusing (80%) was recorded in MS medium contained 3.0 mg/L BAP, 2.0 mg/L NAA and 0.5 mg/L 2, 4-D. It was observed that when hormonal concentration is low the explants produced scanty callus.

Catapan *et al*. (2002) developed an efficient micropropagation protocols for callus culture and root culture of medicinal plant *Phyllanthus urinaria* L. by using single node explants. Maximum multiple shoots *i.e.* 16 to 20 shoots per explants was achieved on MS media supplemented with 5.0 µM kinetin. MS and AR (Anderson Rhododendron) media

promoted good shoot growth and number of shoots per explants. Successful rooting was recorded (93–100%) of the micro shoots on MS medium with absence of growth regulators, although NAA concentration of 1.25–5.0 µM significantly increased the number of roots per explants. After successful acclimatization of regenerated plant about 91% of plantlets survived when transferred to the field. It was observed that micropropagated plants produced flowers after 3 to 4 weeks of acclimatization. High amount of callus formation and growth was achieved when single node explants were inoculated horizontally on MS medium with 5.0 µM IBA. Growth regulators such as 2, 4-D and NAA also induced moderate callusing when used separately. Establishment of root cultures was successful on MS medium containing 1.1 µM NAA.

Chitra *et al*. (2009) standardized the protocol for indirect organogenesis by using internodes and leaf bits of *Phyllanthus amarus*. Callusing of internodes and leaf discs were obtained on MS medium supplemented with NAA and 2, 4-D. The sub culturing of callus was repeatedly done at an interval of three weeks for four cycles. High amount of callusing was obtained on MS medium supplemented with BAP (1.0 mg/L) and glycine (50.0 mg/L). When the callus was sub-cultured on MS medium supplemented with BAP (2.0 mg/L) and GA_3 (0.5 mg/L) showed complete plantlets formation. Rooting (87.09%) was successfully achieved with IBA (0.5 mg/L) and IAA (0.5 mg/L) on half strength MS medium. Regenerated plants were successfully transferred to field after acclimatization.

Victorio *et al*. (2010) developed a protocol for direct and indirect organogenesis of *Phyllanthus tenellus* Roxb. by using nodal segments obtained *in vitro* germination of plantlets. The nodal segments were subculture on modified MS medium along with different plant

growth regulators such as IBA, IAA, KIN and GA_3. The proliferation rate was high when used in combinations like IBA, KIN + gibberellic acid (GA_3) (3.5 mg/L) and IBA + KIN (2.4 mg/L). Rooting was achieved 100% after 45 days when media was supplemented with IBA for rooting. MS medium in combination of 2, 4-D showed the production of friable callus.

Sen *et al*. (2009) developed an *in vitro* regeneration protocol for the medicinally potent plant species *Phyllanthus amarus* by using nodal segment as explants. Maximum number of multiple shoots *i.e.* 15.275±0.96 was recorded on MS medium along with BAP 0.5 mg/L after 3 to 4 weeks of inoculation. The sub culturing was done by separating the shoots from the main cluster for elongation (BAP 0.5 mg/L). *In vitro* rooting was achieved on half strength MS medium in combination with IBA 0.5 mg/L. After acclimatization, the regenerated plants showed 80% survival.

Thilaga *et al*. (2013) developed an efficient protocol to induce high frequency of somatic embryogenesis from *in vitro* derived juvenile leaf tissues of *Phyllanthus emblica* L. on MS medium. A total of twelve combinations of MS medium supplemented with different plant growth regulators such as 2,4-D, IAA, BAP and kinetin in various concentrations. Highest callusing (67.5%) was on media containing 0.45 µM 2,4-D along with 22 µM BAP. After 60 days of inoculation, three distinct types of callus were induced (Type I, II and III). Callus after repeated sub culturing led to somatic embryogenesis. Maturation of somatic embryos was achieved within two weeks when treated with 3.78 µM abscisic acid (ABA). After that matured embryos were transferred to MS medium supplemented with 0.46 µM KIN for the further development into healthy plantlets. After acclimatization about 90% plantlets were transferred to field establishment.

2.8.2. Conservation of Plants Through Vegetative Propagation

Vegetative (asexual) propagation has an advantage over the plants raised through seeds because of zygotic seed formation. Plantlets developed through vegetative propagation has the ability to maintain desirable traits (elite clone) and characters due to maintaining superior genotypes which can increase the yield, disease resistance, survivability and quality of products (Leakey and Newton, 1994; Mudge and Brennan, 1999). Other advantages of vegetative propagation are shortening of flowering, uniform population and act as a source of plant material for propagation when seed set is low or sometimes is the only means of propagation (Mudge and Brennan, 1999). Other advantages of vegetative propagation include shortening of flowering period and uniform distribution of their population. It also acts as source plant material for propagation when seed set is low or sometimes is the only means to propagate.

Vashistha *et al*. (2009) worked on high yielding medicinal and aromatic plant species namely *Angelica glauca* Edgew. and *A. archangelica* L. (Apiaceae) from Himalayan regions. Due to over exploitation their *ex-situ* cultivation is recommended for conservation, sustainable supply of raw material for ethno-medicinal uses and pharmaceuticals companies. Vegetative propagation protocol was standardized using root inducing hormones such as IBA, IAA and GA_3 in different concentrations (100, 200 and 500 ppm). Among the hormones, IBA 100 ppm responded better in both the species.

Eganathan *et al*. (2000) developed a large scale propagation protocol for three mangrove species: *Excoecaria agallocha* L., *Heritiera fomes* Buch.-Ham. and *Intsia bijuga* Kuntze. by using stem cuttings and air layering. IBA alone up to 2500 ppm showed maximum rooting in cuttings and also in air layers of all the three species. The month of October was found to be best for plantation of cuttings and initiation of air layering. After hardening all the plants were transferred into the mangrove forests of Pichavaram, Tamil Nadu, India.

Akwatulira *et al*., (2011) developed a protocol for vegetative propagation of *Warburgia ugandensis* Sprague an important medicinal tree species whose bark is having anti-bacterial and anti-fungal properties. This tree is considered to be threatened and therefore becomes a priority species for conservation and management. During this study, three types of stem cutting were used, viz; hardwood, semi-hardwood and softwood. IBA was used as a root inducing hormone. The data on callusing, rooting, root length and shoot formation was recorded over a period of 93 days. The results showed significant variation ($p < 0.05$) in root and shoot development, number and length of roots and shoots and callus formation for different stem cutting types. The softwood cuttings recorded highest percentage of callusing, rooting and shoot regeneration (46, 49 and 57%) when compared to hardwood and semi-hardwood cuttings.

Danu *et al*. (2015) standardized the vegetative propagation protocol for *Paris polyphylla* Sm. an important medicinal plant of Himalayan region. Due to over exploitation, pressure on the natural habitats required a conservation plan. The propagation method was developed using different combinations of IBA and GA_3 and various soil compositions.

Combination of 100 mg/L IBA and 100 mg/L GA_3 showed maximum rooting percentage along with the potting medium composition of soil: loam: sand (3:2:1).

Nadeem *et al*. (2000) developed a vegetative propagation protocol for *Podophyllum hexandrum* Royle, a medicinal plant and highly valued for podophyllotoxin. Due to its heavy collection and increasing market demand it has been over exploited in the wild. To standardize vegetative propagation protocol, the rhizome segments were treated with NAA and IBA before planting. The root formation started 100 days in all the rhizome segments irrespective of treatment. The results showed that NAA 10 µM (30.0±5.6) and 100 µM (40.0±0.0) and the lower concentration of IBA 10 µM (40.0±4.7) had little effect on rooting percentage when compared with higher concentration of IBA 100 µM (70.0±4.7), control showed 30.0±4.7 rooting percentage.

Chandra *et al*. (2006) developed vegetative propagation protocol using rooting runner cuttings of *Picrorhiza kurrooa* Royle, a medicinal herb from Himalaya and a potential source of patoprotective picrosides, and due to its over exploitation from its natural habitat it has been listed as 'endangered'. Vegetative propagation was carried out by using IBA and NAA. The cuttings treated with 50 µM IBA showed 87% of rooting when compared to other.

2.9. Phylogenetic Studies

In recent years, molecular biological information have provided significant contributions to understand the systematic and solving several evolutionary problems of plants at scales ranging from relationships among the major lineages of land plants to relationships within individual genera (Palmer *et al*., 1988; Taberlet *et al*., 1991; Dayanandan *et al*., 1997;

Olsen and Schaal, 1999; Whitlock and Baum, 1999; Mathews *et al*., 2000). Specific genes can be easily amplified from a wide range of taxa that have levels of sequence variation appropriate to study population level or for studying closely related species (Schaal *et al*., 1998).

The sequences of different genes are most widely used to understand the phylogeny particularly above the genus level (Straub *et al*., 2012; Zimmer and Wen, 2012). Variations in the coding and spacer regions of a number of chloroplast, mitochondrial and nuclear gene sequence are utilized. Most widely used genes are ribulose-bisphosphate carboxylase gene (*rbc*L) and MaturaseK gene (*mat*K), both are coding genes. Internal transcribed spacers (ITS) of the ribosomal DNA is also used to understand the phylogenetic relationship among plant species (Chase *et al*., 1993; Clegg *et al*., 1994; Hsiao *et al*., 1994; Clark *et al*., 1995; Zhang and Wendel, 1995; Alvarez and Wendel, 2003). The data in sequence are cumulative, the potential sizes of informative data sets are immense, and the data are available in public domains like National Center for Biotechnology Information (NCBI). These sequences of data have been used and analyzed by different researchers and taxonomist to address in assessing the plant systematic and phylogeny above the family or even genus level (Donoghue *et al*., 1992; Chase *et al*., 1993; Duvall *et al*., 1993; Shaw *et al*., 2007).

2.9.1. Internal Transcribed Spacers (ITS)

The ITS, nuclear ribosomal DNA (nrDNA) sequences contain potential informative sites and it is a useful molecular marker in studying phylogeny of many taxa mainly endemics and endangered plant species (Lee *et al*., 2006; Vorontsova *et al.*, 2007; Juthatip *et al*. 2010; Luo *et al*. 2011). The ITS region separating 18S and 28S nrDNA and the 5.8S coding

sequences have become widely characterized across the inter-specific and inter-generic level divergence. The high copy number allows easy amplification of the region from the total DNA (Baldwin *et al*., 1995). The ITS region is divided into two *i.e.* ITS-1 and ITS-2, both immediately flanking the 5.8S gene sequence, with former upstream and the latter downstream of that sequence. Both ITS regions (ITS-1, 5.8S, ITS-2) are ranges between 565 and 700 base pairs in almost all angiosperms with some exceptions (Maggini *et al*., 1998).

2.9.2. MaturaseK (*mat*K)

The *mat*K gene, earlier known as *orf*K, is another most widely used. The *mat*K gene also potentially contributes to plant molecular systematic and evolutionary studies (Johnson and Soltis, 1994, 1995; Steele and Vilgalys, 1994; Liang and Hilu, 1996; Kathriarachchi *et al.*, 2005; Samuel *et al.*, 2005). The *mat*K gene with about 1500 base pairs (bp) is located within the intron region of the chloroplast gene *trn*K, on the large single-copy section adjacent to the inverted repeat. The *mat*K gene has been used effectively in studying systematic problems in the families like Saxifragaceae (Johnson and Soltis, 1994, 1995), Polemoniaceae (Steele and Vilgalys, 1994; Johnson and Soltis, 1995), Poaceae (Liang and Hilu, 1996), Orchidaceae tribe Vandeae (Jarrell and Clegg, 1994).

2.9.3. tRNA^{Leu} (UAA) *trn*L

The *trn*L is a chloroplast gene (cpDNA) is one of the most frequently used gene in studying the phylogeny of many less known plant species (de Groot *et al*., 2011). Chen *et al*. (2013) used to identify the fern gametophyte which plays an important role in ferns life cycle. James and Schmidt (2004) used *trn*L gene in identification of potential allergens in specific food crops like peanut, soyabean, canola, potato, wheat, corn and rice. Nepal and Ferguson

(2012) made use of *trn*L gene in studying the phylogeny and to understand natural genetic relationship among 13 species of *Morus* (Tribe: Moreae; Moraceae). Richardson *et al*. (2000) studied the phylogenetic analysis of Rhamnaceae using *trn*L and other gene sequences.

2.9.4. Ribulose-bisphosphate carboxylase gene (*rbc*L)

To understand plant phylogeny of different taxonomic groups, chloroplast DNA (cpDNA) is being extensively used (Clegg and Zurawski, 1991). The direct gene sequencing of polymerase chain reaction (PCR) products has become a rapid expanding area of plant systematics and evolution. The *rbc*L gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase / oxygenase (RUBISCO), which is sequenced from numerous plant taxa, is extensively used in plant phylogeny (Palmer *et al*. 1988; Chase *et al*. 1993 and Clegg *et al*., 1994).

2.9.5. Molecular Phylogenetic Studies in Genus *Phyllanthus*

Lee *et al*. (2006) studied the phylogenetic relationships between 18 *Phyllanthus* species commonly found growing in China. During this study DNA gene sequencing such as ITS1 and ITS2 along with *atp*B (chloroplast gene) and *rbc*L sequences were used. They showed that the cladistic results of this genus is paraphyletic and supported two confusing species, *P*. *niruri* and *P*. *amarus* are two closely related, but they are of different species. Also developed ITS (nrDNA)-based multiplex PCR assay for *P*. *amarus*, *P*. *niruri* and *P*. *urinaria*.

Hoffmann *et al*. (2006) studied the phylogenetic classification of Phyllanthaceae using DNA sequence data of nuclear PHYC (phytochrome C) and plastid *mat*K, *atp*B, *ndh*F (chloroplast NADH dehydrogenase F) and *rbc*L along with its morphological characters.

Using the gene sequences they have developed two new clades which were missing in the earlier classifications.

Vorontsova *et al*. (2007) studied the phylogenetic relationships, morphology and distribution of species *Andrachne cuneifolia* Britton by analyzing the nuclear gene ITS and plastid *mat*K sequences. After the scientific study, dendrogram confirmed that this species belongs to genus *Phyllanthus* and subgenus *Xylophylla*. Morphological characters with closely related species also supported the species placement. Later, it was correctly named as *Phyllanthus cuneifolius* (Britton) Croizat.

Bandyopadhyay and Raychaudhuriy (2010) studied five medicinally important species of genus *Phyllanthus* namely *P. emblica*, *P. reticulatus*, *P. amarus*, *P. fraternus* and *P. urinaria* with the help of ITS 1 and ITS 2 sequences to identify them correctly because of their similar vegetative characters and habitats. The phylogenetic tree generated using ITS 1 sequence was found more potential than ITS 2 in assessing their link at molecular level. Sequence characterized amplified regions (SCAR) markers were designed for *P. urinaria*, *P. amarus* and *P. emblica* based on ITS 1 sequences which helped in distinguishing these three species from other closely related species.

Manissorn *et al*. (2010) analyzed the nucleotide sequences of 56 plant samples which belong to 23 species of *Phyllanthus*. Plants were collected from their natural habitats in Thailand and ITS spacers of ribosomal DNA sequence were used. The phylogenetic trees were constructed for all the *Phyllanthus* species studied. A simple protocol was developed using a PCR-RFLP method to distinguish three main medicinally

important *Phyllanthus* species, namely *P. amarus*, *P. debilis* and *P. urinaria* and successfully applied to analyze the crude drug samples available in Thailand markets.

Luo *et al*. (2011) studied the systematic position of the most widespread species of *Phyllanthus* i.e. *P. reticulatus*, which is being found almost all over the world including Asia, Africa, Australia and Jamaica. By using nuclear ITS sequences, broad treatment of *P. reticulates* has been worked out. The study showed that *P. reticulatus* collected from Jamaica is originally introduced from Asia and not Africa, because the African *P. reticulatus* is entirely different based on the ITS sequences and formed separate clade in the phylogenetic tree. The study revealed that the *P.reticulatus* is introduced to Jamaica without its pollinators (*Epicephala* moth), the pollination does not take place and hence no fruiting is observed and show only vegetative growth phase.

Naik *et al*. (2013) studied the systematic position of *Phyllanthus talbotii* (Phyllanthaceae) using ITS and *mat*K gene sequences. The phylogenetic tree obtained by analysing the ITS and *mat*K confirms its position in subgenus *Eriococcus*.

2.10. Genetic Diversity Studies Using Molecular Markers

In recent years, conservation of endangered plant species is achieved by understanding the genetic profile of particular species in a given population for the specific area and this is the first step for their conservation. The understanding of genetic diversity in a particular species is important in the view point of management and conservation (Geburek, 1997; Rao and Hodgkin, 2002; Mehrotra *et al*., 2012). The level of genetic diversity and variation within and among populations are essential for developing suitable conservation methods and

sustainability (Barton and Keightley, 2001). Population genetic studies have been carried out to understand the loss of genetic diversity and also to restore the threatened populations (Hamrick and Godt, 1996). Loss of genetic variation is the major problem in conservation of particular species, which can prevent a species from natural selection and limit its evolutionary potential (Qamaruz-Zaman *et al*., 1998).

Population genetic diversity studies are essential for conservation program and restoration of threatened populations (Hamrick and Godt, 1996; Chaurasia *et al*., 2009; Kumar *et al*., 2014). The several DNA markers are now used in diversity studies of many plants. The most commonly used marker systems are inter simple sequence repeats (ISSR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and microsatellites or simple sequence repeats (SSR). All these molecular markers are used to characterize DNA variation patterns within the species and among the closely related taxa (Albertson *et al*., 1999; Bakkeren *et al*., 2000; Billotte *et al*., 2001; Wolfe and Randle, 2001; Sica *et al*., 2005; Parab *et al*., 2008; Parab and Krishnan, 2009; Muthusamy *et al*., 2008; Shilpa *et al*., 2013; Kong *et al*., 2014; Linos *et al*., 2014).

2.10.1. Use of RAPD Marker to Understand Genetic Diversity

Plant genetic diversity using RAPD markers have been successful because of the availability of a large number of universal primers and the requirement of small quantity of plant genomic DNA for analysis (Williams *et al*., 1990; Huff *et al*., 1993; Ge *et al*., 1999; Nybom and Bartish, 2000; Kingston *et al*., 2004). RAPD technique is also more suited to endemic and 'CR' plant species since very little is known about the genetic diversity within the

natural populations (Xiaohong *et al*., 2007). RAPD markers have also been commonly used for genetic diversity analysis of plant populations in breeding programme, germplasm collections, genotyping, genome mapping and phylogenetic studies in closely related plants species of same family and genus (Vorontsova *et al*., 2005; Okumus, 2007; Gahlaut *et al*., 2013).

2.10.2. Use of ISSR Marker

ISSR markers are used for the detection of polymorphism in inter-microsatellite loci using specific primers (Wu *et al*., 1994; Zietkiewicz *et al*., 1994; Senapati *et al*., 2011). ISSR markers show dominant inheritance, which are being used as an alternate tool in genetic diversity studies (Tsumura *et al*., 1996). Since, no sequence information of a target DNA of particular species is required, ISSR markers are more suited for the plant species (Xiaohong *et al*., 2007). ISSR analysis is a popular method for estimating genetic diversity of plant populations with several advantages such as speed, low cost and use of small amounts of genomic DNA etc. In recent years, ISSR markers have been applied in genetic studies of many 'RET' plant species. Also, these markers are highly sensitive, reproducible and effective technique for the genetic studies of plants (Luo *et al*., 2007; Parab *et al.,* 2008; Parab and Krishnan, 2009).

2.10.3 Genetic Diversity Studies in Genus *Phyllanthus*

In *Phyllanthus*, number of molecular markers has been used to understand the genetic diversity. Jain *et al*. (2003) studied the genetic variability of *Phyllanthus amarus* found in India by using RAPD analysis. A total of 33 collections from different localities around the country were made covering the states of Maharashtra, Karnataka, Tamil Nadu, Gujarat, West

Bengal, Assam, Tripura, Uttar Pradesh, Punjab and Haryana. Results obtained through un-weighted pair group method (UPGMA) showed up to 65% variation in all 33 samples. The study showed that random hybridization must have taken place across the populations, within the possible cross pollination range.

Li and Zhao (2007) studied the genetic diversity of four populations of *P. emblica* sampled from dry-hot valleys of Yunnan, China, using ISSR markers. A total of 12 ISSR primers were used in the study. Banding pattern showed 135 clear and reproducible DNA fragments, of which 115 were polymorphic (85.19%). The results revealed that the *P. emblica* populations have no significant association between genetic and geographical distances.

Palaniappan and Marappa (2008) investigated the genetic diversity in *Phyllanthus amarus* and its two closely related species, *Phyllanthus virgatus*, and *Phyllanthus debilis* using RAPD and ISSR markers. A total of 54 genotypes of *P. amarus* and 3 genotypes each of *P. virgatus* and *P. debilis* were taken from different parts of Tamil Nadu, India. For the study 16 RAPD and 8 ISSR primers were used. The RAPD and ISSR markers showed the average polymorphism of 68.2% and 69.7% respectively across all 54 genotypes of *P. amarus* and after the addition of the *P. virgatus* and *P. debilis* genotypes, the average polymorphism was increased to 97.5% and 96.5% respectively. The cluster analysis of all the 60 genotypes of all three species using both RAPD and ISSR markers revealed *P. virgatus*, *P. amarus* and *P. debilis* are distinct species of genus *Phyllanthus*.

Theerakulpisut *et al*. (2008) studied the DNA fingerprinting by using RAPD-PCR techniques for *Phyllanthus amarus,* a highly used medicinal plant in traditional medicine in

Thailand with its two intimately related species namely *P. debilis* L. and *P. urinaria* Klein ex Willd., with less effective medicinal properties and these two species can be easily misguided as *P. amarus*. Species specific RAPD markers were first identified and later primers for SCAR were designed from nucleotide sequences of definite RAPD markers. This method was highly specific and rapid for plant identification when analyzed against the DNA of closely related species.

Chaurasia *et al*. (2009) investigated the genetic variability using RAPD markers of commercially cultivated varieties of Indian gooseberry (*Phyllanthus emblica*), widely used as a drug due to its nutraceutical properties. Cluster analysis shown that the three groups were formed within the varieties of *P. emblica* which directly associated to their place of origin. RAPD markers used were also capable to differentiate varieties of same parents and same origin.

Rout and Aparajita (2010) studied the phylogeny of twelve *Phyllanthus* species from India for identification of germplasm and phylogeny study for conservation with the use of molecular markers mainly ISSR and RAPD. Nei's similarity index varies from 0.26 to 0.81 for ISSR and 0.23 to 0.76 for RAPD marker. Cluster analysis was performed by UPGMA. The phylogenetic tree obtained from both ISSR and RAPD markers formed two groups: Group I comprising of only one species *Phyllanthus angustifolius* (Sw.) Sw. Group II was with remaining 11 species *P. nivosus*, *P. flaternus*, *P. reticulus*, *P. acidus*, *P. nivosus* "*Varigata*", *Phyllanthus* spp "Àcc No.1", *P. rotundifolius*, *P. emblica*, *P. uninaria*, *P. virgatus*, *P. amarus*, thus showing the comparable to notable morphological characteristics and genetic variation among all twelve species of *Phyllanthus.*

Bandyopadhyay and Raychaudhuri, (2013) developed SCAR markers by employing RAPD and AFLP for the identification of five species genus *Phyllanthus* which are being used in traditional medicine and herbal drug industries. The results showed that RAPD data separated the *P. urinaria* from the remaining four species, but however AFLP method was successful in distinguishing all the herbs, *i.e.*, *P. amarus*, *P. fraternus* and *P. urinaria*.

Upadhyay *et al*. (2014) studied the clonal fidelity of synthetic seed-derived from *Phyllanthus fraternus* plantlets by comparing with the mother plant using RAPD and ISSR. The results showed no changes in molecular profiles of the synthetic seed-derived plantlets and mother plant, thus confirming the genetic stability in regenerates.

Singh *et al*. (2014) studied the genetic diversity of eight varieties of *Phyllanthus emblica*, the Indian gooseberry, found in arid regions by using RAPD markers. The ten RAPD primers used in the investigation exhibited 56.18 % of polymorphism and the study reveals the existence of genetic diversity among the eight varieties of *P. emblica*.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study was carried out as a part of Ph.D. programme of Department of Botany, Goa University, during the period from April 2009 to April 2015. Details of the methodology followed for the study is presented in this chapter.

3.1. Study Area

The study was carried out in the Western Ghats regions of Goa, Karnataka and Tamil Nadu, India. *Phyllanthus talbotii* Sedgw. is mostly confined to riverine habitats (fresh water river and stream beds) represented by deciduous, mixed deciduous and a few patches of lowland of dry evergreen forests at an elevation ranging from 20 m to 608 m in Goa and Karnataka. *Pseudoglochidion anamalayanum* Gamble occurence is very much restricted to the evergreen forest areas of Anamalai Tiger Reserve (1100–1400 m), Valparai, Tamil Nadu.

Field trips and surveys were carried out with the help of Forest Department, Goa and Tamil Nadu. These regions were thoroughly surveyed and sampled for the collection of both the plant species during the study period.

3.2. Survey and Estimation of Populations

Systematic field trips and surveys were carried out in Western Ghats regions of Goa, Karnataka and Maharashtra for the location of different population of *P. talbotii.* Field survey was also carried out to Anamalai Tiger Reserve, Valparai, Tamil Nadu for the location of the population of *P. anamalayanum*. During field survey both the species were collected from their naturally occurring population by referring earlier literature and

herbarium specimens. The species were photographed in their natural habitats. Seeds were collected, grown and maintained in poly-house at Department of Botany, Goa University, Panaji- Goa. Collected plant species from new populations were identified by using standard literature including local / regional floras and with herbarium specimens. During the field survey latitude and longitude of different population were recorded using handheld Global Positioning System (GPS). In addition, altitude, size of the population, number and height of the plants were recorded. In case of *P. talbotii* the number of ramets per plant was counted.

3.3. Study of the Ecology, Collection of Environmental and Phenological Data 3.3.1. Soil Analysis

To understand the soil profile of *P. talbotii* and *P. anamalayanum,* the rhizosphere soil samples were collected from all the population sites. From each population three soil samples were collected in the place where plants are growing abundantly and made as one composite sample and used for the analysis of pH, electrical conductivity (EC), macro and micro nutrient contents etc. based on standard references provided in Tables 1, 2, 3 (Bangar and Zende, 1978; Gajbhiye, 1985; Caruso *et al*., 2007). Soil samples were brought to laboratory, dried and sieved for further analysis. Soil testing was carried out in Soil Testing Laboratory of Farmers Training Centre, Ela Farm, Directorate of Agriculture, Old Goa, Goa.

Table 1. Minimum requirement of micronutrients (Fe, Mn, Zn, Cu) in soil, Gajbhiye (1985) (Standard).

Micronutrients	Critical limits (ppm)
Fe	6.0
Mn	15.0
Zn	0.50
Cu	0.80

Table 2. Berger-Parker index for soil macro-nutrients (standard).

Nitrogen	Ratings [kg/ha^{-1}]
Very low	< 140
Low	141-280
Medium	281-420
Moderately low	421-560
High	561-700
Very High	>701`
Potassium	**Ratings [kg/ha^{-1}]**
Very low	< 100
Low	101-150
Medium	151-200
Moderately low	201-250
High	251-300
Very High	>300
Phosphorus	**Ratings [kg/ha^{-1}]**
Very low	< 7
Low	7.1-14
Medium	14.1-21
Moderately low	21.1-28
High	28.1-35
Very High	>35

Table 3. Range and overall average of the pH and organic carbon, Banger and Zende (1978) (Standard).

pH	Ratings
Extremely alkaline	> 9.0
Strongly alkaline	8.4-9.0
Moderately alkaline	7.6-8.3
Slightly alkaline	7.1-7.5
Nearly Neutral	7.6-7.0
Slightly acid	6.0-6.5
Moderately acid	5.3-5.8
Strongly acid	4.5-5.2
Extremely acid	< 4.5
Organic Carbon	**Ratings [per cent]**
Very low	< 0.20
Low	0.21-0.40
Medium	0.41-0.060
Moderately low	0.061-0.080
High	0.081-1.00
Very High	>1.00

3.3.2. Phenology

The periodic field visits were carried out for the understanding of flowering and fruiting period. Vegetative and reproductive phases were recorded for both the species for the duration of four years from May 2009 to May 2013. The plant species associated with *P*. *talbotii* and *P*. *anamalayanum* were also recorded.

3.4. Vegetative Propagation of *Phyllanthus talbotii*

3.4.1. Stem Cuttings

Fresh stem cutting were collected from the natural populations which showed good and luxuriant vegetative growth with high number of individuals in the population and brought to plant nursery for providing hormonal treatments. Each stem cutting with two to three nodes and about 10–15 cm in length were obtained from healthy plants. The bottom portion of the cuttings was cut at a 45° angle just below the node as this considered being good for root initiation and top portion of the cutting was with straight cut. The bottom portion of the cuttings which was considered good for root initiation was cut at 45° angle just below the node and the top portion was with straight cut.

3.4.2. Hormonal Treatments

Plant hormones such as indole-3-butric acid (IBA), indole-3-acetic acid (IAA) and commercially available rooting powder namely rootex were used for induction of roots in stem cuttings. Stem cuttings were treated with 50, 100, 150, 200, 250 and 300 ppm of IAA and IBA for 24 hours. For rootex treatment, the stem cuttings at the base were made wet with water and then dipped in rootex powder and transferred to root trainers. The steps involved in vegetative propagation are provided in the form of flow-chart (Fig. 1).

Vegetative propagation

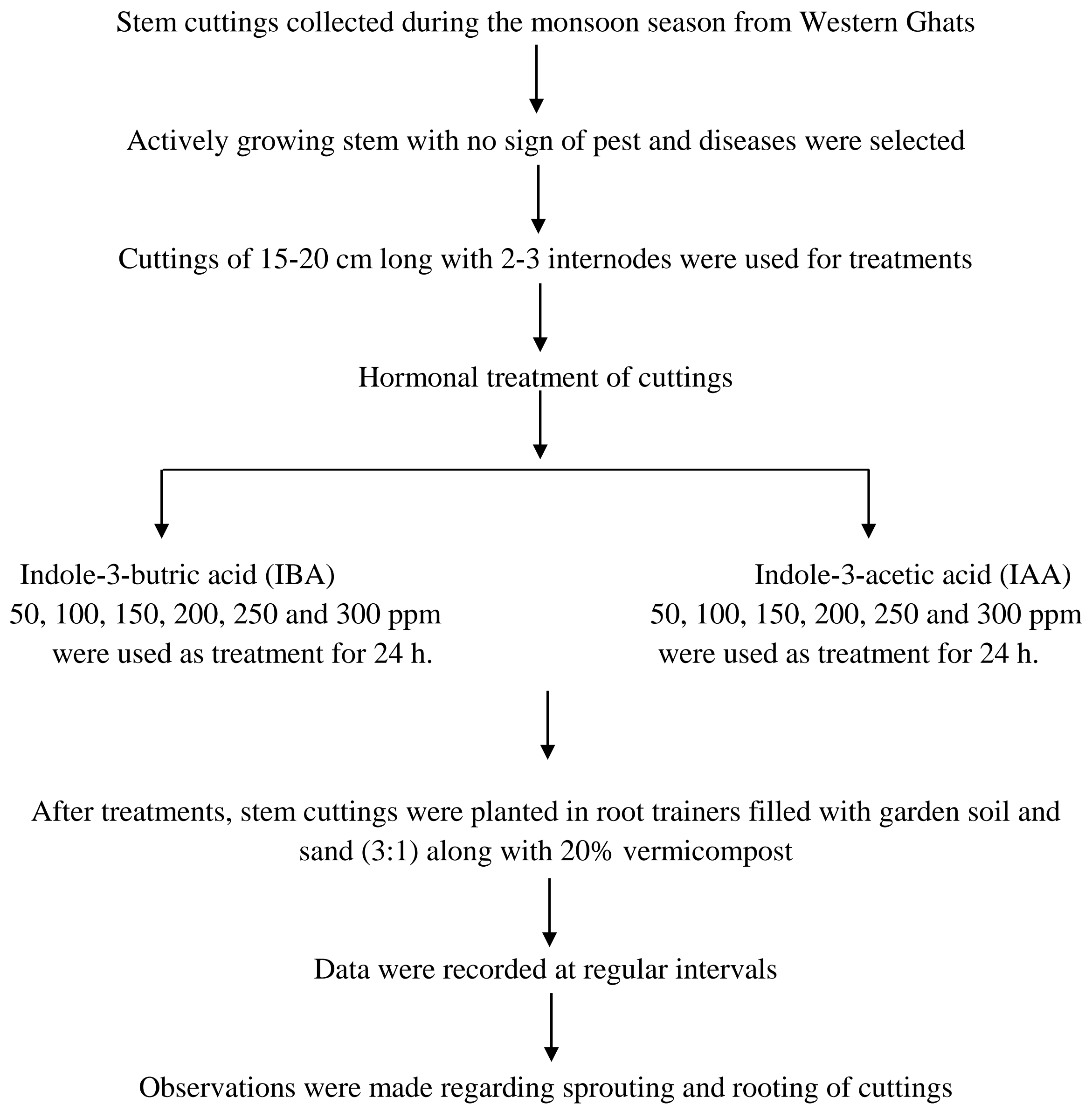


Fig. 1. Flow-chart of steps involved in vegetative propagation using stem cuttings.

3.5. Vegetative Propagation of *Pseudoglochidion anamalayanum*

3.5.1. Stem cuttings

The vegetative propagation methods followed for *P. anamalayanum* was same as that of *P. talbotii* as provided in section 3.4. except the changes in the hormonal concentrations.

3.5.2. Hormonal Treatment

A total of 400 stem cuttings were treated with hormones such as indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) of different concentrations (100, 250, 500, 750 and 1000 ppm).

3.5.3. Air-Layering

For the air-layering of *P. anamalayanum,* healthy stem bark region was scraped using grafting knife. The hormonal solutions of 1000 ppm of (IBA), (IAA) and commercial rootex were applied on the exposed region. Then handful of damp sphagnum moss mixed with coco peat and vermicompost in the ratio of (3:1:1) was placed around the cut surface of the stem. Before using the sphagnum moss was soaked in water for several hours to ensure that it holds sufficient moisture. Excess water was squeezed out from the sphagnum moss since it results in decay and deterioration of the plant tissue. Moss covered portion was wrapped using a polyethylene film with holes in them and both ends were tied using strong thread to ensure a tight seal.

3.5.4. Root Suckers

Root suckers are characteristics of *P. anamalayanum*. Around 40 to 50 root suckers were observed in each matured plant. A total of 555 root suckers were carefully removed from the main roots without damaging them. These root suckers were immediately transplanted directly in the field.

3.6. *In Vitro* Propagation of *P. talbotii*

3.6.1. Sterilization Procedure

3.6.1.1. Sterilization of Glassware and Culture Vials

The glassware and culture vials used for in vitro culture were first washed in tap water, soaked in dilute hydrochloric acid solution for the duration of two hours, followed by a detergent. Then they were washed and rinsed with distilled water and drained. Glasswares were sterilized by exposing to hot dry air oven at 100–120°C for 4 to 6 hours.

3.6.1.2. Sterilization of Inoculation Instruments

Instruments used for inoculation were sterilized by using wet sterilization method. Forceps, surgical blade holders, surgical blades and cotton etc., were covered in aluminum foil and kept for sterilization in an autoclave at 121°C and 15 psi pressure for 20 minutes. After the sterilization they were removed and transferred to laminar air-flow chamber and exposed to germicidal ultra violet (UV) light (253.7 nm) for 30 minutes before inoculation.

3.6.1.3. Sterilization of Culture Room

The culture room was maintained sterile by fumigating the area once in a month by vapourising 1:2 ratio of ethanol and formaldehyde solution. The vapours were allowed to remain in the closed culture room for about 24 hours. After the fumigation, formalin

vapours were absorbed by placing ammonia solution in few Petri plates in the culture room.

3.6.1.4. Laminar Air-Flow System

The inoculation of explants was carried out in a laminar air-flow system. Prior to inoculation, inner surface of laminar air-flow system was swabbed with disinfectant solution (dettol) and followed by cleaning using alcohol with cotton. Then the laminar air-flow hood was exposed to germicidal UV (253.7 nm) for about 20–25 minutes. The materials required for inoculation such as culture medium, explants etc. were then brought to the laminar air-flow system.

3.6.2. Media Preparation

Murashige and Skoog's (1962) (MS), Woody Plant medium (1981) (WPM) were used for in vitro culture. The medium consisted of micronutrients, macronutrients, vitamins, amino acids, carbon source, plant growth regulators and a gelling agent agar. The chemicals required for the preparation of medium were weighed with the help of digital weighing balance.

The compositions of chemicals used for the preparation of both MS and WPM medium are provided in Tables 4, 6. For MS medium, separate iron stock solution was prepared by weighing desired quantity of sodium ethylenediaminetetraacetic acid (EDTA) and iron sulfate dissolved independently in 50 ml of double distilled water. Later, after complete dissolving, both solutions were combined and stored in amber colored bottle at 4°C. The details of MS stock solution are given in Table 5.

Table 4. The chemical composition of MS medium (Murashige and Skoog, 1962).

Nutrients	Constituents	(mg/L)
Major salts	Ammonium nitrate (NH_4NO_3)	1650
	Potassium nitrate (KNO_3)	1900
	Calcium chloride ($CaCl_2.2H_2O$)	440
	Magnesium sulfate ($MgSO_4.7H_2O$)	370
	Potassium dihydrogen orthophosphate (KH_2PO_4)	170
Minor salts	Boric acid (H_3BO_3)	6.2
	Manganese sulfate ($MnSO_4.4H_2O$)	22.3
	Zinc sulfate ($ZnSO_4.7H_2O$)	8.6
	Potassium iodide (KI)	0.83
	Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25
	Cobalt chloride ($CoCl_2.6H_2O$)	0.025
	Copper sulfate ($CuSO_4.5H_2O$)	0.025
Iron sources	Ferrous sulfate ($FeSO_4.7H_2O$)	27.84
	Sodium ethylenediaminetetraacetic acid ($Na_2EDTA.2H_2O$)	37.24
Organic supplements	Thiamine HCl	0.10
	Nicotinic acid	0.50
	Pyridoxine HCl	0.50
	Glycine	2.00
	Myo-inosital	100

Table 5. Stock Solutions of MS Medium.

Stocks	Constituents	Amount (g/L)	Strength of Stocks	Stocks to be taken for 1 liter medium
A	NH_4NO_3 KNO_3	16.5 19.0	10X	100 ml
B	$MgSO_4.7H_2O$ $MnSO_4.4H_2O$ $ZnSO_4.7H_2O$ $CuSO_4.5H_2O$	37.0 2.23 0.86 0.0025	100X	10 ml
C	$CaCl_2.2H_2O$ KI $CoCl_2.6H_2O$	44.0 0.083 0.0025	100X	10 ml
D	$KH_2\ PO_4$ $H_3\ BO_3$ $Na_2MoO_4.2H_2O$	17.0 0.62 0.0025	100X	10 ml
E	$FeSO_4.7H_2O$ Na_2 EDTA. $2H_2O$	2.784 3.724	100X	10 ml
F	Thiamine HCl Nicotinic acid Pyridoxine HCl Glycine Myo-inosital	0.010 0.050 0.050 0.200 10.0	100X	10 ml

Table 6. The Composition of Woody Plant Medium (Lloyd and McCown, 1981).

Nutrients	Constituents	(mg/L)
Macronutrient	Ammonium nitrate (NH_4NO_3)	400
	Calcium nitrate ($Ca(NO_3)_2.4H_2O$)	556
	Calcium chloride ($CaCl_2.2H_2O$)	96
	Potassium dihydrogen orthophosphate (KH_2 PO_4)	170
	Magnesium sulfate ($MgSO_4.7H_2O$)	370
Micronutrient	Boric acid (H_3BO_3)	62
	Manganese sulfate ($MnSO_4.4H_2O$)	22.3
	Zinc sulfate ($ZnSO_4.4H_2O$)	86
	Copper sulfate ($CuSO_4.5H_2O$)	0.25
	Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25
Iron sources	Ferrous sulfate ($FeSO_4.7H_2O$)	27.84
	Sodium ethylene diamine tetra acetic acid (Na_2 EDTA. $2H_2O$)	37.24
Organic supplements	Thiamine HCl	1.00
	Nicotinic acid	0.50
	Pyridoxine Hcl	0.50
	Glycine	2.00
	Myo- inositol	100

3.6.2.1. Preparation of MS medium (Murashige and Skoogs, 1962)

Stock solutions of A and B 100 ml each, 10 ml of stock solutions C and F, 1 ml of stock solutions of D and G and 0.1 ml of stock solution E was added to 400 ml of double distilled water in 2 L flask and mixed well. Then, 30 g of sucrose was added and made the volume to 900 ml with distilled water. After adding required amount of auxins and cytokinins, medium pH was adjusted to 5.7 ± 0.2 using 0.1 N NaOH or 0.1 N HCl. Then 8 g agar was added and made the volume one liter with double distilled water. The medium was allowed to boil in microwave oven, cooled to 60°C, dispensed into culture vials and autoclaved.

3.6.2.2. Preparation of Woody Plant Medium (WPM) (Lloyd and McCown, 1981)

Stock solutions of 100 ml each of A, B, C, D and E was added to 200 ml of warm double distilled water in two liter beaker. Then 10 ml of stock solutions of F, G and H was added and mixed well with glass rod (Table 7). Later, 30 grams of sucrose as carbon source was added and mixed using glass rod. Volume of the medium was made to 900 ml with distilled water. Medium pH was adjusted to 5.7 ± 0.2 using 0.1N NaOH or 0.1 N HCl, after adding required amount of plant growth regulators (auxins or cytokinins) alone or in combinations. Then 8 grams of agar was added and made the volume to one liter with double distilled water. Medium was made to boil, cooled to 60°C, 15-20 ml of media dispensed into culture vials and plugged with non-absorbent cotton. Medium containing culture vials were autoclaved at 121°C and 15 psi pressure for 20 minutes. Basal medium without any plant growth regulators was used as control. IAA was filter-sterilized and added just before solidification of medium.

Table 7. Stock solutions of Woody Plant Medium (WPM).

Stocks	Constituents	Amount (g/L)	Strength of Stocks	Stocks to be taken for 1 litre medium
A	NH_4NO_3	40.0	10X (Macronutrient)	100 ml
B	$Ca(NO_3)_2.4H_2O$	55.6	10X (Macronutrient)	100 ml
C	$CaCl_2.2H_2O$	9.6	10X (Macronutrient)	100 ml
D	$KH_2\ PO_4$	17.0	10X (Macronutrient)	100 ml
E	$MgSO_4.7H_2O$	37.0	10X (Macronutrient)	100 ml
F	H_3BO_3 $MnSO_4.H_2O$ $ZnSO_4.4H_2O$ $CuSO_4\ 5H_2O$ $Na_2MoO_4.2H_2O$	0.620 2.230 0.860 0.025 0.025	100X Micronutrient	10 ml
G	$FeSO_4.7H_2O$ $Na_2EDTA.2H_2O$	2.780 3.740	100X (Iron source)	10 ml
H	Thiamine HCl Nicotinic acid Pyridoxine HCl Glycine Myo- inositol	0.10 0.05 0.05 0.20 10.0	100X Organic supplements	10 ml

3.6.2.3. Preparation of Stock Solution of Plant Growth Regulators (PGRs)

The plant growth regulators (PGR's) such as cytokinins: 6-benzylaminopurine (BAP), kinetin (KIN), and auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D) stock solutions (mg/ml) were prepared (Table 8). All plant growth regulators were obtained from Sigma-Aldrich Chemical Company, USA. For the preparation of stock solutions, 100 mg powder of each hormone was weighed and dissolved in 1–2 ml of 1 M NaOH. Then 100 ml sterile double distilled water was used to make the final volume and stored in amber coloured bottles at 4°C.

3.6.3. Culture Condition

After inoculation, culture vials were placed in culture room with the temperature of 25 ± 2°C under white fluorescent light (40 μ mole m^2 S^{-1}) with 16 hours of photoperiod. The cultures were examined at regular intervals under microscope as well as visually for their growth and regeneration.

3.6.4. Plant Materials

Healthy plants of *P. talbotii* were developed through vegetative propagation and maintained in the poly-house at Department Botany, Goa University, Goa. Explants were also collected from the natural population.

3.6.4.1. Explants

Seeds, nodal segments, leaflets and shoot tips were collected from healthy plants of *P. talbotii* and used for *in vitro* propagation.

Table 8. Plant growth regulators (PGRs) used for *in vitro* regeneration.

Plant growth regulators	Molecular weight (g/mol^{-1})	Solubility	Stock (mg/100ml)
6-Benzylaminopurine (BAP)	225.25	1M NaOH	100
Kinetin (KIN)	215.22	1M NaOH	100
Indole-3-acetic acid (IAA)	175.19	1M NaOH	100
Naphthalene acetic acid (NAA)	186.21	1M NaOH	100
Indole-3-butyric acid (IBA)	203.24	1M NaOH	100
2,4-dichlorophenoxyacetic acid (2,4-D)	221.04	1M NaOH	100
TDZ - Thidiazuron (N-phenyl-N′-1,2,3-thiadiazol-5-yl) urea	220.25	0.1N KOH	50
Zeatin	219.24	1M NaOH	50

3.6.4.2. Surface Sterilization of Explants

The collected explants were first cleaned with water and running water. Then the explants were soaked in aqueous solution of detergent (Teepol/ Tween-20) for 15 minutes. Any dirt or other adhering particles on the surface were removed during this process. Later, the explants were sterilized in absolute alcohol for about 20 seconds. Later, explants were transferred to sterile Petri plate containing filter paper.

3.6.5. Inoculation

Seeds were inoculated directly after the sterilization process. However, nodal segments were given fresh cut on both the sides and placed vertically, one side touching the medium. Leaf-lets were also given fresh cuts before placing it on the medium. After inoculation, culture tubes were sealed with sterile cotton plugs and labeled. Inoculated culture vials were maintained in the plant tissue culture room.

3.6.6. Regeneration Medium

MS and WPM supplemented with different plant growth regulators (PGRs) at various concentrations either singly or in combination, were tried for multiple shoots induction (Table 9, 10). A total of ten treatment were tried, which includes (i) kinetin (KIN), (ii) 6-benzylaminopurine (BAP); (iii) 2,4-dichlorophenoxy acetic acid (2,4-D); (iv) Zeatin; (v) Thidiazuron (N-phenyl-N′-1,2,3-thiadiazol-5-yl) urea (TDZ) alone in various concentrations and combinations of (vi) 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA); (vii) 6-benzylaminopurine (BAP) and 1-naphthalene acetic acid (NAA); (viii) 6-benzylaminopurine (BAP) and TDZ; (ix) kinetin (KIN) and 1-naphthalene acetic acid (NAA); (x) indole-3-acetic acid (IAA) and Zeatin. Explants were inoculated on the

Table 9. Plant growth regulators used for direct multiple shoot formation.

Treatment (I)	**Treatment (II)**	**Treatment (III)**	**Treatment (IV)**	**Treatment (V)**
KIN mg/L	BAP mg/L	2, 4-D mg/L	TDZ mg/L	Zeatin mg/L
0.5	0.5	0.5	0.5	0.5
1.0	1.0	1.0	1.0	1.0
1.5	1.5	1.5	1.5	1.5
2.0	2.0	2.0	2.0	2.0
2.5	2.5	2.5	2.5	2.5
3.0	3.0	3.0	3.0	3.0
3.5	3.5	3.5	3.5	3.5
4.0	4.0	4.0	4.0	4.0
4.5	4.5	4.5	4.5	4.5
5.0	5.0	5.0	5.0	5.0

Table 10. Various combinations of plant growth regulators tried for direct multiple shoot formation.

Treatments	KIN mg/L	BAP mg/L	IAA mg/L	NAA mg/L	TDZ mg/L	ZEA mg/L
(VI)		0.5	1.0	-	-	-
		1.0	1.0	-	-	-
		1.5	1.0	-	-	-
		2.0	1.0	-	-	-
		2.5	1.0	-	-	-
		3.0	1.0	-	-	-
		3.5	1.0	-	-	-
		4.0	1.0	-	-	-
		4.5	1.0	-	-	-
(VII)		0.5	-	1.0	-	-
		1.0	-	1.0	-	-
		1.5	-	1.0	-	-
		2.0	-	1.0	-	-
		2.5	-	1.0	-	-
		3.0	-	1.0	-	-
		3.5	-	1.0	-	-
		4.0	-	1.0	-	-
		4.5	-	1.0	-	-
(VIII)		0.5	-	-	1.0	-
		1.0	-	-	1.0	-
		1.5	-	-	1.0	-
		2.0	-	-	1.0	-
		2.5	-	-	1.0	-
		3.0	-	-	1.0	-
		3.5	-	-	1.0	-
		4.0	-	-	1.0	-
		4.5	-	-	1.0	-
(IX)	0.5		1.0			
	1.0		1.0			
	1.5		1.0			
	2.0		1.0			
	2.5		1.0			
	3.0		1.0			
	3.5		1.0			
	4.0		1.0			
	4.5		1.0			
(X)			1.0			0.5
			1.0			1.0
			1.0			1.5
			1.0			2.0
			1.0			2.5
			1.0			3.0
			1.0			3.5
			1.0			4.0
			1.0			4.5

regeneration medium with the quantity of 15–20 ml each, made slanted in rimless culture tubes of size 150×25 mm (Borosil Glass Works Limited, India).

3.6.7. Data Collection and Statistical Analysis

The cultures were observed and monitored at regular intervals (every 4 days) on the basis of visual observation and under the stereo-zoom light microscope to understand the morphogenesis. Ten replications were maintained for every treatment. Experiments were repeated for three times. Effect of plant growth regulators on callus induction and shoot regeneration were calculated on the basis of percentage of cultures showing callus or shoot bud formation. After 42 days of inoculation, the percentage of regeneration was recorded for each treatment. Close-up photographs of cultures were taken using *Nikon D90 DSLR* camera attached with *Nikon* 60 mm macro lens (Nikon Corporation, Tokyo, Japan).

3.7. *In-Situ* Conservation of *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum*

3.7.1. *Phyllanthus talbotii*

Vegetative stem cuttings were collected from different population present in the Western Ghats regions of Goa during September 2009, October 2010 and September 2012. Stem cuttings were treated with various concentrations of root inducing hormone *i.e.* IBA, IAA and commercial rootex. The detailed protocol is provided in Section 3.4. For *in-situ* conservation 12,083 vegetative stem cuttings grown in root trainers were reintroduced into 25 localities in Goa either into the existing population to enrich them or planted in new sites to establish new populations. For selecting new sites, soil parameters which closely matched with natural habitats were considered. Transplantation was carried out in the month of April in the years 2010, 2011 and 2012, prior to monsoon season in Goa. The height of plants varied from 60 to 70 cm at the time of transplanting. The survivability was

checked and the height of the plants was re-measured in the month of January 2011 for the cuttings transplanted in April 2010, height was re-measured in January 2012 for the cuttings transplanted in April 2011 and height of the plants was re-measured in January 2013 for those transplanted in April 2012.

3.7.2. *Pseudoglochidion anamalayanum*

As a first set of experiment, in the year 2010, a total of 200 stem cuttings of 30 cm height of *P. anamalayanum* were reintroduced (planted directly) at two different locations of Anamalai Tiger Reserve, Tamil Nadu, by giving sharp horizontal cut at the bottom with a quick dip in root inducing hormone (IBA 500 ppm) during the monsoon season (June and July). The second set of experiment was carried out during the month of July 2012, a total of 555 root suckers of *P. anamalayanum* were transplanted by using crow-bar pitting methods for *in-situ* conservation in 6 different locations of Anamalai Tiger Reserve, Tamil Nadu. During the third set of experiment, a total of 796 suckers were transplanted in to two different locations of Anamalai Tiger Reserve, Tamil Nadu. Re-introduced root suckers were periodically monitored for their survival.

3.8. Genetic Diversity Studies

The study involves screening of twenty two ISSR primers for the evaluation of genetic diversity between different population and within single population of *P. talbotii* and *P. anamalayanum*. Details of primers used and their sequences are provided in Table 11.

3.8.1. Plant Material

Field trips were carried out for the collection of plant material. For DNA isolation, leaf samples were collected from a healthy (diseased free) plants and brought to laboratory

Table 11. List of random ISSR primers used for screening of genomic DNA of both *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum*.

Sr. No.	Oligo Name	Primer Sequence 5'-3'
1.	807	AGA GAG AGA GAG AGA GT
2.	808	AGA GAG AGA GAG AGA GC
3.	810	GAG AGA GAG AGA GAG AT
4.	811	GAG AGA GAG AGA GAG AC
5.	812	GAG AGA GAG AGA GAG AA
6.	817	CAC ACA CAC ACA CAC AA
7.	823	TCT CTC TCT CTC TCT CC
8.	826	ACA CAC ACA CAC ACA CC
9.	828	TGT GTG TGT GTG TGT GA
10.	834	AGA GAG AGA GAG AGA GYT
11.	835	AGA GAG AGA GAG AGA GYC
12.	836	AGAGAGAGAGAGAGAGYA
13.	844	CTCTCTCTCTCTCTCCTRC
14.	848	CACACACACACACACARG
15.	855	ACACACACACACACACYT
16.	861	ACCACCACCACCACCACC
17.	868	GAAGAAGAAGAAGAAGAA
18.	873	GACAGACAGACAGACA
19.	876	GATAGATAGATAGATA
20.	881	GGGTGGGGTGGGGTG
21.	888	BDBCACACACACACACA
22.	890	VHVGTGTGTGTGTGTGT

Supporting information: Y = (C,T); B = (C,G,T); D = (A,G,T); H = (A,C,T); V = (A,C,G); R = Purine; Y= Pyrimidine.

(Department of Botany, Goa University). The materials were washed, cleaned, dried and stored at -20°C deep freezer for further use.

3.8.2. Isolation and Purification of Genomic DNA

For the isolation and purification of genomic DNA, CTAB method (cetyl trimethyl ammonium bromide) was employed (Edwards *et al*., 1991). Since, the leaf tissue of both *P. talbotii* and *P. anamalayanum* contains high amount of secondary metabolites such as polyphenolic compounds etc., and mucilage in *P. talbotii*, a modified CTAB method was used (Doyle and Doyle 1990; Ramage, *et al*., 2004). Detailed procedure used during this study is given below.

For each sample, 5 g of leaf tissue without mid-rib region was grounded to a fine powder using liquid nitrogen. This was then suspended in 25 ml of preheated extraction buffer (20 mM EDTA at pH 8.0, 100 mM Tris-HCl at pH 8.0, 1.5 M NaCl, 4% CTAB and 100 µl of βmercaptoethanol). The suspension was mixed by inverting the tubes three to four times and incubated at 60°C for 60 minutes. Every 10 minutes centrifuge tube was opened to release the gas and also samples were mixed by inverting 3 to 4 times. After 60 minutes, equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12000 rpm for 20 minutes at 4°C. Supernatant was carefully transferred to the new centrifuge tube and 0.6 volume of ice cold isopropanol was added. The centrifuge tubes were incubated at -20°C for 4 h. Then the DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was discarded and the pellets containing DNA was dissolved in 700 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and the contents were transferred to new Eppendorf tube. After complete dissolution, the purification of DNA was carried out by adding equal volume of phenol: chloroform mixture (1:1) and the tubes were inverted three to four times followed by

centrifugation at 8000 rpm for 10 minutes at room temperature (RT). Later, upper transparent layer was transferred to a new Eppendorf tube. Then, equal volume of chloroform was added and content was mixed three to four times and centrifuged at 8000 rpm for 10 minutes at RT and this step was repeated once again. After centrifugation, upper layer was transferred to a new Eppendorf tube. Later, 40 µl of 3 M sodium acetate (pH 5.2) and three volumes of 100% ethanol (1 ml) were added. Then the tubes were incubated at -20°C for 12 h (overnight) followed by centrifugation at 8000 rpm for 15 minutes at RT. Supernatant was discarded and pellet was re-suspended in 1 ml of ethanol (70%), gently vortexed and centrifuged at 8000 rpm for 10 minutes at RT. After centrifugation, supernatant was decanted and DNA pellet was air dried or incubated at 37°C for 15 minutes till no water droplets present in the pellet. Then the DNA pellet was dissolved in 100 µl of TE buffer and stored at -20°C for further use. The concentration of DNA was estimated by agarose gel electrophoresis and staining with ethidium bromide and also by spectrophotometric methods. To check the purity of the DNA, the optical density (OD) was taken by measuring the samples at two different wave lengths i.e. 260 and 280 nm. A ratio obtained below 1.8 denotes that the DNA is contaminated with protein and or other UV absorbers in the sample. A ratio higher than 2.0 indicates that the sample may be contaminated with chloroform or phenol (CIMMYT, 2005).

3.8.3. Determination of Quality of DNA by Agarose Gel Electrophoresis

The quality of DNA was checked by agarose gel containing ethidium bromide. Agarose power of 0.8 g of was added to 100 ml of 1X TBE buffer and melted using micro-wave oven. On slight cooling 3 µl of ethidium bromide (stock 10 mg/ml) was added to the agarose solution, mixed well, poured in to gel-casting tray with proper comb setting and kept for 30 minutes for solidification of the gel. Agarose gel was carefully removed from

gel casting tray and placed in electrophoresis unit containing 1X TBE buffer and the comb was slowly taken out from the gel. Gel loading dye of 1 µl of 6X was taken in 0.5 ml Eppendorf tube and placed in ice box. Then, 10 µl of DNA sample was added and mixed well with loading dye. The DNA samples were carefully loaded into the wells and run initially at 50 volt for 10 minutes. Then the voltage was increased to 100. When the DNA samples reached three-fourth length of the gel, the electrophoresis unit was switched off. Later, the gel was observed and photographed using the gel documentation system (Alpha DigiDoc^{TM}, Alpha Innotech Corporation, California, USA).

3.8.4. Inter Simple Sequence Repeat (ISSR) Analysis

Genetic diversity studies were carried out using genomic DNA of both plant species collected from different populations and within the population. ISSR analysis was performed using universal random oligonulceotide primers. For primer synthesis, primer sequences were provided to Sigma Chemical Company, USA, and the specific primers were obtained. List of primers used during this study are summarized in Table 11.

3.8.4.1. Amplification of Genomic DNA

The genomic DNA was amplified using polymerase chain reaction (PCR). Amplification reaction was carried with the final total volume of 25 µl, containing 30-50 mg of genomic DNA, 2.5 µl of 10X Taq assay buffer, 3 µl of 25 mM $MgCl_2$, 2.5 µl of 10 mM dNTPs mix, 15 ng of primers, 0.5 units of Taq polymerase and final volume made using sterile distilled water. All PCR chemicals were obtained from Merck-Genei, Bangalore, India.

3.8.4.2. Amplification Condition and Scoring of Bands

The PCR amplification was performed following the protocol described by Parab *et al*. (2008) using a Mastercycler gradient PCR (Eppendorf AG, Hamburg, Germany). Initial denaturation step of 5 minutes at 94°C, followed by 35 cycles of 94°C for 1 minute (denaturation) and 72°C for 2 minutes (extension) and 10 minutes of final extension step at 72°C. The amplified products were analysed using 2% agarose gels in 1X TBE buffer. Ethidium bromide was used for staining the gel, visualized under UV and photographed using Gel Documentation System (AlphaDigiDoc™, Alpha Innotech Corporation, California, USA). The number of bands present in the samples was visually scored.

3.8.4.3. Analysis of Data

Primer code was used for naming of ISSR amplified fragments. All informative ISSR bands were scored independently as 1 for presence and 0 for absence of bands. To calculate the percentage of polymorphism, the proportion of polymorphic bands over the total number of bands were taken in consideration. Dendrogram and genetic distance were made by clustering using BioDiversity Pro software a statistical package programme.

3.9. Systematic Positions of *P. talbotii* and *P. anamalayanum* Using Gene Sequences

3.9.1. Sample Collection

Periodic field survey and collection trips were carried out to different parts of Goa and Karnataka for collection of *P. talbotii* plant materials for Herbarium and study taxonomy. For molecular gene sequencing *P. talbotii* sample was collected from Saccordem, Goa. Similarly, regular field survey and collection trips were made to Anamalai hills, Valparai, Coimbatore District, Tamil Nadu, for the collection of *P. anamalayanum* for genetic diversity studies and molecular gene sequencing.

3.9.2. Herbarium Preparation

The collected samples from different natural populations of both plant species were processed for herbarium using standard method (Vogel, 1987; Jain and Rao, 1977). All herbarium specimens are deposited in Herbarium, Department of Botany, Goa University, Panaji - Goa.

3.9.3. Systematic Position Based on Gene Sequencing Data

To ascertain the taxonomic position of *P. talbotii* and *P. anamalayanum*, the universal ITS, *mat*K and *rbc*L primers were used (Table 12). A silica dried leaf samples of both species were supplied to Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala. For *P. talbotii*, ITS and *mat*K sequences and for *P. anamalayanum,* ITS, *mat*K and *rbc*L gene sequences were obtained.

3.9.4. Phylogenetic Tree Construction

Along with the gene sequences obtained from RGCB, for *P. talbotii* and *P. anamalayanum*, an identical gene sequences were retrieved from National Center for Biotechnology Information (NCBI) and arranged in FASTA format and aligned with the query sequences. The sequence alignment was carried out using clustalW (ver. 1.6). The individual Maximum likelihood analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) version 5.05, software (Tamura, *et al*., 2004; 2011). 1000 replicates of bootstrapping for clades were performed for assessing the confidence limit (bootstrap percentage) (Felsenstein, 1985).

Table 12. Primers used for sequencing of ITS, *mat*K and *rbc*L genes from *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum*.

Target	Primer Name	Direction	Sequence (5' to 3')	Reference/Remarks
***mat*K**	3F-KIM-F	Forward	CGTACAGTACTTTT GTGTTTACGAG	CBOL Plant Working Group (http://www.barcoding.si.edu /plant_working_group.html)
	1R-KIM-R	Reverse	ACCCAGTCCATCTG GAAATCTTGGTTC	
***rbc*L**	*rbc*La-F	Forward	ATGTCACCACAAAC AGAGACTAAAGC	CBOL Plant Working Group (http://www.barcoding.si.edu /plant_working_group.html)
	*rbc*L724-R	Reverse	GTAAAATCAAGTCC ACCRCG	
ITS	ITS5-F	Forward	GGAAGTAAAAGTCG TAACAAGG	CBOL Plant Working Group (http://www.barcoding.si.edu /plant_working_group.html)
	ITS4-R	Reverse	TCCTCCGCTTATTGA TATGC	

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

RESULTS

4. RESULTS

The results reported here are based on the investigation of population studies, ecology, propagation, conservation and molecular studies of two critically endangered plant species namely *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum*. During this study, (i) Western Ghats of Goa, Karnataka, Tamil Nadu and adjacent regions were surveyed for population studies and specimens collected for laboratory experiments and processed for herbarium. (ii) Developed and standardized protocols for vegetative propagation using various plant growth regulators for both *P. talbotii* and *P. anamalayanum*. (iii) Developed and standardized protocols for *in-vitro* regeneration and mass multiplication of *P. talbotii*. (iv) *In-situ* conservation of two critically endangered plant species *P. talbotii* and *P. anamalayanum* by reintroduction into their natural habitats to establish new populations, enrich the existing natural populations and monitor their growth and performance. (v) Studied the genetic diversity and variation within and among the populations of each of these two critically endangered plant species using ISSR molecular markers, and assessed its taxonomic position using ITS, *mat*K and *rbc*L gene sequencing.

4.1. Taxonomic Description

4.1.1. *Phyllanthus talbotii*

Phyllanthus talbotii Sedgw. Journ. Ind. Bot. 2. 124. 1921. (as '*talboti*'); Singh & Kulkarni, *In*: *Red Data Book of Indian plants* 3: 124. 1990. Balakrishnan & Chakrabarty. *Family Euphorbiaceae in India* 374. 2007.

A monoecious sub-shrub, up to 150 cm high; stem terete, suffruticose, brown, often with vertical streaks with prominent nodes; branchlets up to 25 cm long, with up to 100

Plate 1. *Phyllanthus talbotii* Sedgw. a. Habitat; b. Habit; c. Staminate flowers (insert is close-up); d. Pistillate flowers (insert is close-up); e. Seeds; f. Capsule.

leaves, hirsute. Stipules triangular to deltoid, *c.* 1.5 × 1 mm, margin white or brown acuminate at apex. Petiole *c.* 0.5 mm long; lamina oblong, up to 5.9 × 3 mm, rounded at base, entire, acute or slightly apiculate at apex, midvein prominent, secondary veins 4 – 5 pairs, often invisible, sparsely hairy on both surfaces. Staminate flowers: axillary, solitary or in clusters of 2 – 3, *c.* 2.5 × 2 mm. Bracts ovate, shorter than stipule; bracteoles subulate. Pedicel capillary, *c.* 2 mm long, slightly dilated at apex. Sepals 4, obovate to elliptic, *c.* 2 x 1.3 mm, acute to rounded at apex, green in the middle with a broad white wavy margin, one veined. Disc of 4 distinct units, yellow. Staminal column *c.* 0.7 mm long; anthers 4, in two pairs, *c.* 0.5 mm across, yellow. Pistillate flowers: solitary, axillary, on the same branchlet as staminate flowers, *c.* 3 mm across when open. Bracteoles lanceolate to ovate, up to 1 mm long. Pedicel *c.* 2 mm long, dilated at apex. Sepals 6, ovate to elliptic, 1.5 – 2 × 1 mm, entire, acute to acuminate at apex, greenish and long in the middle and white to creamy on either side. Disk yellow, wavy, slightly 5-lobed. Ovary 3-lobed, *c.* 0.5 × 1 mm; styles 3, each distinctly forked. Fruiting pedicel *c.* 3 mm long, dilated at apex. Capsule *c.* 3 × 4 mm, 3- lobed, hirsute; cocci 3; seeds 2 in each cocci, *c.* 1.8 × 1 mm, 3-angled, curved on dorsal side, smooth brown in colour (Plate 1).

Flowering and Fruiting: August – November.

Distribution: Western Ghats regions of Goa and Karnataka, along the fresh water streams and rivers.

Specimens examined: INDIA, **Goa**; North Goa District, Sanguem , 23.9.1997, *V. Joshi and S. Rajkumar*, 1022, 1070; Selaulim reservoir site, 30.8.2010, *S.S. Naik*, 4001, 4002, Collem stream, 11.9.2010, *S. S. Naik*, 4003, 4004, Waddem stream, 12.10.2010, *S. S. Naik*, 4005, 4006, 4007; Dudhsagar waterfalls, 11.9.2010, *S. S. Naik*, 4008, 4009, 40010 (All the herbarium specimens are deposited at Goa University Herbarium).

4.1.2. *Pseudoglochidion anamalayanum* Gamble

Pseudoglochidion anamalayanum Gamble in Kew Bull. Misc. Inf. Kew 1925: 329. 1925. Balakrishnan & Chakrabarty, Family Euphorbiaceae in India 377. 2007. *Pseudoglochidion anamalayanum* Gamble and Fl. Madras 2 (7): 1285. 1925; Chandrabose in Henry *et al*., Fl. Tamil Nadu Ser. 1, 2: 239. 1987; Nayar and Sastry, Red Data Book of Indian Plants 3: 126. 1990**.**

A monoecious tree, up to 10 m high; branches slender, irregular; branchlets phyllanthoid. Leaves 10 – 14 per branchlet, distichous, ovate to lancelolate, 4 – 9 x 1.5 - 3 cm, rounded to acute at base, entire along margins, acute to acuminate at apex, glabrous, lateral nerves regular on both the sides; stipules subulate, up to 4 mm long; petiole up to 2.5 mm long. Flowers either axillary or on special leafless branchlets; flowering branchlets up to 14 cm long, might continue to grow and produce leaves after flowering. Staminate flowers: in clusters; bracts and bracteoles subulate, at the base of pedicel; pedicel filiform, 5 – 6 mm long; sepals 6, biseriate, imbricate, ovate-lanceolate, *c.* 3 × 1 mm; petals 0; stamens 3, joined at base by short filaments; anthers 3, *c.* 2 × 0.5 mm with prominent apiculate connective, yellow, extrorse; glands 6, conspicuous. Pistillate flowers: solitary or in clusters of 2 – 3, pedicel *c.* 1 mm long, thick; sepals 6, biseriate, as in staminate flowers, often unequal; ovary 3-chambered, ovules 2 in each; styles fused to form a thick column of up to 3 mm when young; stigma 6-toothed; disc minute, annular. Capsules 3-lobed, 8 – 10 mm in diameter, subglobose, glabrous, depressed at apex. Seeds three sided, dorsally curved, 4 – 5 mm long, dotted, hilum black spotted (Plate 2).

Flowering and Fruiting: Throughout the year.

Distribution: Restricted to Anamalai Tiger Reserve of Anamalais (1,100–1,400 m), Valparai, Coimbatore district, Tamil Nadu (India).

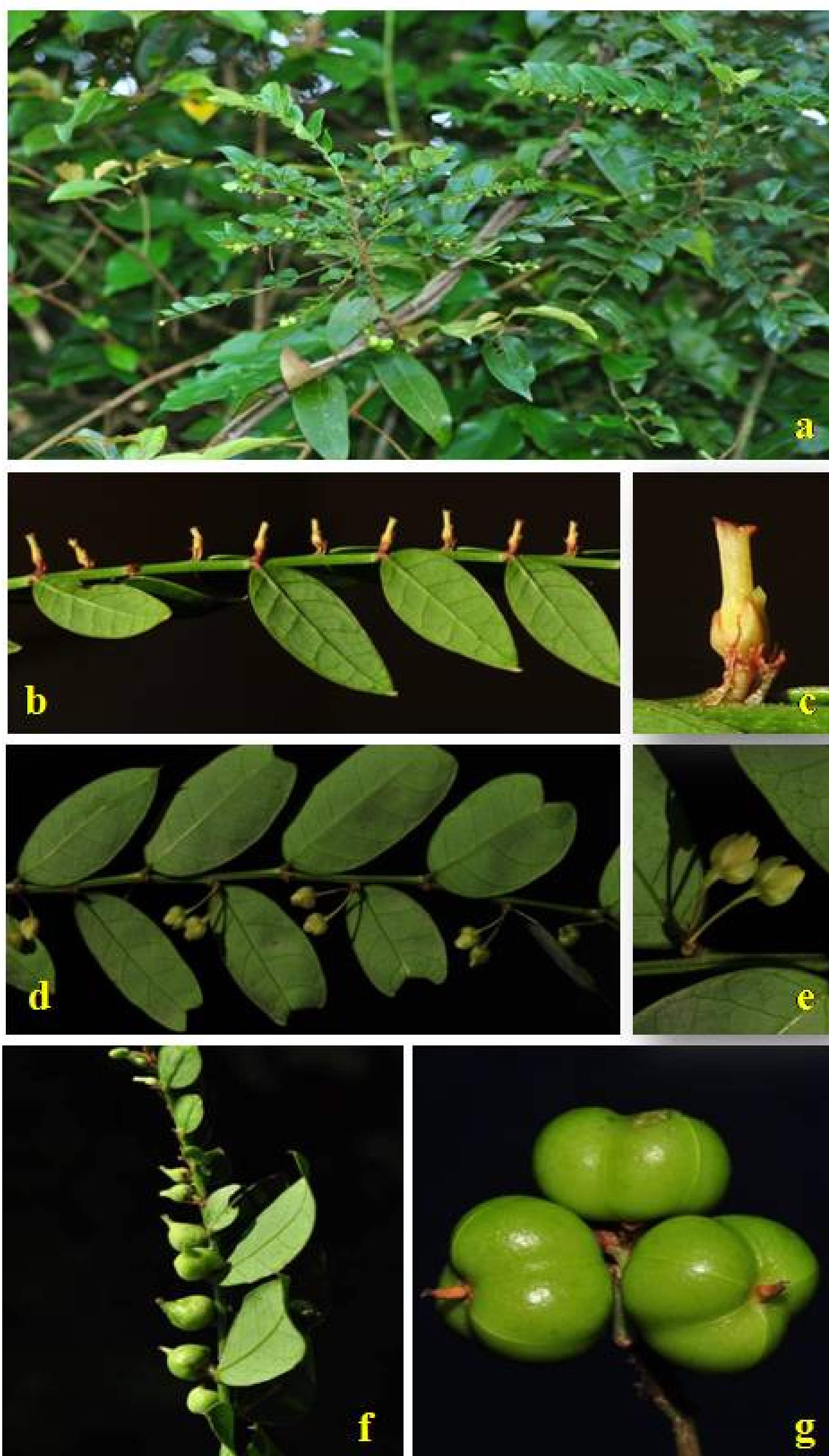


Plate 2. ***Pseudoglochidion anamalayanum*** **Gamble. a. Branchlet; b. Pistillate flowers; c. Close-up of pistillate flower; d. Staminate flowers; e. Close-up of staminate flowers; f. Immature fruits; g. Matured fruits.**

Specimens examined: INDIA, Tamil Nadu; Coimbatore district, Valparai, Iyerpadi, 23.1.2010, 4951, 4952, 4953, 4954, 4955, 4956, 4957, 4958, 4959. All specimens are collected by *M. K. Janarthanam*, *S. Krishnan* and *S. S. Naik*; all herbarium specimens are deposited at Goa University Herbarium.

4.2. Survey and Estimation of Natural Populations and Phenology

4.2.1. Survey and Estimation of Populations of *Phyllanthus talbotii*

The systematic field trips and survey were carried out in around Western Ghats regions of Goa and Karnataka to locate the populations of *P. talbotii.* Different populations located in the State of Goa and Karnataka is provided in Plate 3. Some of the habitats of the populations are given in Plate 4. During the field visits latitude and longitude of different populations were taken using handheld GPS. The latitude and longitude (location), altitude, approximate size of each population, number of plants, ramets in each plant and height of the plants for each population is given below:

1. Saccordem, Dharbandora, Goa (Plate 4a)

Location : N15°23.684' E74°04.881'
Altitude : 51 m
Size : Approximately 700 m^2
Number of Plants : 303
Ramets in each plant: 02–28
Height of the plants : 45–110 cm

This population has been found distributed along the sides of the stream and in the middle wherein water flows throughout the year. The plants were always seen in shallow parts of the stream. The population receives very bright sun light and plants showed the accumulation of large amount of anthocyanin pigments.

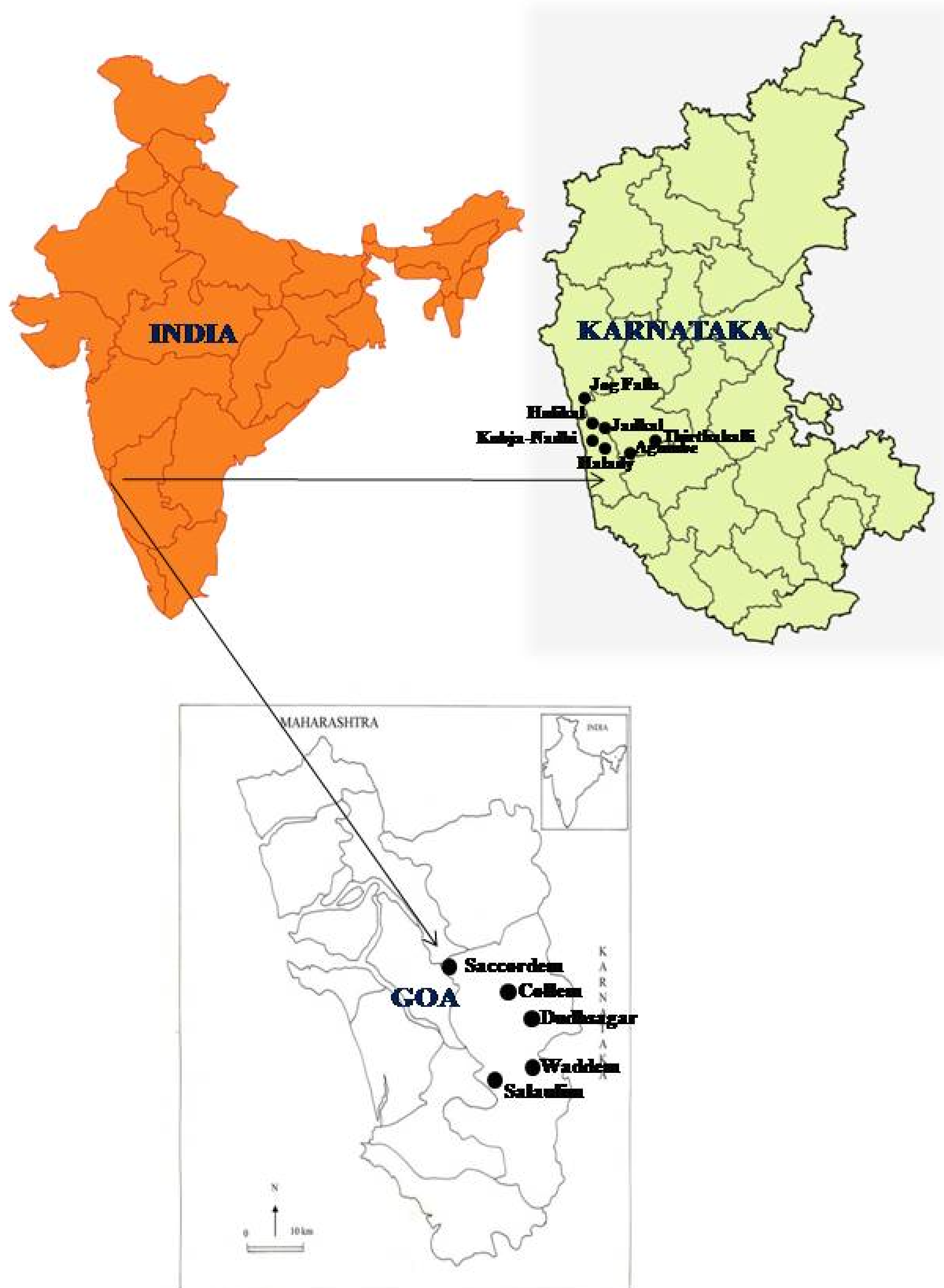


Plate 3. Map showing the location of *Phyllanthus talbotii* Sedgw. in the study area.

Plate 4. **Populations of *Phyllanyhus talbotii* with different substratum. a. Saccordem; b. Waddem; c. Collem; d. Dudhsagar; e. Salaulim Dam; f. Halady; g. Jog Falls; h. Agumbe.**

2. Waddem, Sanguem, Goa (Plate 4b)

Location : N15°09.143' E74°13.649'

Altitude : 44 m

Size : Approximately 600 m^2

Number of Plants : 185

Ramets in each plant: 02–14

Height of the plants : 55–65 cm

This population is located in a stream wherein water flows throughout the year. The stream is very shallow and not too many large trees are present in the area, the sun light is more and the plants showed lots of anthocyanin pigments. Threat to this population is mainly due to the washing of mining trucks which leads to spillage of oil and other pollutant.

3. Collem, Dharbandora, Goa (Plate 4c)

Location : N 15°20.290' E 074°14.767'

Altitude : 55 m

Size : Approximately 600 m^2

Number of Plants : 327

Ramets in each plant: 02–45

Height of the plants : 50–60 cm

Collem is located at the foot-hills of the Western Ghats of Goa. Single population of *P. talbotii* is found growing in a stream which comes from Dudhsagar. The stream is shallow and perennial. The size of the population is comparatively larger than other populations. People from neighboring areas come here for picnic which adds threat to this population. Other threats are washing of trucks (mainly mining trucks) and washing of clothes by the villagers very close to this population.

4. Dudhsagar, Dharbandora, Goa (Plate 4d)

Location : N15°18.700' E74°18.843'

Altitude : 205 m

Size : Approximately 700 m^2

Number of Plants 157

Ramets in each plant: 02–36

Height of the plants : 40–50 cm

This population is located exactly at the tourist spot where water from Dudhsagar falls. The people mainly come for picnic to this place which causes threat to the population. In this population, plants are found scattered on either side of the stream and also found growing in the crevices of big boulders and rocks**.**

5. Salaulim Dam, Sanguem, Goa (Plate 4e).

Location : N15°12.807' E74°10.624'

Altitude : 45 m

Size : Approximately 300 m^2

No. of Plants 32

Ramets in each plant: 02–45

Height of the plants : 55–65 cm

One population is found near Salaulim water reservoir with a very few number of plants

6. Jog falls, Karnataka (Plate 4g)

Location : N 14°13. 942' E 074°49. 149'

Altitude : 470 m

Size : Approximately 10,000 m^2

Number of Plants : 157

Ramets in each plant: 03–26

Height of the plants : 55–120 cm

Jog falls is located in Uttar Kannada district of Karnataka. Plants of *P. talbotii* are found growing in river and incidentally this is one of the type locality (syntypes from Supa and Jog falls was mentioned in literature). The river flows throughout the year with very little flow in summer. In this population, plants are found scattered on either side of the stream and also found growing in the crevices of big boulders and rocks. Among the surveyed area, about 50–60% area is occupied with water and boulders. Within few hundred meters of this population the world famous Jog falls is present along the same river. After the falls, no population of *P. talbotii* is found.

7. Agumbe, Karnataka (Plate 4h)

Location : N13°30.595' E75°05.190'

Altitude : 636 m

Size : Approximately 250 m^2

Number of Plants : 116

Ramets in each plant: 03–22

Height of the plants : 55–80 cm

This is a small population but it is undisturbed.

8. Tunga River, Thirthahalli, Karnataka

Location : N13°41.084' E75°14.640'

Altitude : 608 m

Size : Approximately 750 m^2

Number of Plants : 127

Ramets in each plant: 03–26

Height of the plants : 55–80 cm

Phyllanthus talbotii is found growing in Tunga river bed in a place called Thirthahalli in Shimoga district, Karnataka. The river flows throughout the year.

9. Madanakka Village, Karnataka

Location : N13°30.408' E75°01.262'

Altitude : 91 m

Size : Approximately 400 m^2

Number of Plants : 208

Ramets in each plant: 03–27

Height of the plants : 55–80 cm

This is also an undisturbed population.

10. River near Halady, Karnataka

Location : N13°34.926' E74°51.471'

Altitude : 25 m

Size : Approximately 600 m^2

Number of Plants : 307

Ramets in each plant: 02–28

Height of the plants : 55–90 cm

This population is located in Dakshina Kannada of Karnataka at the bank of river called Netravati near the village Halady. In this population, plants are found scattered on either side of the stream.

11. Kubja-Nadi bridge, village, Karnataka

Location : N13°39.910' E74°47.296'

Altitude : 20 m

Size : Approximately 450 m^2

Number of Plants : 172

Ramets in each plant: 03–26

Height of the plants : 55–80 cm

This population is located in the bank of river called Netravati near the village Bijre. Plants are found on either side of the stream.

12. Jadkal, Karnataka

Location : N13°47.731' E74°48.274'

Altitude : 32 m

Size : Approximately 500 m^2

Number of Plants : 235

Ramets in each plant: 02–25

Height of the plants : 50–120 cm

This population is located near the village Jadkal in Udupi District of Karnataka.

13. River near Hulikal, Karnataka

Location : N13°43.455' E75°00.541'

Altitude : 578 m

Size : Approximately 350 m^2

Number of Plants : 192

Ramets in each plant: 03–25

Height of the plants : 55–90 cm

In this population, plants are found on either sides of the stream.

4.2.2. Phenology of *Phyllanthus talbotii*

In *P. talbotii* senescence of leaves starts from the month of April and continues through May and complete leaf shedding was observed in the month of June to July. Depending on the monsoon rain, during the month of August when water level comes down new shoots are seen and flowering starts. The flowering, fruiting and seed setting were observed from August to November.

4.2.3. Survey and Estimation of Populations of *Pseudoglochidion anamalayanum*

The periodic field trips and survey were carried out in Anamalai Tiger Reserve, Valparai, Tamil Nadu, for population study, plant samples collected for laboratory experiments and processed for herbarium. Total of eight populations were located and

distributional geographic area is provided in Plate 5. Some of the populations in Anamalai Tiger Reserve are shown in Plate 6. Details of latitude and longitude (location), altitude, total number of plants, total number of saplings through seeds or from root suckers, plant height, numbers of plants fruiting and flowering are provided below:

1. **37th hair pin bend (Plate 6c)**

Location: N10°22.191' E76°59.503'

Altitude: 1350 m

Total number of Plants	217
Total number of saplings	467
Number of plants with the height ≤ 3m	08
Number of plants with the height > 3m	209

Number of plants in flowering and fruiting on the day of survey: 122

The plants are found growing between 36th and 37th hair pin bends at the right hand side when coming down to 37th hair pin bend**.** The plants are very healthy and show luxuriant growth with thick branching and foliage. The soil near the plant is very loamy and black brown in colour covered with dry leaf litter. The plants receives fair amount of sun light.

2. **Opposite side of 37th hair pin bend**

Location: N10°22.329' E76°59.593'

Altitude: 1361 m

Total number of Plants	29
Total number of saplings	38
Number of plants with the height ≤ 3m	08

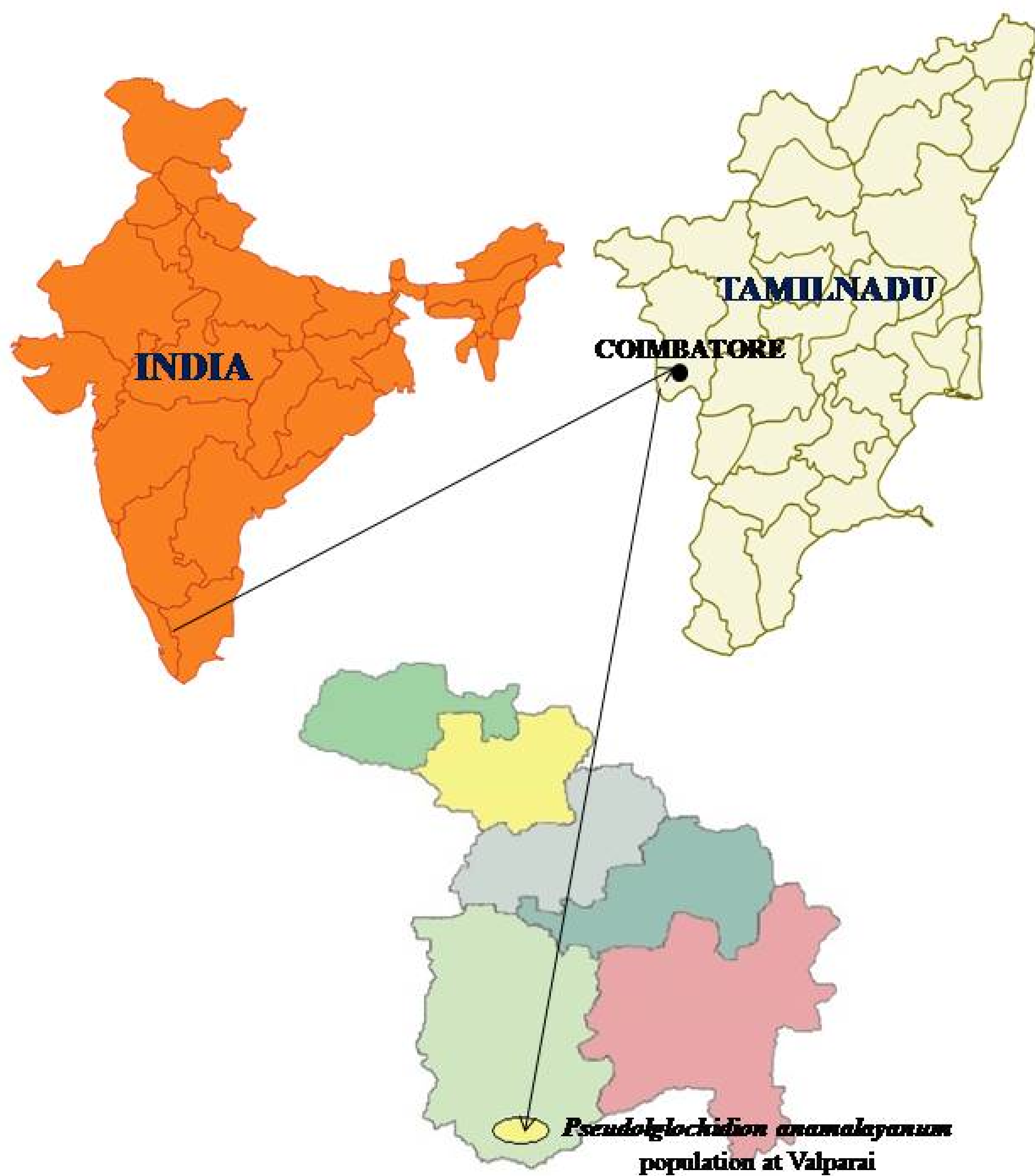


Plate 5. Map showing the location of *Pseudoglochidion anamalayanum* in the study area.

Plate 6

Plate 6. **Habit, habitat and different populations of *Pseudoglochidion anamalayanum* at Anamalai Tiger Reserve, Valparai, Tamil Nadu.**

a, b. **Anamalai Tiger Reserve Forest.**

c. **Population at 37th hair pin bend.**

d. **Population in between 39th-40th hair pin bend.**

e. **Population behind Nature Conservation Foundation (NCF) Center, Iyerpady IInd Division Field.**

f. **Population close to PKT tea estates.**

g. **Population opposite to PKT tea estates.**

h. **Population in Sirukundra Tea Estate.**

ANAMALAI TIGER RESERVE
VALPARAI RANGE
WELCOMES YOU
a

b

c

d

e

f

g

h

Number of plants with the height > 3m 21

Number of plants in flowering and fruiting on the day of survey: 62

The plants are found growing to the upper side of 37th hair pin bend at the right hand side when coming down to 38th hair pin bend. The plants are very healthy and show luxuriant growth. The soil near the plant is very loamy and black brown in colour covered with dry leaf litter. The plants receives fair amount of sun light.

3. In between 39-40 hair pin bend (Plate 6d)

Location: N 10°21.847' E 076°59. 841'

Altitude: 1298 m

Total number of Plants 09

Total number of saplings 12

Number of plants with the height $\leq$ 3m 04

Number of plants with the height > 3m 05

Number of plants in flowering and fruiting on the day of survey: 04

The plants are found growing in between 39th and 40th hair pin bend when coming down to 40th hair pin bend and very close to the road side. Many plants were damaged and less luxuriant growth was observed. The plant receives moderate amount of sun light. Since the plants are found growing very close to the road side; the possibility of land slide affecting plant is noticed.

4. Behind Nature Conservation Foundation (NCF) Centre, Iyerpady IInd Division (Plate 6e)

Location: N 10°22.223' E 076°58.947'

Altitude: 1295.4 m

Total number of Plants	469
Total number of saplings	698
Number of plants with the height ≤ 3m	343
Number of plants with the height > 3m	126

Number of plants in flowering and fruiting on the day of survey: 148

The plants are found growing in the periphery of tea estates from where reserve forest starts. The plants are very healthy and show luxuriant growth with thick branching and foliage. The soil near the plant is very loamy and black brown in colour covered with dry leaf litter. The plants receive fair amount of sun light.

5. Close to the Peria Karamalai Tea (PKT) estates

Location: N 10°21.917' E 076°58.049'

Altitude: 1275 m

Total number of Plants	42
Total number of saplings	151
Number of plants with the height ≤ 3m	10
Number of plants with the height > 3m	32

Number of plants in flowering and fruiting on the day of survey: 08

The plants are found growing near PKT tea estates at the left hand side (10 m interior into the forest from the road) when going towards PKT tea estates. The plants are very healthy and show luxuriant growth with thick branching and foliage. The soil near the

plants is very loamy and black brown in colour covered with dry leaf litter. The plants receive fair amount of sun light.

6. In PKT tea estates

Location: N 10°21.373' E 076°59.940'

Altitude: 1280 m

Total number of Plants	16
Total number of saplings	04
Number of plants with the height ≤ 3m	14
Number of plants with the height > 3m	02

Number of plants in flowering and fruiting on the day of survey: 21

All plants are found growing just a meter away at left hand side of the road when going towards PKT tea estates along the slopes and shows very poor growth but flowers and fruits are in plenty, this may be due to more sunlight it receives as compared to other populations.

7. Opposite to PKT tea estates (Plate 6g)

Location: N 10°21.917' E 076°58.049'

Altitude: 1275 m

Total number of Plants	72
Total number of saplings	51
Number of plants with the height ≤ 3m	15
Number of plants with the height > 3m	57

Number of plants in flowering and fruiting on the day of survey: 13

This population is present to the opposite side of PKT tea estates at the right hand side (20 m away from the road) when going towards Balaji temple. This is the only population found growing in an open area with some shrubs and grasses. It was also observed that Indian Gaur often comes to this area for grazing and it was noted that they sometimes graze on *P. anamalayanum* may be due to which plants are very short with less than 4 m in height and moderate foliage. The soil near the plants is very loamy and black brown in colour covered with dry leaf litter. The plants receive good sun light.

8. **Sirukundra Tea Estate, Valparai (Plate 6h)**

Location: N 10°18.542' E 076°57.593'

Altitude: 1118 m

Total number of Plants	641
Total number of saplings	742
Number of plants with the height ≤ 3m	310
Number of plants with the height > 3m	331

Number of plants in flowering and fruiting on the day of survey: 363

This population is found growing in private forest property of Sirukundra tea estate. The plants are very healthy and show luxuriant growth with thick branching and foliage. The soil near the plant is very loamy and black brown in colour covered with dry leaf litter. The plant receives good sun light.

4.2.4. Phenology of *Pseudoglochidion anamalayanum*

P. anamalayanum is an ever green tree. Flowering and fruiting was observed throughout the year. It was observed in the field that the fruits were severely damaged by fruit borers. Fruit borers invariably damaged the fruits starting from early stages to

maturity. When the damaged fruits are cut open, it was observed that entire seed including the embryo was eaten by the larva. The various developmental stages of damaged fruits and a larva which was found inside the fruit are depicted in Plate 7.

4.3. Environmental and Ecological Data

4.3.1. *Phyllanthus talbotii*

It is mostly found growing along the shallow stream and river beds. Substratum varied from sandy to loamy soil, lateritic rocks, and rock crevices (Plate 4). They are normally found growing in patches due to the dehiscent capsules and shedding seeds near the mother plant. They germinate and establish in the vicinity of the mother plant. Flowering and fruiting is from August to November. The seedlings are seen in the month of November and December and start growing luxuriantly from January onwards. It was observed that during the rainy season the plants are almost submerged in the water with no leaves and survive in the form of leafless sticks in the month of June and July. The new leaves start appearing in the months of August to September. The plant has strong root system to hold the soil strongly thus preventing soil erosion as well as preventing plant from getting uprooted in strong water currents. *P. talbotii* mostly associated with other plants species such as *Homonoia riparia*, *Rotula aquatica*, *Syzygium heyneanum* and *Osmunda regalis*. Major threats for *P. tallbotii* are sand quarry, construction of check dams, pollution of streams and other anthropogenic activities (Plate 8).

Soil Analysis

Soil samples collected from each population of *Phyllanthus talbotii* were made as composite sample and used for the soil analysis. Data revealed that the soil pH and electrical conductivity (EC) is varying between different locations, however the pH

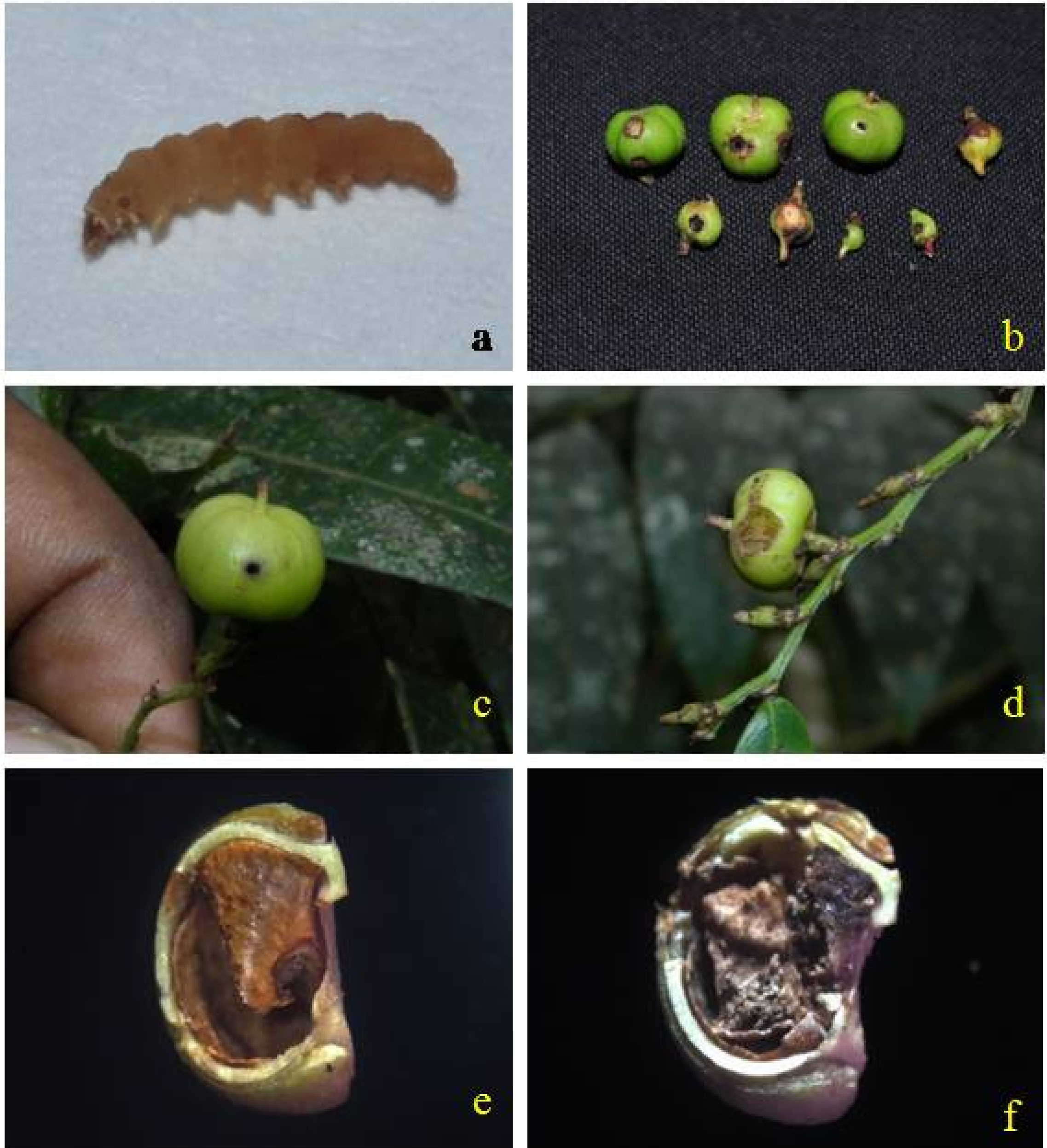


Plate 7. Fruits of *Pseudoglochidion anamalayanum* damaged by insect larvae. a. Insect larvae that damages the fruit; b. Different developmental stages of fruits infected by insect larvae; c. Hole seen after the damage caused by insect larvae; d. Different kind of infection observed in the fruit; e, f. Completely damaged matured fruit including embryo.

Plate 8. **Various threats noted where *Phyllanthus talbotii* populations exist. a, b. Cashew 'feni' distillation unit set-up at Saccordem near sugar factory (arrow indicate the population and a plant; c-f. Sand quarry activity at different sites; g, h. Habitat loss due to check dam construction.**

recorded showed slight to moderate acidic nature of the soil both in Goa as well as Karnataka populations (pH ranged from 5.6 to 6.4). Electrical conductivity also varied from medium to high. The soil showed the presence of high to moderate amount of organic carbon contents which are essential for healthy plant growth. It was observed that Goa populations are rich in organic carbon when compared to the populations found in Karnataka having moderate amount of organic carbon. The soil was with very less phosphorus, while potassium content was found to be medium to very low. All other micronutrients are found to be under required limit. Details of soil analysis are provided in Table 13.

4.3.2. *Pseudoglochidion anamalayanum*

P. anamalayanum is located in the evergreen forests of Anamalai Tiger Reserve, Valparai, Coimbatore district of Tamil Nadu. The plants found growing in loamy soil with pH ranging from 5–6. In general, flowering and fruiting was observed throughout the year, however, the plants found growing on the edge of the forest showed with gregarious flowering since they are exposed to more sunlight. The plants growing deep in the forest have not been observed either in flowering or fruiting. In these cases good numbers of plants have been observed to be arising from suckers. It was also observed that the direct germination of seeds have given rise to very few seedlings, which was found growing beneath the mother plants. Very often capsules are attacked by insect borer and seeds are found to be damaged (Plate 7). *P. anamalayanum* found growing with other associated plants such as *Clerodendrum viscosum*, *Cullenia exarillata*, *Elaeocarpus munronii*, *Elaeocarpus serratus*, *Mallotus tetracoccus*, *Mesua ferrea* and *Nageia wallichiana.* The threats noticed includes chopping of tree branches which extends to the private tea estates. In addition, these plants are also eaten by Indian Gaur (*Bos gaurus*) which has been noted.

Table 13. Soil analysis of natural populations of *Phyllanthus talbotii*.

Locations	pH	E.C. (mmhos/cm)	Macro Nutrient Kg/ha			Micro Nutrients ppm				
			*Nitrogen/ Org. C (%)	P	K	Zn	Fe	Mn	Cu	B
Goa										
Saccordem	5.6	0.128	0.64	4.4	82.6	1.43	58.45	51.82	3.37	0.19
Waddem	6.1	0.138	0.77	6.4	132.7	2.62	51.34	57.28	2.76	0.18
Collem	5.9	0.179	1.33	4.2	81.45	2.26	52.33	58.63	2.18	0.12
Dudhsagar	5.7	0.163	1.12	3.7	93.72	2.09	43.89	45.39	2.11	0.16
Salaulim	6.1	0.195	1.17	4.1	98.87	2.85	55.65	48.13	3.3	0.19
Karnataka										
Jog Falls	6.0	0.290	2.2	Tr.	100.8	2.57	56.92	40.18	4.04	0.15
Agumbe	5.7	0.198	1.9	Tr.	87.32	2.2	46.47	38.66	3.08	0.08
Thirthahalli	6.4	0.121	0.48	Tr.	11.2	1.09	45.26	32.48	2.71	0.13
Madanakka village	6.2	0.116	0.38	Tr.	11.18	1.16	46.29	34.67	2.98	0.16
Halady	5.6	0.302	0.78	Tr.	67.2	0.59	48.31	7.17	3.09	0.09
Kubja-Nadi bridge	5.9	0.143	0.87	0.3	68.23	2.13	51.46	36.86	1.93	0.19
Jadkal	5.7	0.934	1.39	0.5	108.6	1.63	53.88	36.43	3.25	0.04
Halkal	5.7	0.749	1.33	0.4	128.6	1.88	51.77	39.47	3.05	0.06

* Nitrogen is one tenth of the organic carbon

Several herds of elephants and Indian Gaur have damaged the newly planted cuttings / saplings by trampling (Plate 9).

Soil Analysis

Soil samples collected from each population of *Pseudoglochidion anamalayanum* were made as composite sample and used for the soil analysis. Data revealed that the soil pH and electrical conductivity (EC) is varying between different locations, however the pH recorded showed slight to moderate acidic nature of the soil both in all the populations (pH ranged from 5.6 to 6.4). Electrical conductivity was medium. The soil showed the presence of high amount of organic carbon contents which is because of high amount of leaf litter and other biomass decompositions. The soil showed very low to trace amount of phosphorus, while potassium content was found to be very high in most of the populations, except low (134.4 kg/Ha.) at Iyerpady near PKT tea estates. All micronutrients are found to be under required limit except manganese (Mn) which was very low at Iyerpady near PKT tea estates (1.12 ppm), behind NCF Information center (1.38 ppm) and Sirukundra (4.56 ppm) populations. Details of soil analysis are provided in Table 14.

Table 14. Soil analysis of natural populations of *Pseudoglochidion anamalayanum*.

			Macro Nutrient Kg/ha			Micro Nutrients ppm				
Locations	**pH**	**E.C. (mmhos/ cm)**	***N/ Org. C (%)**	**P**	**K**	**Zn**	**Fe**	**Mn**	**Cu**	**B**
37th Hair pin bend	6.1	0.198	2.12	Tr.	470.4	5.87	38.78	17.43	0.59	1.16
39th Hair pin bend	5.7	0.117	2.54	11.9	313.6	7.88	62.76	20.52	0.072	0.67
Iyerpady near PKT tea estates	5.5	0.059	0.35	Tr.	134.4	4.86	43.39	1.21	0.093	0.11
Behind NCF Information center	5.3	0.098	2.05	Tr.	593.6	6.27	50.16	1.38	0.31	0.44
Sirukundra	5.4	0.139	2.68	Tr.	313.6	3.71	52.04	4.56	0.33	0.89

Plate 9. Threats observed for the populations of *Pseudoglochidion anamalayanum*. a, b. Wild animal (Indian Gaur and elephants) movements case damage to the population; c-f. Cutting of trees for road widening (arrow indicate the cut tree); g. Most of the populations growing near tea estates.

4.4. Vegetative Propagation

4.4.1. Response of Stem Cuttings in *Phyllanthus talbotii*

During this study, various concentrations of IBA (indole-3-butric acid) and IAA (indole-3-acetic acid) were used for initiation of roots in vegetative stem cuttings of *P. talbotii*. Stem cuttings started sprouting in all treatments of IBA and IAA after 15–20 days of planting. Out of thirteen treatments used, both IBA and IAA showed similar results in respect to the average shoot length (45 cm). Similarly, both IBA and IAA revealed the survival rate of stem cuttings of about 90–95% after 15 days of planting in the root-trainer under poly-house condition.

The number of primary root formation varied with different concentrations of IBA and IAA. Among the IBA concentrations, maximum numbers of primary roots (9.8±1.3) were observed in 150 ppm, followed by 100 ppm (9±1.8). In various concentrations of IAA tried, 200 ppm revealed with maximum numbers of primary roots (10.2±0.8) followed by 150 ppm (7.8±0.8) as compared to control (3.6±0.5). The length of root was almost similar in 100 ppm of IBA (33.7±0.5cm) and 150 ppm of IBA (32.1±0.9 cm). Among IAA concentrations, 200 ppm showed the maximum length of root (21.9±1.4 cm) as compared to control (7.8±0.3 cm). Rootex was found to be effective when compared to control, however, the survival rate was low (44.62%) (Table 15; Plates 10–12).

4.4.2. Response of Stem Cuttings in *Pseudoglochidion anamalayanum*

A total of 400 stem cuttings were used for vegetative propagation. Out of 400, about 10–15% of stem cuttings induced roots and shoot development (Plate 13–14). The low percentage of rooting was observed may be due to the non-availability of poly-house facility at Valparai (Tamil Nadu) where the experiment was conducted in an open area.

Table 15. Effects of IBA, IAA and Rootex hormones on induction of roots in stem cutting of *Phyllanthus talbotii*.

Plant Growth Regulators	Conc. (ppm)	Cuttings Responded (%)	Avg. no. of Primary roots (180 days)	Avg. Roots length in cm. (180 days)
IBA	50	91	6±1.0	21.9±1.4
	100	94	9±1.8	33.7±0.5
	150	96	9.8±1.3	32.1±0.9
	200	93	6.8±0.8	25.7±0.6
	250	92	4.8±0.8	17.8±0.5
	300	89	3.8±0.8	16.7±0.3
IAA	50	93	7.2±0.8	27.1±0.9
	100	98	7.2±0.8	28.4±0.5
	150	96	7.8±0.8	25.5±1.1
	200	94	10.2±0.8	21.9±1.4
	250	89	5.8±0.8	29.1±0.5
	300	87	3.8±0.8	15.8±0.4
Rootex	Powder	45	9.2±0.3	25±0.4
Control	Distilled water	58	3.6±0.5	7.8±0.3

Date represents mean ± standard errors for five replicates.

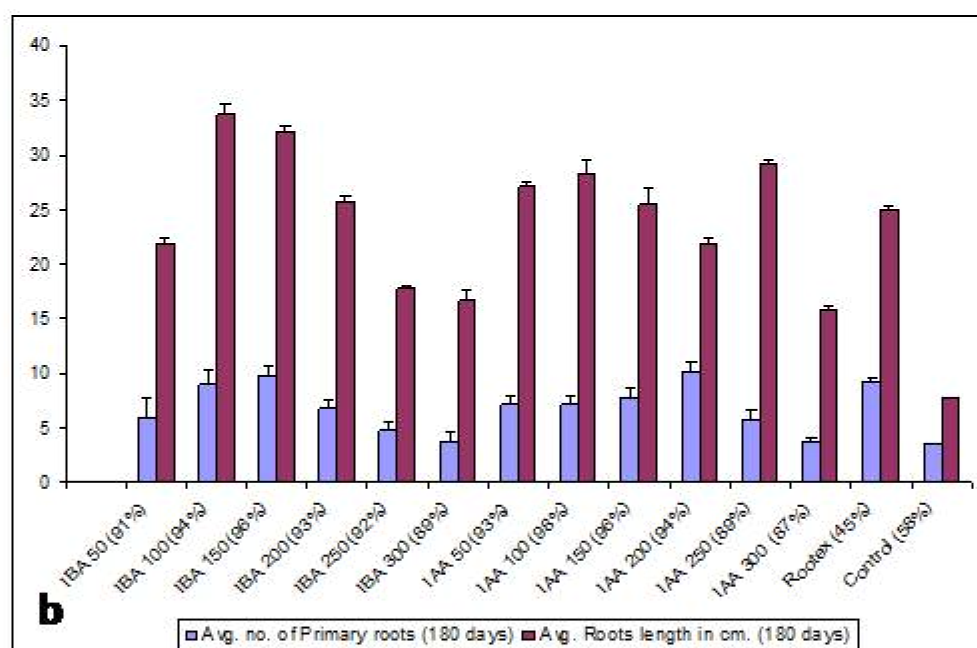


Plate 10. **Vegetative propagation of *Phyllanthus talbotii*: a. Rooting of vegetative stem cuttings in response to various plant growth regulators i. control; ii. Rootex; iii. IAA 50 ppm; iv. IAA 100 ppm; v. IAA 150 ppm; vi. IAA 200 ppm; vii. IAA 250 ppm; viii. IAA 300 ppm; ix. IBA 50 ppm; x. IBA 100 ppm; xi. IBA 150 ppm; xii. IBA 200 ppm; xiii. IBA 250 ppm; xiv. IBA 300 ppm; B. Comparative account of effects of plant growth regulators on rooting of stem cuttings.**

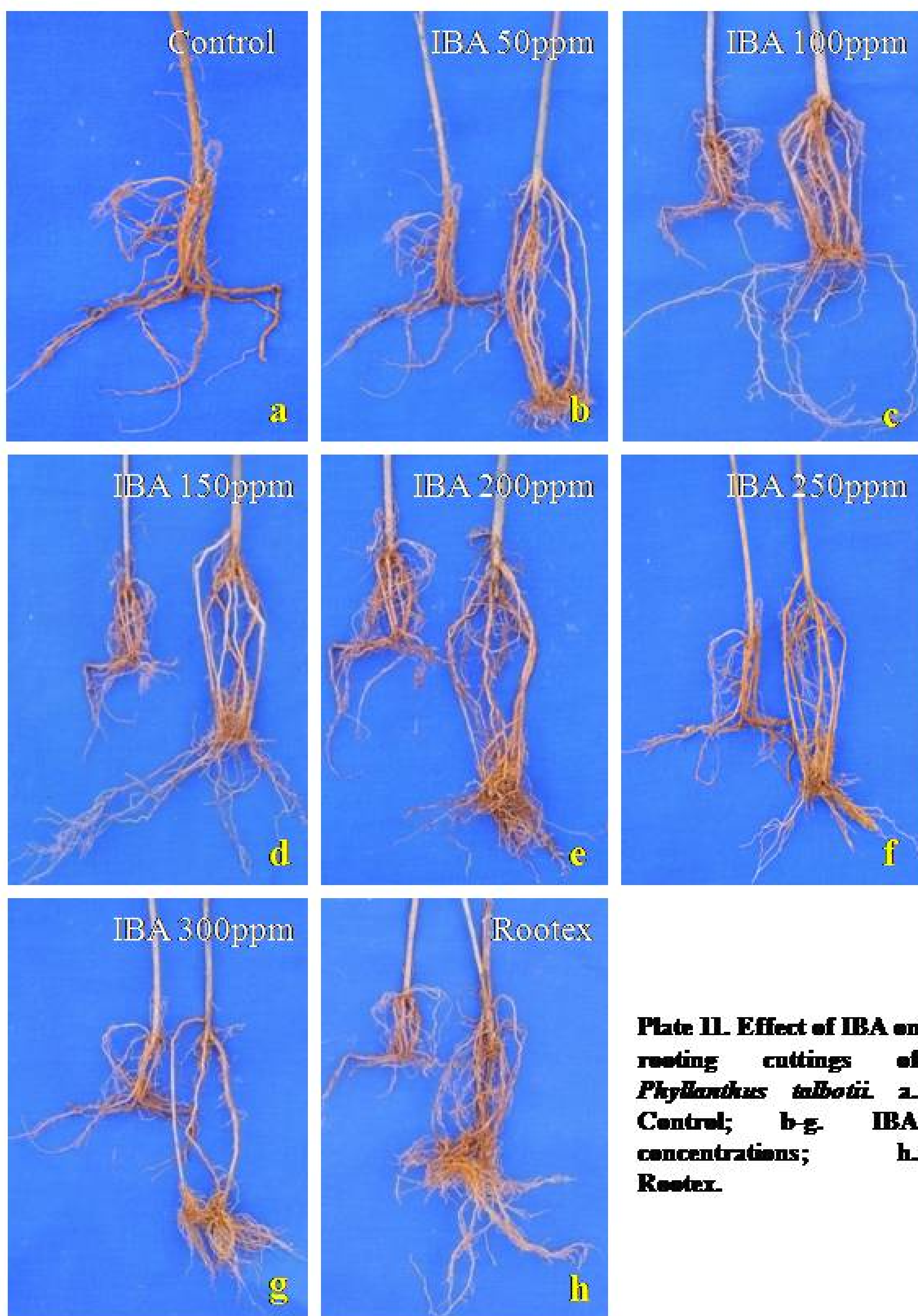


Plate 11. Effect of IBA on rooting cuttings of *Phyllanthus talbotii*. a. Control; b-g. IBA concentrations; h. Rootex.

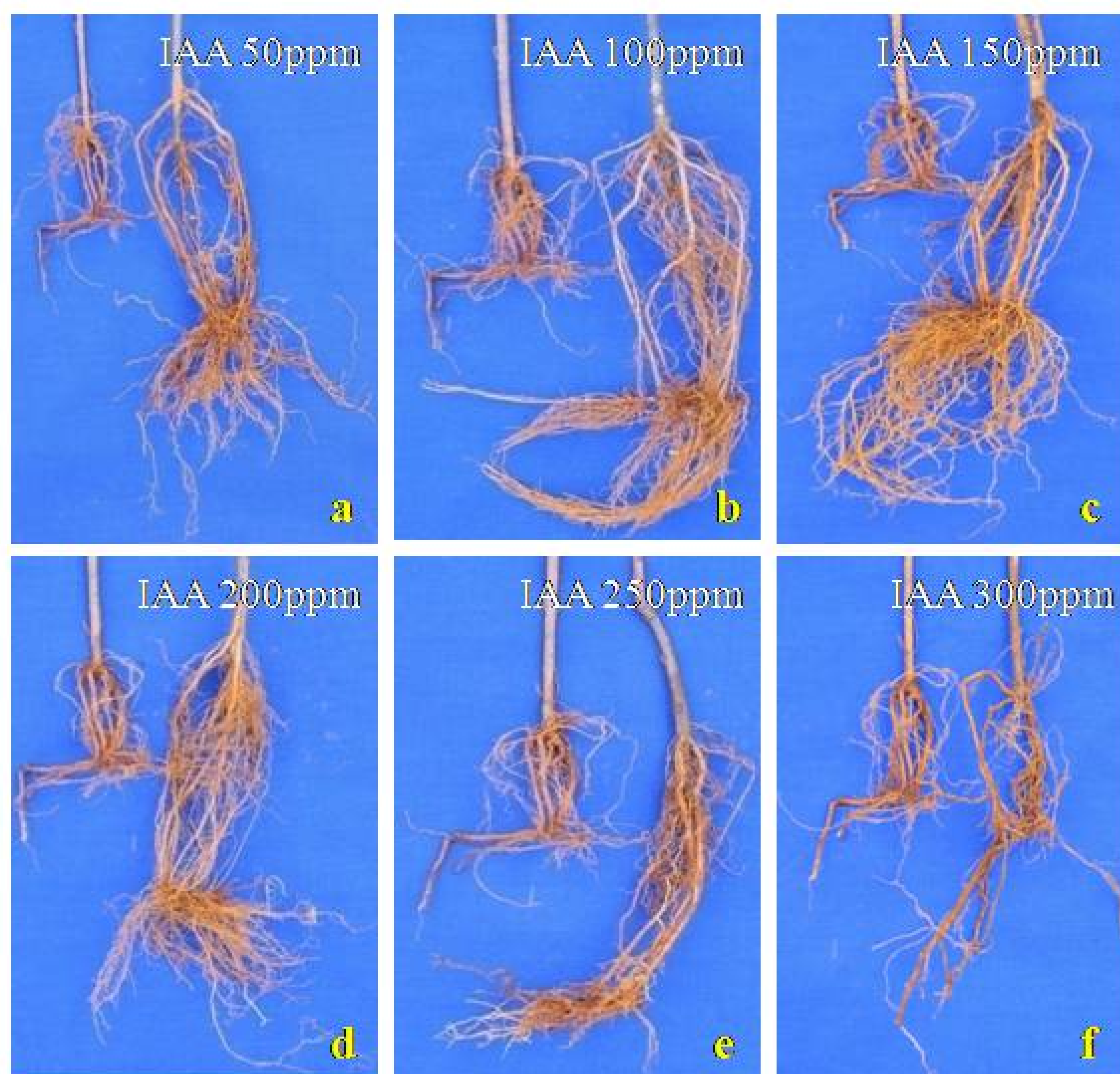


Plate 12. Effect of IAA on rooting of stem cuttings of *Phyllanthus talbotii*. a-f. Different concentrations of IAA used.

Plate 13. a-d. Vegetative propagation of *Pseudoglochidion anamalayanum* through stem cuttings using various concentrations of plant growth regulators IBA and IAA.

Plate 14. Survival of stem cuttings of *Pseudiglochidion anamalayanum*. a, b. Rooted stem cutting brought from Valparai to Goa University polyhouse; c-h. Stem cuttings transferred to pots showing healthy growth in polyhouse at Goa University.

Rooted stem cuttings of four months old of about 25 numbers were brought from Valparai, Tamil Nadu to Goa University and placed in poly-house condition for further growth (Plate 14a, b). Later these plants were transferred to bigger pots with garden soil and farm-yard manure in the ratio of 1:1 (Plate 14c–f). All these stem cuttings responded well and showed luxuriant growth with random flowering. Presently, these plants have grown to the height of 1.5 to 2 m as recorded during the month of March 2015 (Plate 14g, h).

4.4.3. Air-layering of *Pseudoglochidion anamalayanum*

Air-layering technique was successful when compared to vegetative propagation by stem cuttings. Air-layered branch showed the formation of fibrous and white root system. Before the initiation of roots on the cut surface, swelling and callus formation was observed. Air-layered portion was opened after 75 days and checked for rooting. The air-layered branches were observed with profuse thick rooting (Plate 15a–g). A total of 70 branches were air-layered. Among them, 55 were rooted and survived. Among the treatment, rootex showed highest percentage of rooting and survival, followed by IBA and IAA respectively. However, control showed with 40% of rooting and survival (Table 16). Subsequently, the rooted branches were transferred to poly-bags and then transported to Research Nursery, Nature Conservation Foundation (NCF), Old Valparai, Tamil Nadu (Plate 15h).

Table 16. Effect of plant growth regulators on air layering of *Pseudoglochidion anamalayanum*.

Treatment	Total no. of air layered plants	No. of air-layered branches rooted and survived after 75 days	% of air-layered branches rooted and survived after 75 days
Control	10	4	40
IBA	20	17	85
IAA	20	16	80
Rootex	20	18	90
Total	70	55	-

Plate 15. **Vegetative propagation by air-layering in *Pseudiglochidion anamalayanum*. a-g. Root formation in air layered branch; h. Rooted air-layered branch transferred to poly-bags for further growth and placed in nursery at Valparai.**

4.5. *In-Vitro* Regeneration of *Phyllanthus talbotii*

4. 5. 1. Responses of Explants

Different explants such as shoot tip, leaf, nodal segments and seeds were used for *in vitro* regeneration. The vegetative propagated stem cuttings were also brought to the poly-house and maintained for their active growth. These plants were used as source plants to obtain explants regularly for tissue culture study. Among the explants seeds have formed maximum quantity of callus and followed by nodal explants. However, none of the explants or callus has regenerated in to shoots even after several sub-cultures.

4.5.2. *In-Vitro* Seed Germination

Sterilized seeds were inoculated aseptically on full and half strength Murashige and Skoog's medium (MS) without any plant growth regulators (Basal medium). The seeds were swollen after 3 to 4 days of inoculation and germinated within a week (Plate 16a, b). The half strength medium supported more for seed germination than full strength MS medium. Cotyledons emerged after 10 days seedlings growth, during that time seedlings were measured *c.* 2 cm in height (Plate 16c; Table 17). However, further seedling growth was not observed and *in vitro* seedlings died due to shoot necrosis.

4.5.3. Effects of Plant Growth Regulators on Callus Formation

The various plant growth regulators such as auxin and cytokinins were tried individually or in combinations for callus induction. Percentage of regeneration and callus induction for each treatment was scored after one month of culture.

Seed and nodal explants were cultured on MS medium containing 2, 4-D at 0.5–5 mg/L showed callus formation after 7–14 days of culture. For seed explants, swelling of seeds were observed in 3 to 4 days of culture. Initiation of callus was observed in seeds

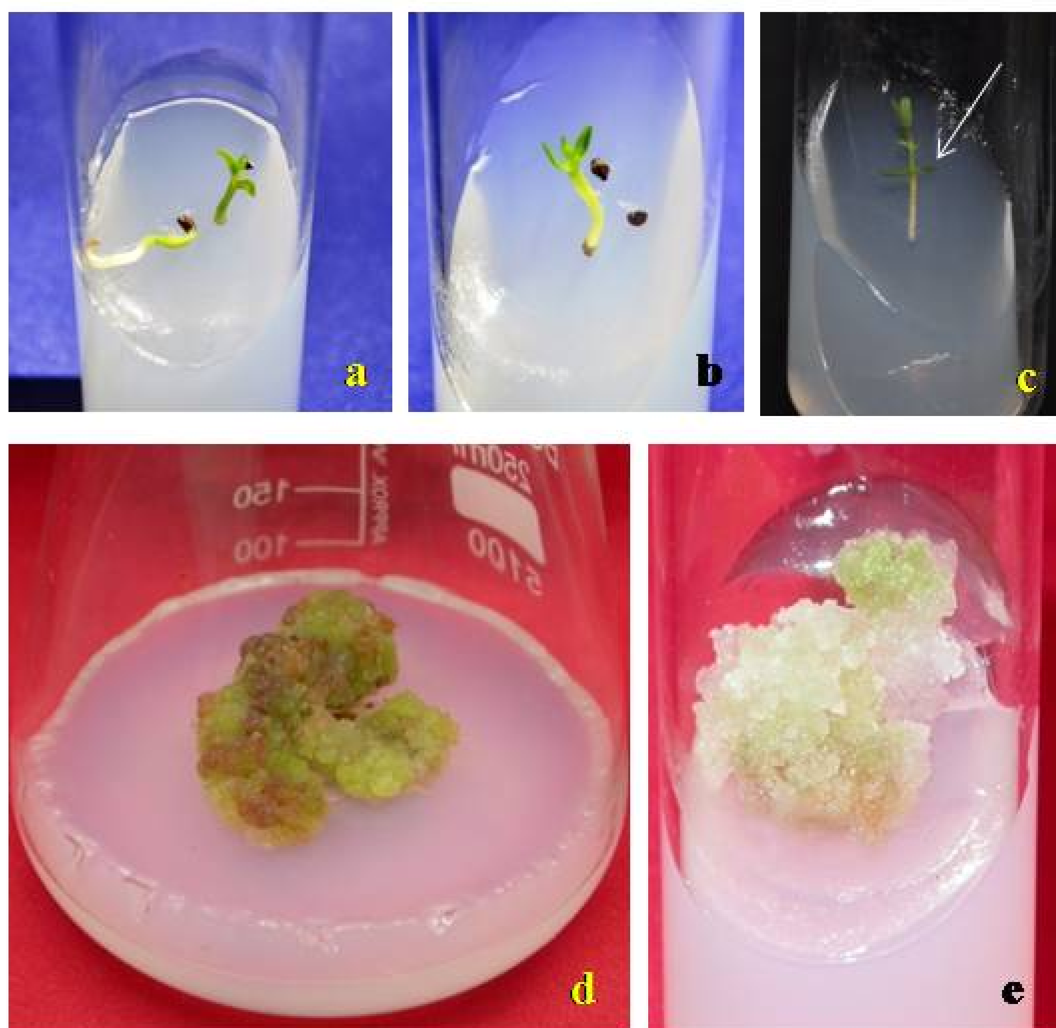


Plate 16. ***In vitro*** **regeneration of** ***Phyllanthus talbotii.*** **a.** ***In vitro*** **seed germination on ½ strength MS basal medium; b.** ***In vitro*** **seed germination on full strength MS basal medium; c. Formation of cotyledons on media containing MS salt and B5 vitamin; d. Extensive callus formation observed in nodal explants on MS with 1 mg/L kinetin; e. Callus formation achieved from seed explants on MS with 1 mg/L 2,4-D.**

Table 17. Responses of *in-vitro* seed germination of *Phyllanthus talbotii*.

MS Basal Medium	Days taken for germination	Seedling height after 10 days (cm)	Seed Germination (%)
Full Strength	6-7	1.8	65
Half Strength	5-7	2.1	78

Table 18. Influence of 2, 4-D on callus induction of seed and nodal segments explants in *Phyllanthus talbotii.*

MS + 2,4-D (mg/L)	Seed Explants		Nodal Explants	
	Culture Responded (%)	Response	Culture Responded (%)	Response
Control	-	-	-	-
0.5	53.33	C	63.33	EC
1.0	56.66	EC	70.00	EC
2.0	46.66	EC	60.00	EC
3.0	40.00	C	56.66	EC
4.0	36.66	C	33.33	C
5.0	22.66	C	23.33	C

C: Explant produced callus   EC: Explant produced extensive callus

after one week of culture. Extensive callus formation was noted in 1 and 2 mg/L 2, 4-D., after one month inoculation (Plate 16e). In nodal segment explants, 2, 4-D., 0.5 to 3 mg/L have produced substantial quantity of callus after 30 days of culture (Table 18; Plate 16d). However, later the callus turned to pinkish colour due to the development of anthocyanin and no further regeneration was observed.

Among the cytokinins BAP, KIN and Zeatin 0.5 mg/L produced extensive quantity of callus (Table 19). In the nodal segments initiation of callus was observed prominently at the cut surface of the explants. It was observed that, when the callus developed from nodal segment it was compact, greenish in colour; while, when derived from seeds it was loose in appearance and whitish in colour. However, both callus did not show any further regeneration of plants when sub-cultured on cytokinin medium (Plate 17).

4.5.4. Direct Shoot Regeneration

For the direct multiple shoot formation explants such as shoot tips and nodal segments were used. Both auxins and cytokinins were used individually and in combinations with MS and WPM medium. Among the cytokinins BAP and KIN showed some response especially the concentrations 0.5 and 1 mg/L (Plate 18a-f). Among the media evaluated, MS medium was found to be more responsive than WPM. However, no multiple shoot formations were observed in any of the cytokinins and auxin treatments.

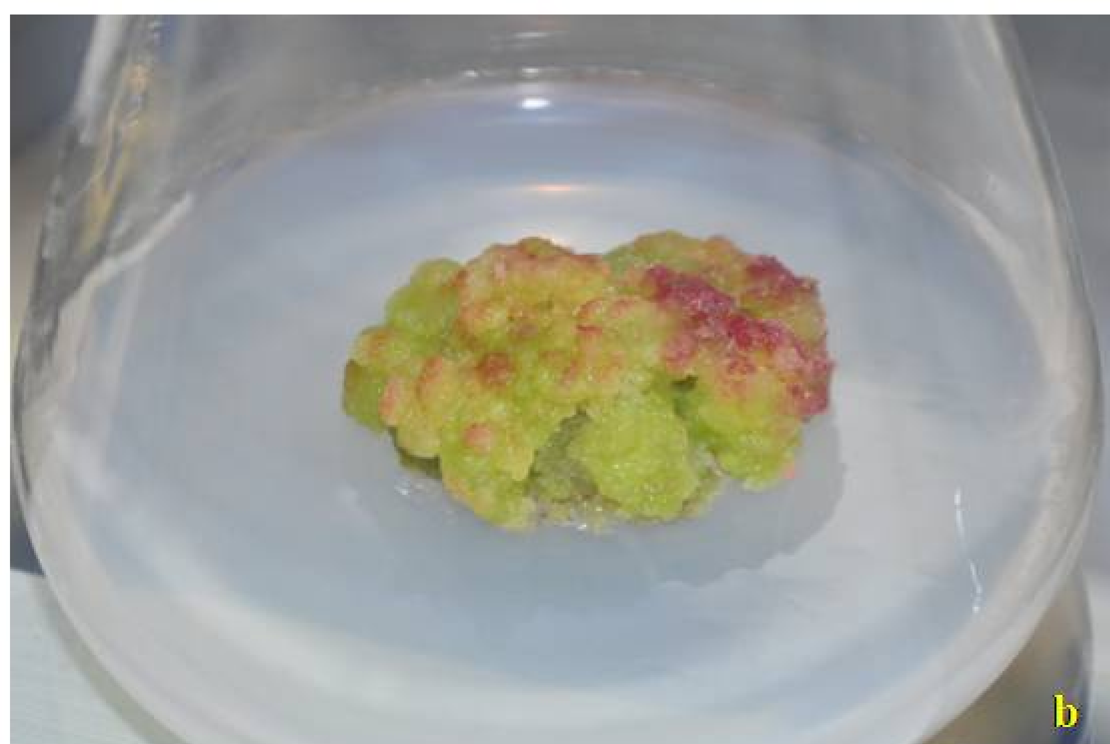


Plate 17. **a. Sub culturing of *Phyllanthus talbotii* callus on different regeneration media in culture flasks. b. Callus turned green and showed the formation of anthocyanin pigments on MS medium with BAP 1 mg/L.**

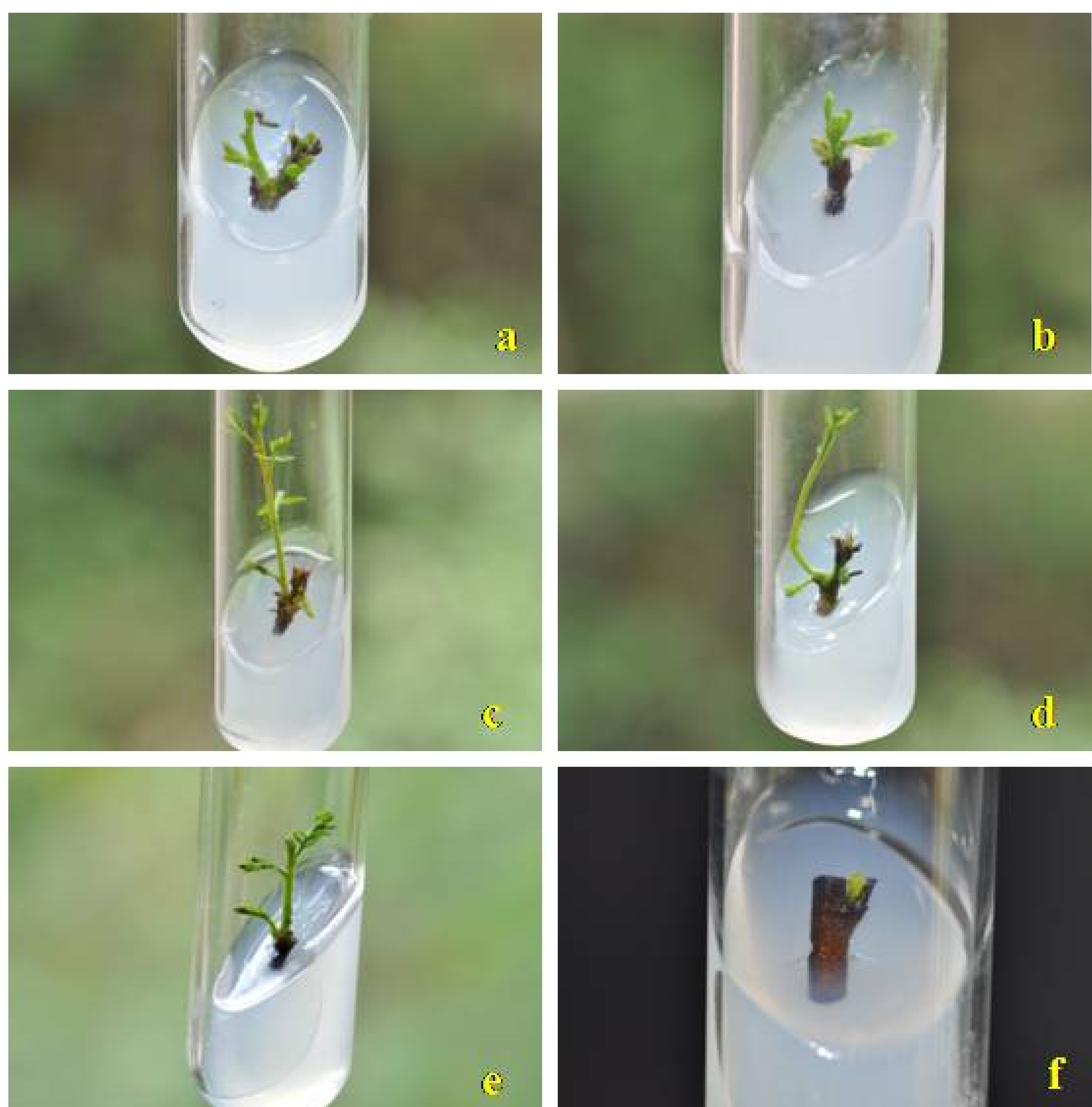


Plate 18. ***In vitro*** **studies of** ***Phyllanthus talbotii.*** **a, b. Nodal segment cultured on MS medium with BAP 1 mg/L; c. BAP 0.5 mg/L; d. BAP 1 mg/L; e. kinetin 1 mg/L; f. TDZ 0.5 mg/L.**

Table 19. Seed and nodal segments derived callus of *Phyllanthus talbotii* sub-cultured on MS medium supplemented with cytokinins for regeneration.

MS + Cytokinins (mg/L)	Seed Explants		Nodal Explants	
	Culture Responded (%)	**Response**	**Culture Responded** (%)	**Response**
Control	-	-	-	-
BAP (0.5)	51.66	EC	74	EC
BAP (1.0)	66.66	C	70	C
KIN (0.5)	65.00	EC	72	EC
KIN (1.0)	73.33	C	69	C
Zeatin (1.0)	63.33	C	70	C
Zeatin (0.5)	68.33	EC	72	EC

C: Explant produced callus EC: Explant produced extensive callus

4.6. *In-Situ* Conservation

4.6.1. *Phyllanthus talbotii*

For *in-situ* conservation of *P. talbotii*, river and stream beds of Western Ghats region of Goa was surveyed. Based on the survey, four existing natural populations and 21 new suitable locations were identified for re-introduction. Soil analysis data was also taken in consideration for the selection of new locations and suitability of the same for re-introduction. The soil analysis study ensured that the soil characteristics of both natural habitats as well as the newly selected sites for the reintroduction are almost similar (Table 20).

Reintroduction of *P. talbotii* was carried out in three successive years (April 2010 to April 2012). During this study, a total of 12,083 cuttings were transplanted (reintroduced) to twenty five sites in Goa. Among the re-introduced sites, four were existing populations (enrichment of existing populations) and 21 sites were new locations of similar habitats for the reintroduction. Out of 12,083 cuttings reintroduced 7,260 (60.08%) cuttings survived. For the enrichment of existing populations cuttings from the same populations or sites were used. At the end of nine months, survival percentage was 68.53% for the plants introduced during 2010 into the existing population and a new population established in Valkinim has shown 61.41% survivability. During 2011 and 2012 more populations were established as well as existing population were enriched. Among the cuttings planted in April 2011-12 the survival percentage is more than 60% except for six populations (Table 21). At Dhuner and Zambaulim the survival percentage of the cuttings was very low (22.88 and 09.39%). The sites such as Ganjem, Nanus and Bhutpal also showed very less survival rate (35, 20.62 and 24.16%). However, wherever cuttings survived, height increased over 50% to 90–110 cm and also produced 6-8 ramets

Table 20. Soil analysis of newly established populations of *Phyllanthus talbotii.*

			Macro Nutrient Kg/ha			Micro Nutrients ppm				
Locations	pH	E.C. (mmhos/cm)	*Nitrogen/ Org. C (%)	P	K	Zn	Fe	Mn	Cu	B
Valkinim	5.8	0.132	0.84	6.5	101.2	2.12	44.13	61.92	2.11	0.28
Dhuner	6.1	0.138	0.77	6.4	132.7	2.62	51.34	57.28	2.76	0.18
Selaulim Garden	6.0	0.158	1.33	6.2	71.2	3.31	53.82	38.73	3.98	0.06
Kodra	5.9	0.147	1.26	4.7	121.3	1.43	41.55	61.72	3.11	0.53
Ganjem	6.3	0.173	1.41	3.9	77.5	2.62	45.13	59.45	2.98	0.15
Nanus	5.7	0.257	2.93	5.6	152.4	1.38	57.62	58.32	3.13	0.07
Ansolem	5.9	0.128	0.98	7.2	133.1	2.32	48.22	64.21	3.81	0.37
Kahkdi	5.7	0.184	1.14	6.8	83.1	2.14	50.12	43.31	2.15	0.17
Satrem	6.1	0.318	2.21	6.3	79.3	2.43	49.15	57.61	4.61	0.24
Valpoi	6.3	0.242	2.08	3.7	123.3	1.83	47.32	49.41	2.54	0.21
Waddem Bridge	6.1	0.152	0.72	6.3	121.4	1.98	46.38	71.72	2.02	0.51
Netravali	5.7	0.183	0.93	3.4	143.2	2.88	51.47	57.83	3.17	0.43
Kazara	6.4	0.262	2.23	5.8	138.6	2.64	50.11	63.54	3.32	0.35
Zambaulim	5.9	0.273	2.85	8.9	198.3	3.41	52.43	70.14	4.14	0.04
Balli	6.2	0.306	0.83	14.9	179.2	2.58	49.66	40.12	3.79	0.05
Condimar	6.5	0.261	0.88	9.8	188.1	3.31	56.98	62.73	4.54	0.38
Ghorwawal	6.1	0.289	0.79	12.7	165.7	2.39	52.46	61.15	3.34	0.11
Barazan	5.8	0.189	0.81	9.7	154.4	2.43	51.32	60.34	4.53	0.24
Butpal	6.0	0.321	2.63	4.8	156.3	1.93	42.34	53.31	4.01	0.21
Potrem	5.8	0.192	2.16	11.5	168.2	1.73	50.11	69.34	2.69	0.43
Wedia	5.6	0.131	0.65	4.3	82.8	1.97	58.22	51.54	3.08	0.32

* Nitrogen is one tenth of the organic carbon

Table 21. Reintroduction of *Phyllanthus talbotii* in the year 2010, 2011 and 2012.

Sr. No.	Place Names	Lat.	Long.	Alt. (ft)	Date	No. of Planted Cuttings	No. of survived plants	Survival %
1	Waddem (E)	N15°06.186'	E74°12.444'	143	02.04.10	1500	1028	68.53
2	Valkinim (N)	N15°12.275'	E74°14.744'	115	04.04.10	1003	616	61.41
3	Selaulim Dam (E)	N15°13.091'	E74°14.077'	148	03.04.11	850	524	61.64
4	Saccordem (E)	N15°24.710'	E74°48.234'	179	08.04.11	954	582	61
5	Collem (E)	N15°20.290'	E74°14.767'	181	08.04.11	355	223	62.81
6	Dhuner (N)	N15°09.125'	E74°13.664'	128	21.04.11	1112	254	22.88
7	Selaulim (N)	N15°12.973'	E74°10.816'	148	03.04.11	200	93	46.05
8	Kodra (N)	N15°23.826'	E74°04.549'	179	21.4.12	275	178	64.72
9	Ganjem (N)	N15°28.011'	E74°05.014'	17	21.4.12	320	112	35
10	Nanus (N)	N15°27.745'	E74°04.553'	16	21.4.12	160	33	20.62
11	Ansolem (N)	N15°29.775'	E74°08.654'	79	22.4.12	522	336	64.36
12	Kahkdi (N)	N15°30.190'	E74°08.223'	40	22.4.12	386	232	60.1
13	Satrem (N)	N15°36.604'	E74°12.921'	311	22.4.12	396	241	60.85
14	Valpoi (N)	N15°32.176'	E74°08.931'	297	22.4.12	100	63	63
15	Waddem (N)	N15°06.358'	E74°12.618'	135	23.4.12	180	124	68.88
16	Netravali (N)	N15°05.676'	E74°13.147'	112	23.4.12	600	478	79.66
17	Kazara (N)	N15°05.199'	E74°10.309'	263	23.4.12	540	434	80.37
18	Zambaulim (N)	N15°11.386'	E74°05.741'	102	23.4.12	330	31	9.39
19	Balli (N)	N15°09.741'	E74°01.720'	43	24.4.12	120	96	80
20	Condimar (N)	N15°08.261'	E74°01.471'	85	24.4.12	420	361	85.95
21	Ghorwawal (N)	N15°07.275'	E74°02.086'	194	24.4.12	450	276	61.33
22	Barazan (N)	N15°04.382'	E74°01.649'	144	24.4.12	120	81	67.5
23	Bhutpal (N)	N14°59.969'	E74°05.126'	49	24.4.12	120	29	24.16
24	Potrem (N)	N15°12.087'	E74°15.087'	84	25.4.12	540	412	76.29
25	Wedia (N)	N15°09.962'	E74°15.202'	208	25.4.12	530	423	79.81
TOTAL						**12083**	**7260**	**60.08**

E - planted for enriching the existing population; N - planted for establishing new population

Plate 19. **a-g. Vegetative mass propagation of *Phyllanthus talbotii* at Goa Forest Department Research Nursery, Valkinim, Goa; h. Field monitoring before planting the rooted stem cuttings.**

Plate 20. **Reintroduction of *Phyllanthus talbotii* at different sites in Goa for *in situ* conservation. a, b. Waddem; c. Collem; d, e. Potraem; f. Selaulim dam; g. Saccordem; h. Monitoring of reintroduced plants.**

Plate 21. **Established stem cuttings of *Phyllanthus talbotii* in the introduced sites of Goa after 8 months of plantation. a. Potrem; b. Condimar; c-f. Waddem; g. Wedia; h. Balli.**

Waddem

Collem

Valkinim

Condimar

Plate 22. Reintroduced sites of *Phyllanthus talbotii* before and after 8 months of reintroduction.

thus establishing well. Thus the populations of *P. talbotii* have been established at different localities (Table 21; Plates 19–22).

4.6.2. *Pseudoglochidion anamalayanum*

In situ conservation of *P. anamalayanum* was carried out by using vegetative stem cuttings and root suckers. In the first set of experiment, a total of 200 stem cuttings of approx. 30 cm height were cut directly from the trees and planted (reintroduced) in two different sites. One site was selected near PKT tea estate and another site was near 39th hair-pin bend of Anamalai Tiger Reserve, Valparai, Coimbatore district, Tamil Nadu. The survival of the planted stem cuttings were checked after 35 days, it was observed that these directly planted cuttings failed to establish and dried.

As a second set of experiment of *in-situ* conservation for *P. anamalayanum*, root suckers collected from the different natural populations where they were found growing abundantly. In case of *P. anamalayanum*, roots suckers formed an important vegetative propagule for *in-situ* conservation since it produces profuse coppicing (formation of many young shoots as new plantlets from the main roots) during their growth. These young saplings (roots suckers) are directly formed from adventitious root buds and are good source of planting material if separated carefully from the mother plants without damaging roots. During this study, a total of 555 root suckers were planted in the year 2012 at six different locations of Anamalai Tiger Reserve. Transplanted suckers were monitored periodically for their successful establishment and survival in new sites. After checking for their survival it was found that transplanted suckers established well and observed to be healthy this showed an overall survival of 252 (45.40%) root suckers after eight months of transplantation (Table 22; Plates 23, 24).

Table 22. Reintroduction of *Pseudoglochidion anamalayanum* root suckers in the year 2012 and their survival at Valparai, Tamil Nadu.

Sr. No	Place Names	GPS Coordinates	Altitude (meters)	Planted Suckers	Survival in December 2012	Survival in March 2013
1	**PKT Opposite**	N10°21.242' E76°59.917'	1235 m	65	54 (83.07%)	51(78.46%)
2	**PKT Top**	N10°21.242' E76°59.917'	1239 m	40	08 (20.00%)	5 (12.5%)
3	**Old Kadambarai Road**	N10°21.847' E74°59.841'	1350 m	100	78 (78.00%)	72 (72%)
4	**Upper Aliyar Dam**	N10°26.130' E77°60.074'	860 m	110	84 (76.36%)	72 (65.45%)
5	**Iyerpady Top**	N10°21.917' E76°58.049'	1365 m	130	–	–
6	**Manambolli**	N10°21.499' E76°15.661'	596 m	110	87 (79.09%)	52(47.27%)
Total				**555**	**311 (56.04%)**	**252 (45.4%)**

Plate 23. ***In situ*** **conservation of** ***Pseudoglochidion anamalayanum*** **using root suckers at Anamalai Tiger Reserve, Valparai, Tamil Nadu.**

Plate 24. Survival of root suckers of *Pseudiglochidion anamalayanum* after 8 months of reintroduction in the natural habitats.

Based on the success seen in the root suckers, an additional 796 root suckers were transplanted during the onset of monsoon in the year 2013 at two new different localities (Table 23). The survival rate of March 2013 transplanted root suckers could not be assessed due to expiry of the working permit to work at Anamalai Tiger Reserve.

Table 23. Reintroduction of *Pseudoglochidion anamalayanum* root suckers in the year 2013 at Valparai, Tamil Nadu.

Sr. No	Place Names	GPS Coordinates	Altitude (meters)	Planted Suckers
1	**Upper Aliyar Dam**	N10°26.130' E77°60.074'	859 m	57
2	**Manambolli**	N10°21.499' E76°15.661'	595 m	739
Total				**796**

4.7. Genetic Diversity Studies

4.7.1. Genetic Diversity among the Populations of *Phyllanthus talbotii*

4.7.1.1. ISSR Band Pattern

Twenty two ISSR primers were initially screened using genomic DNA isolated from *Phyllanthus talbotii*. Out of 22 primers used for amplification of genomic DNA, 12 primers did not amplify for any of the samples. Remaining 10 ISSR primers showed clear and reproducible banding pattern were used during this study. ISSR analysis was carried out in eight populations collected from different regions of Goa and Karnataka. Banding patterns of amplified primers are shown in (Plate 25a-d). A total of 85 ISSR bands were obtained from ten different primers and for each primer, the number of bands ranged from 05 to 12 with an average of 8.50 bands per primer. Of these sixty-one bands were polymorphic with an average of 6.10 bands per primer. Average polymorphism across all the samples was 71.85%, thus showing good genetic diversity in *P. talbotii* populations in the State of Goa and Karnataka. The primer details, percentage of polymorphism and other details are provided in Table 24**.**

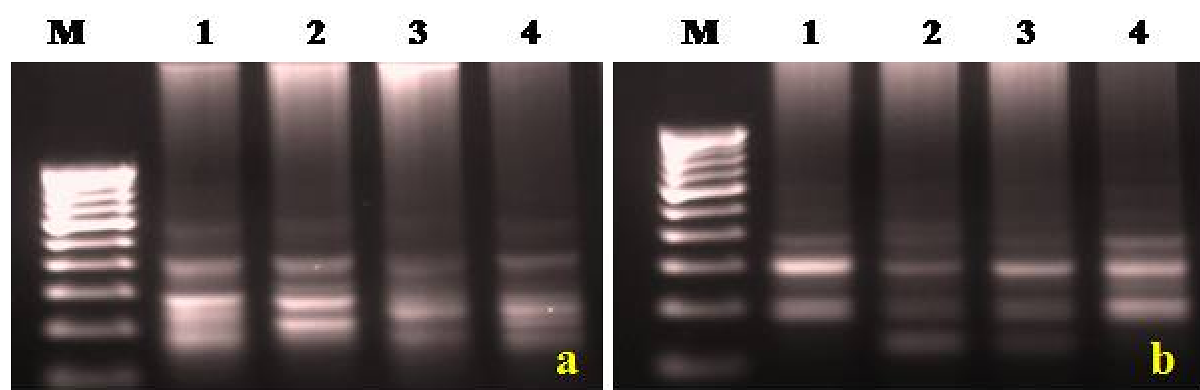
4.7.1.2. Genetic Identity and Cluster Analysis

Pair wise genetic similarities were computed from ISSR data using BioDiversity Pro software. The similarity matrix value obtained for the populations are given in (Table 25). ISSR data of eight different populations of *P. talbotii* was used to generate dendrogram by using Bray-Curtis cluster analysis (single link) method through BioDiversity Pro software. Dendrogram separated the populations based on the geographic locations. The populations of Karnataka did not form any cluster and each population stayed separately which may be due to different altitudinal ranges and distant geographical locations of each population. However, populations of Goa, Saccordem and Waddem,

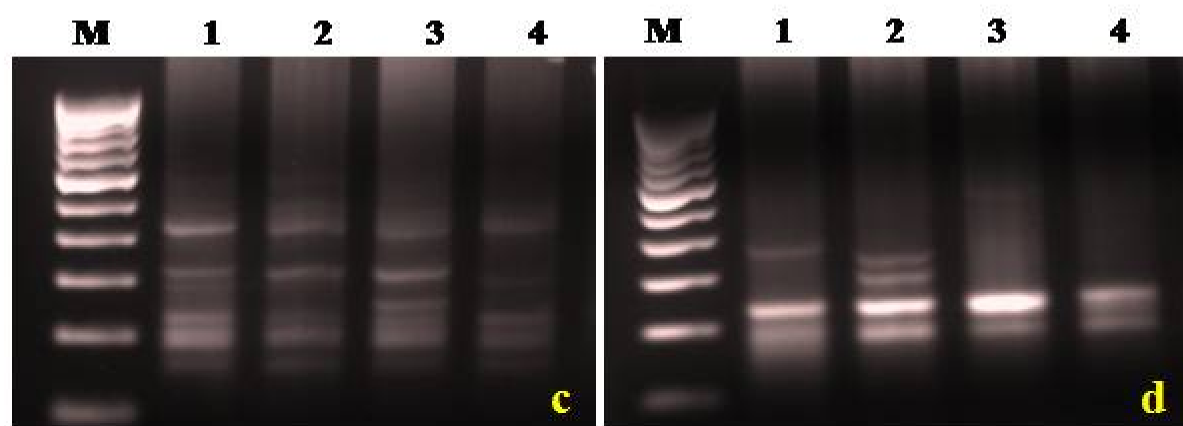
Plate 25 a, b. **ISSR banding pattern in 4 populations of *Phyllanthus talbotii* from the State of Goa obtained from PCR amplification by ISSR primer a. Oligo 807 and b. Oligo 817. M indicates standard DNA marker; Lane 1. Dudhsagar; Lane 2. Collem; Lane 3. Waddem and Lane 4. Saccordem.**

c, d. **ISSR banding pattern in 4 populations of *Phyllanthus talbotii* from the State of Karnataka obtained from PCR amplification by ISSR primer c. Oligo 807 and d. Oligo 817. M indicates standard DNA marker; Lane 1. Jadkal; Lane 2. Halady, Lane 3. Jog Falls and Lane 4. Thirthahalli.**

e, f. **ISSR banding pattern in 8 samples from single population of *P. talbotii* from Waddem, Sanguem, Goa obtained from PCR amplification by ISSR primer e. Oligo 807 and f. Oligo 817. M indicates standard DNA marker; Lanes 1 to 8 are samples from different individuals from single population.**

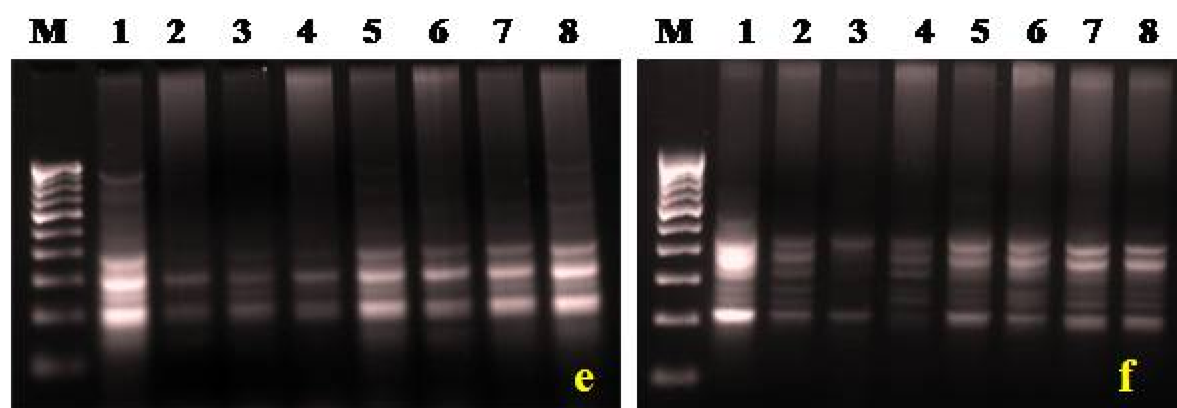


Primer: UBC 807 **Goa** Primer: UBC 817



Primer: UBC 807 **Karnataka** Primer: UBC 817

Waddem, Sanguem taluka-Goa



Primer: UBC 807 Primer: UBC 817

Plate 25. **ISSR primers banding profile of *Phyllanthus talbotii* within and among the populations.**

Table 24. Amplified, polymorphic bands, percentage of polymorphism and unique bands in ISSR analysis among the populations of *Phyllanthus talbotii* from Goa and Karnataka.

Primer	Primer sequence	Total No. of bands	No. of Polymorphic bands	Polymorphism Percentage	No. of unique bands
807	AGA GAG AGA GAG AGA GT	10	07	70	-
817	CAC ACA CAC ACA CAC AA	11	08	72.72	01
828	TGT GTG TGT GTG TGT GA	06	04	66.66	-
835	AGA GAG AGA GAG AGA GYC	08	04	50	02
844	CTC TCT CTC TCT CTC CTR C	07	07	100	-
848	CAC ACA CAC ACA CAC ARG	09	06	66.66	-
868	GAA GAA GAA GAA GAA GAA	08	03	37.50	-
873	GAC AGA CAG ACA GAC A	09	09	100	-
876	GAT AGA TAG ATA GAT A	12	09	75	01
881	GGG TGG GGT GGG GTG	05	04	80	-
Average		**85**	**61**	**-**	**04**
Mean		**8.5**	**6.1**	**71.85**	**-**

Table 25. Similarity matrix among the populations of *Phyllanthus talbotii* based on ISSR analysis.

Similarity Matrix								
	Saccordem	Collem	Dudhsagar	Waddem	Jog Fallsa	Thirthahalli	Halady	Jadkal
Saccordem	*	78.2609	70.00	91.6667	76.1905	76.1905	70.00	50.00
Collem	*	*	85.7143	80.00	72.7273	81.8182	66.6667	58.8235
Dudhsagar	*	*	*	63.6364	63.1579	84.2105	66.6667	57.1429
Waddem	*	*	*	*	78.2609	69.5652	72.7273	44.4444
Jog Falls	*	*	*	*	*	80.00	73.6842	66.6667
Thirthahalli	*	*	*	*	*	*	73.6842	66.6667
Halady	*	*	*	*	*	*	*	71.4286
Jadkal	*	*	*	*	*	*	*	*

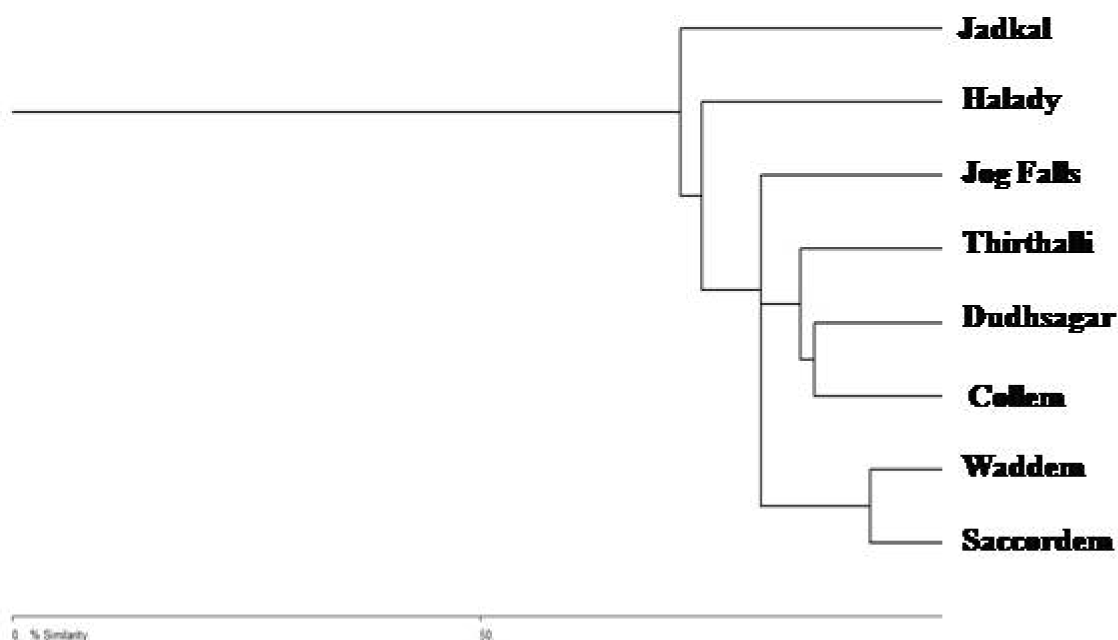


Fig. 2. **Dendrogram of Bray-Curtis cluster analysis of genetic variation and similarities among eight populations of *Phyllanthus talbotii* from Goa and Karnataka using ISSR data.**

Dudhsagar and Collem formed the clusters, this may be due to closer geographical locations and similar altitudinal ranges (Fig. 2).

4.7.1.3. Genetic Diversity within the Population *Phyllanthus talbotii*

Ten primers were selected for the amplification of genomic DNA based on the earlier screening as mentioned in 4.7.1.1. ISSR analysis was carried out for eight plant samples collected within the single population of Waddem, Sanguem, Goa. Banding profile of amplified ISSR primers is presented in (Plate 25 e-f). A total of 91 ISSR bands were obtained from eight different randomly selected individual plants. For each primer, the number of bands ranged from 05 to 09 with an average of 9.10 bands per primer. Of these sixty-eight bands were polymorphic with an average of 6.80 bands per primer. Average polymorphism across all the samples was 75.12%, thus showing good genetic diversity in *P. talbotii* population at Waddem (Table 26).

4.7.1.4. Genetic Identity and Cluster Analysis

Pair wise genetic similarities were computed from ISSR data. Similarity matrix value obtained for the populations are given in Table 27. ISSR data of eight individual plants from single population of Waddem, Goa, was used to generate dendrogram. Dendrogram showed two major clusters with sub-clade (Fig. 3). Even though the population is small approximately with an area of 600 m^2 substantial genetic diversity shown between the individuals within the single population.

Table 26. Amplified, polymorphic bands, percentage of polymorphism and unique bands in ISSR analysis within single of *Phyllanthus talbotii* from Waddem, Sanguem, Goa.

Primer	Primer sequence	Total No. of bands	No. of Polymorphic bands	Polymorphism Percentage	No. of unique bands
807	AGA GAG AGA GAG AGA GT	10	08	80	-
817	CAC ACA CAC ACA CAC AA	09	07	77.77	-
828	TGT GTG TGT GTG TGT GA	08	05	62.50	-
835	AGA GAG AGA GAG AGA GYC	11	09	81.81	-
844	CTC TCT CTC TCT CTC CTR C	09	08	88.88	01
848	CAC ACA CAC ACA CAC ARG	10	05	50	-
868	GAA GAA GAA GAA GAA GAA	08	05	62.50	-
873	GACAGACAGACAGACA	09	07	77.77	01
876	GATAGATAGATAGATA	10	07	70	-
881	GGGTGGGGTGGGGTG	07	07	100	-
Average		**91**	**68**	**-**	**02**
Mean		**9.1**	**6.8**	**75.12%**	**-**

Table 27. Similarity matrix within single population of *Phyllanthus talbotii* based on ISSR analysis.

	Waddem-1	Waddem-2	Waddem-3	Waddem-4	Waddem-5	Waddem-6	Waddem-7	Waddem-8
Waddem-1	*	76.9231	66.6667	76.9231	96.7742	93.3333	100.00	100.00
Waddem-2	*	*	88.8889	90.00	80.00	83.3333	76.9231	76.9231
Waddem-3	*	*	*	77.7778	69.5652	72.7273	66.6667	66.6667
Waddem-4	*	*	*	*	80.	83.3333	76.9231	76.9231
Waddem-5	*	*	*	*	*	96.5517	96.7742	96.7742
Waddem-6	*	*	*	*	*	*	93.3333	93.3333
Waddem-7	*	*	*	*	*	*	*	100.
Waddem-8	*	*	*	*	*	*	*	*

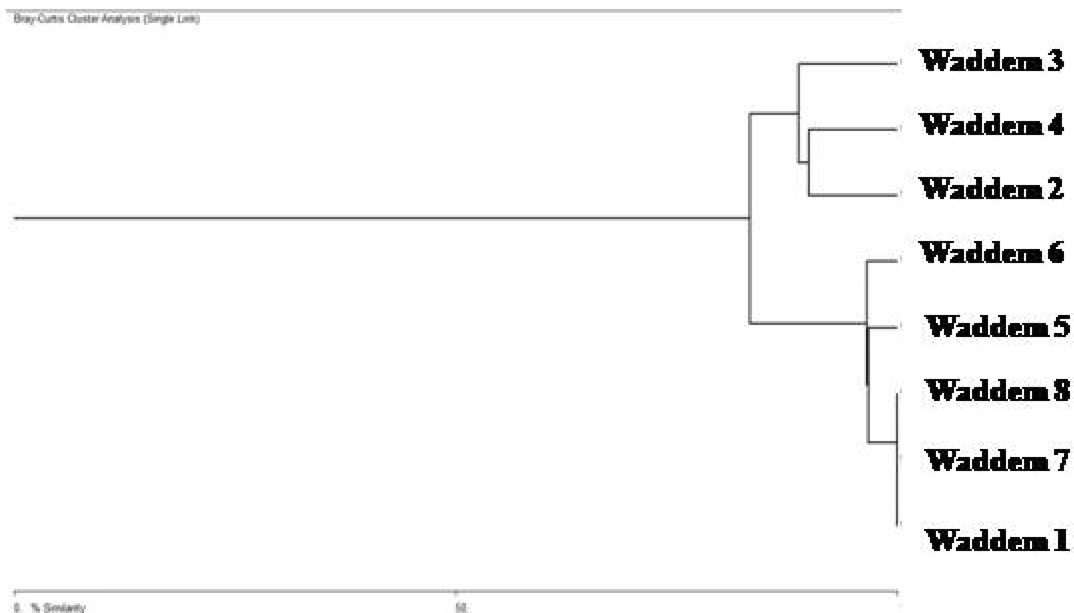


Fig. 3. **Dendrogram of Bray-Curtis cluster analysis of genetic variation and similarities within single population of *Phyllanthus talbotii* at Waddem, Sanguem, Goa based on ISSR data.**

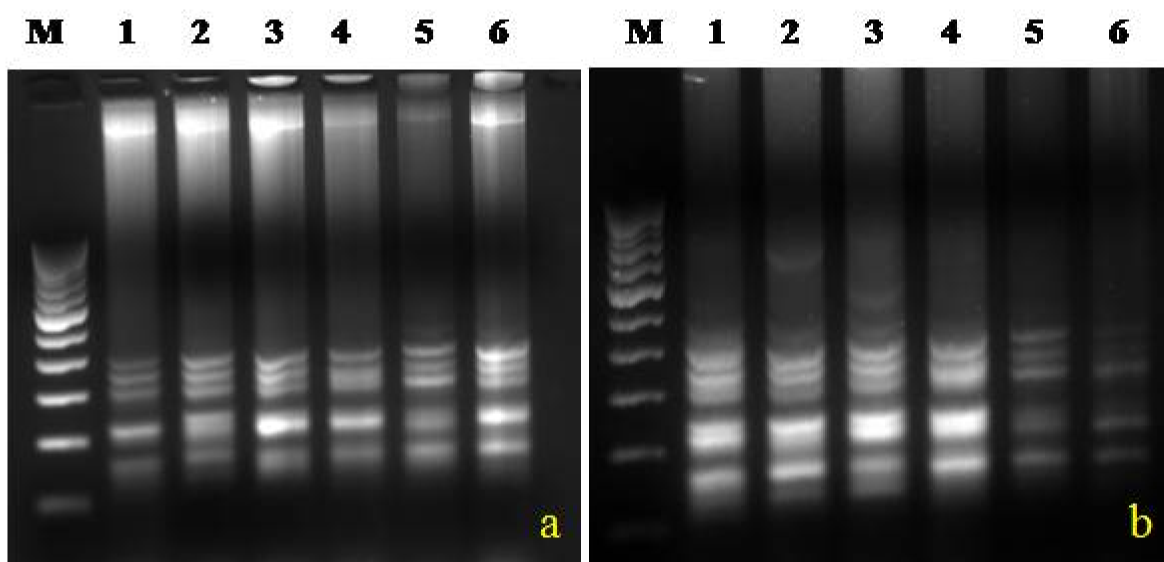
4.7.2. Genetic Diversity among the Populations *Pseudoglochidion anamalayanum*

4.7.2.1. Band Pattern

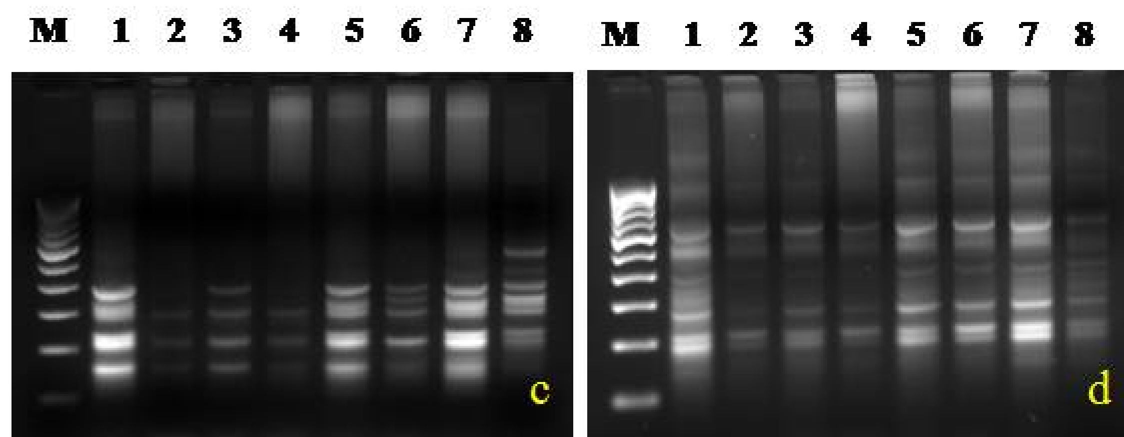
Twenty two ISSR primers were initially screened using genomic DNA isolated from *P. anamalayanum* and among them 11 primers did not amplify. Remaining 11 ISSR primers showed reproducible banding patterns and used during this study. For ISSR analysis, leaf samples were collected from eight different populations. Banding patterns of amplified primers are shown in (Plate 26a, b). A total of 88 ISSR bands were obtained from eleven different primers. For each primer, the number of bands ranged from 05 to 10 with an average of 5.27 bands per primer. Of these, 58 bands were polymorphic with an average of 5.27 bands per primer Average polymorphism across all the populations was 65.73%, thus showing good genetic diversity among the populations of *P. anamalayanum* (Table 28).

4.7.2.2. Genetic Identity and Cluster Analysis

Pair wise genetic similarities were computed from ISSR data. The similarity matrix value obtained for the populations are presented in (Table 29). ISSR data of eight different populations are used to generate the dendrogram. The population 37th hair pin bend and 39th hair pin bend formed a cluster while other populations stood independently. However, PKT populations linked with 37th and 39th hair pin bend populations since they are found within the closer geographical locations. Srikundra, 39th hair pin bend right and Iyerpadi IInd division populations not clustered, this may be due to the geographical separations mainly due to the tea estates which make them as distinct populations (Fig. 4).



Primer: Oligo 835 Primer: Oligo 844



Primer: Oligo 835 Primer: Oligo 868

Plate 26. **ISSR banding pattern in 6 populations of *Pseudoglochidion anamalayanum* obtained from PCR amplification by ISSR primer. a. Oligo 835; b. Oligo 844; M indicates standard DNA marker; lane 1. 37 Hairpin Bend; Lane 2. PKT,; Lane 3. 39 Hairpin bend (Right); Lane 4. Srikundra; Lane 5. Iyerpadi 2nd Division,; Lane 6. 39th Hairpin Bend (Left).**

c and d. ISSR banding pattern of 08 samples from single population of *Pseudoglochidion anamalayanum* obtained from PCR amplification by ISSR primer c. Oligo 835; d. Oligo 868; M indicates DNA size marker; 1 to 8 are samples from 8 different individuals.

Table 28. Amplified, polymorphic bands, percentage of polymorphism and unique bands in ISSR analysis among the populations of *Pseudoglochidion anamalayanum* from Valparai, Tamil Nadu.

Primer	Primer sequence	Total No. of bands	No. of Polymorphic bands	Polymorphism Percentage	No. of unique bands
808	AGA GAG AGA GAG AGA GC	09	05	55.55	-
810	GAG AGA GAG AGA GAG AT	05	02	40	-
812	GAG AGA GAG AGA GAG AA	06	04	66.66	-
817	CAC ACA CAC ACA CAC AA	09	04	44.44	-
828	TGT GTG TGT GTG TGT GA	08	07	87.5	-
835	AGA GAG AGA GAG AGA GYC	08	03	37.50	-
844	CTC TCT CTC TCT CTC CTR C	10	03	30	-
848	CAC ACA CAC ACA CAC ARG	07	05	71.42	-
868	GAA GAA GAA GAA GAA GAA	07	07	100	02
873	GAC AGA CAG ACA GAC A	09	09	100	01
881	GGG TGG GGT GGG GTG	10	09	90	03
Total		**88**	**58**	**-**	**06**
Mean		**8**	**5.27**	**65.73%**	**-**

Table 29. Similarity matrix among the populations of *Pseudoglochidion anamalayanum* based on ISSR analysis.

Similarity Matrix	Lane 1	Lane 2	Lane 3	Lane4	Lane5	Lane6
	37 Hairpin Bend	PKT	39 Hairpin Bend (Right)	Srikundra	Iyerpadi 2nd Division	39 Hairpin Bend (Left)
37 Hairpin Bend	*	80.00	71.4286	73.3333	64.5833	85.2459
PKT	*	*	78.3333	79.6875	65.3846	83.0769
39 Hairpin Bend (Right)	*	*	*	69.0909	74.4186	75.00
Srikundra	*	*	*	*	70.2128	80.00
Iyerpadi 2nd Division	*	*	*	*	*	68.75
39 Hairpin Bend (Left)	*	*	*	*	*	*

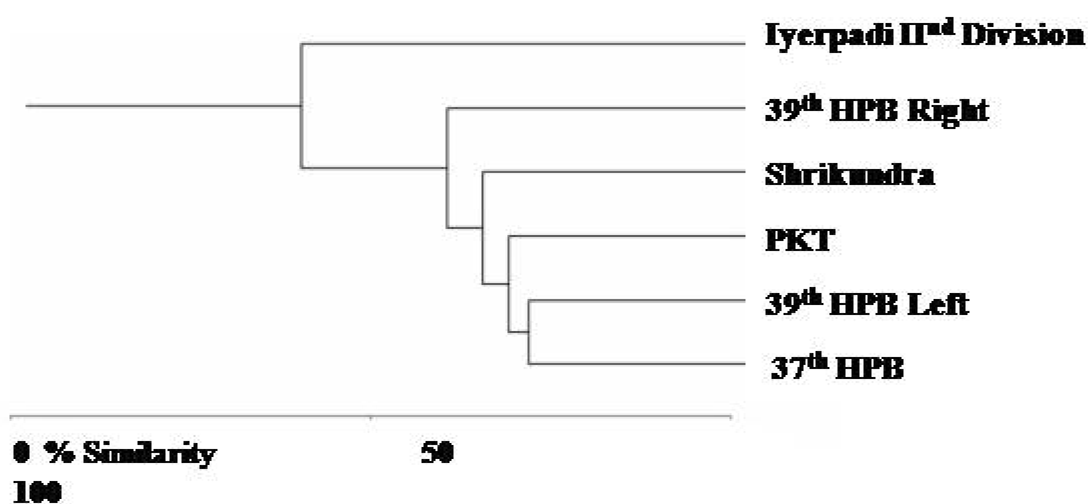


Fig. 4. **Dendrogram of Bray-Curtis cluster analysis of genetic variation and similarities among six populations of *Pseudoglochidion anamalayanum* from Anamalai Tiger Reserve, Valparai, Tamil Nadu, based on ISSR data.**

4.7.3. Genetic Diversity within the Population *Pseudoglochidion anamalayanum*

4.7.3.1. ISSR Band Pattern

For understanding the genetic diversity within the single population of *P. anamalayanum*, leaf samples from eight individual plants were collected from single population at PKT tea estate area of Anamalai Tiger Reserve. Eleven ISSR primers were used and banding patterns are shown in (Plate 26c, d). A total of 88 ISSR bands were obtained from eleven different primers. For each primer, the number of bands ranged from 04 to 10 with an average of 5.27 bands per primer. Of these, 58 bands were polymorphic with an average of 7.72 bands per primer. Average polymorphism across all the individual plant samples was 70.67%, thus showing the existence of good genetic diversity within the single population (Table 30).

4.7.3.2. Genetic Identity and Cluster Analysis

Pair wise genetic similarities were computed using ISSR data. The similarity matrix value obtained for these individual plants of single population is provided in (Table 31). ISSR data of eight different individual plants randomly selected from single population of *P. anamalayanum* was used to generate the dendrogram. These eight individual plants from single population formed two major clusters and sub-clades indicating the presence of good genetic diversity within the single population (Fig. 5).

Table 30. Amplified, polymorphic bands, percentage of polymorphism and unique bands in ISSR analysis within single population of *Pseudoglochidion anamalayanum* from PKT.

Primer	Primer sequence	Total No. of bands	No. of Polymorphic bands	Polymorphism Percentage	No. of unique bands
808	AGA GAG AGA GAG AGA GC	08	03	62.50	-
810	GAG AGA GAG AGA GAG AT	07	06	85.71	-
812	GAG AGA GAG AGA GAG AA	08	04	50	-
817	CAC ACA CAC ACA CAC AA	09	07	77.77	-
828	TGT GTG TGT GTG TGT GA	04	04	100	-
835	AGA GAG AGA GAG AGA GYC	10	06	60	02
844	CTC TCT CTC TCT CTC CTR C	05	04	80	01
848	CAC ACA CAC ACA CAC ARG	07	05	71.42	-
868	GAA GAA GAA GAA GAA GAA	10	04	40	-
873	GAC AGA CAG ACA GAC A	08	04	50	-
881	GGG TGG GGT GGG GTG	09	09	100	-
Total		**88**	**85**	**56**	**03**
Mean		**8**	**7.72**	**70.67%**	

Table 31. Similarity matrix within single populations of *Pseudoglochidion anamalayanum* based on ISSR analysis.

Similarity Matrix								
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
Sample 1	*	61.2613	61.2613	63.7168	94.8718	85.7143	96.8153	92.9936
Sample 2	*	*	85.2941	88.5714	60.177	65.9794	59.6491	57.8947
Sample 3	*	*	*	80.00	60.177	68.0412	59.6491	59.6491
Sample 4	*	*	*	*	62.6087	70.7071	62.069	62.069
Sample 5	*	*	*	*	*	85.9155	96.8553	93.0818
Sample 6	*	*	*	*	*	*	86.7133	82.5175
Sample 7	*	*	*	*	*	*	*	93.75
Sample 8	*	*	*	*	*	*	*	*

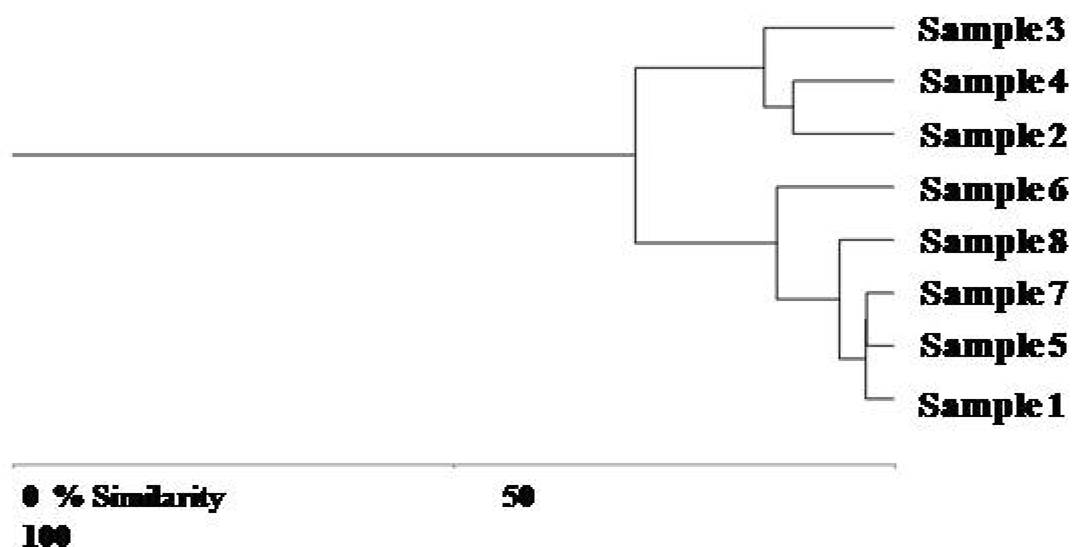


Fig. 5. **Dendrogram of Bray-Curtis cluster analysis of genetic variation and similarities within single population of *Pseudoglochidion anamalayanum* from PKT tea estate, Anamalai Tiger Reserve, Valparai, Tamil Nadu, based on ISSR data.**

4.8. Systematic Positions Using Gene Sequencing

4.8.1. Analysis of Gene Sequences

Phyllanthus talbotii

Based on the morphological characters *P. talbotii* was placed in the subgenus *Eriococcus* of *Phyllanthus*. During this study, sequencing of ITS and *mat*K of *P. talbotii* were carried out and sequences of 641 and 808 base pairs (bp) respectively were aligned using Basic Local Alignment Search Tool (BLAST) with highly similar sequences. The BLAST analysis involved *Phyllanthus* nucleotide sequences of 13 species for ITS and 15 species for *mat*K along with query sequence. The phylogenetic tree generated using both ITS and *mat*K gene sequences in the present study revealed the position of *P. talbotii* (Figs. 6, 7). Tree showed that it is closely related to *P. cinereus*, a plant found in Sri Lanka. These species come under the subgenus *Eriococcus*. Both *P. talbotii* and *P. cinereus* forms a major clade along with other species some of which fall under the same subgenus of *Eriococcus*. The position of other species in the clade is not known in literature, these species are distributed from North East India to South East Asia. Other species of subgenus from India are mostly narrow endemics in Southern Western Ghats for which sequences are not available to carry out analysis.

Pseudoglochidion anamalayanum

Pseudoglochidion anamalayanum, due to its intermediate characters of genus *Phyllanthus* and *Glochidion* the efforts were made to analyse its taxonomic position using ITS, *mat*K and *rbc*L gene sequence. The ITS, *mat*K and *rbc*L sequences of *P. anamalayanum* of 658, 783 and 655 bp respectively obtained during this study and BLAST analysis was carried out to get the highly similar sequences (above 90% similarity) from NCBI. Further analysis was carried out using the highly similar nucleotide

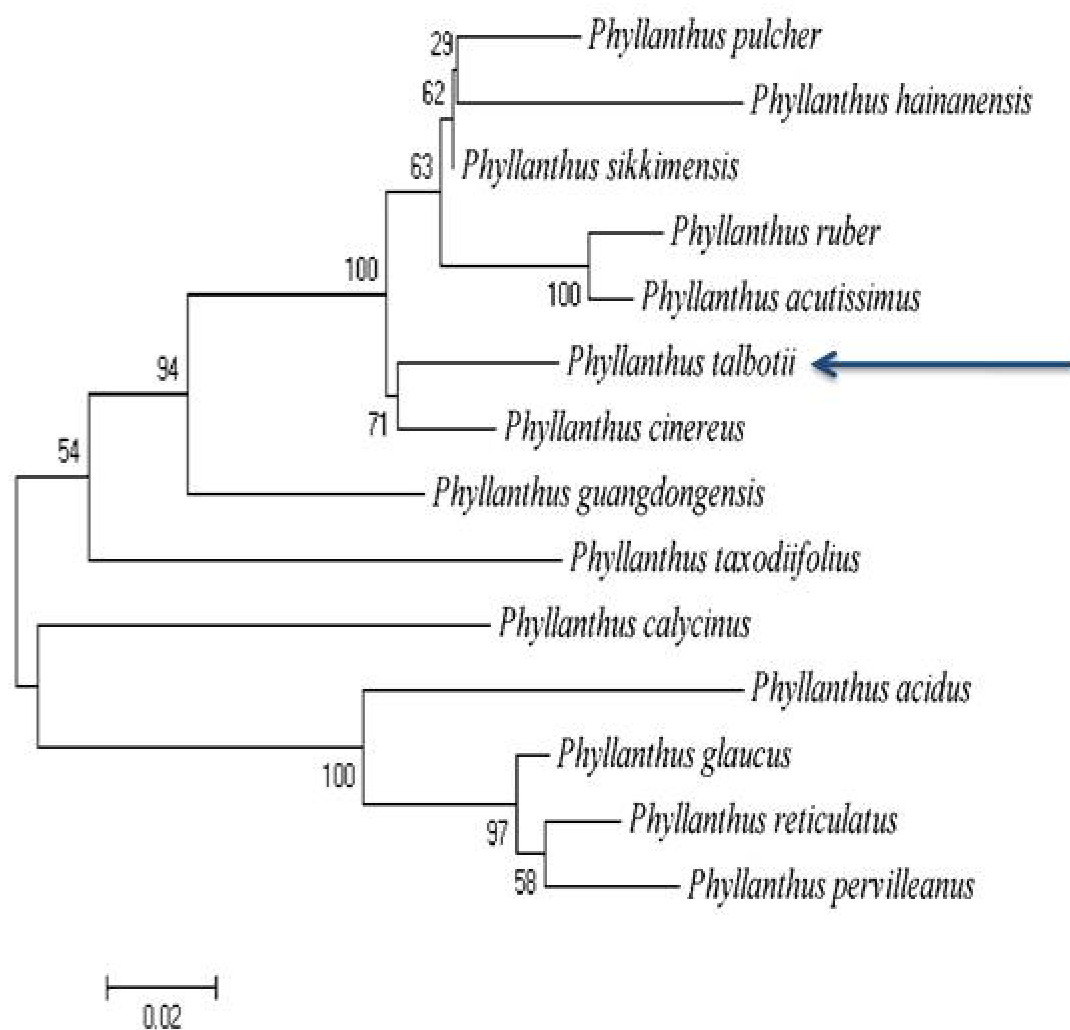


Fig. 6. **Maximum likelihood tree using ITS sequences of *Phyllanthus talbotii* (Bootstrap percentage is indicated).**

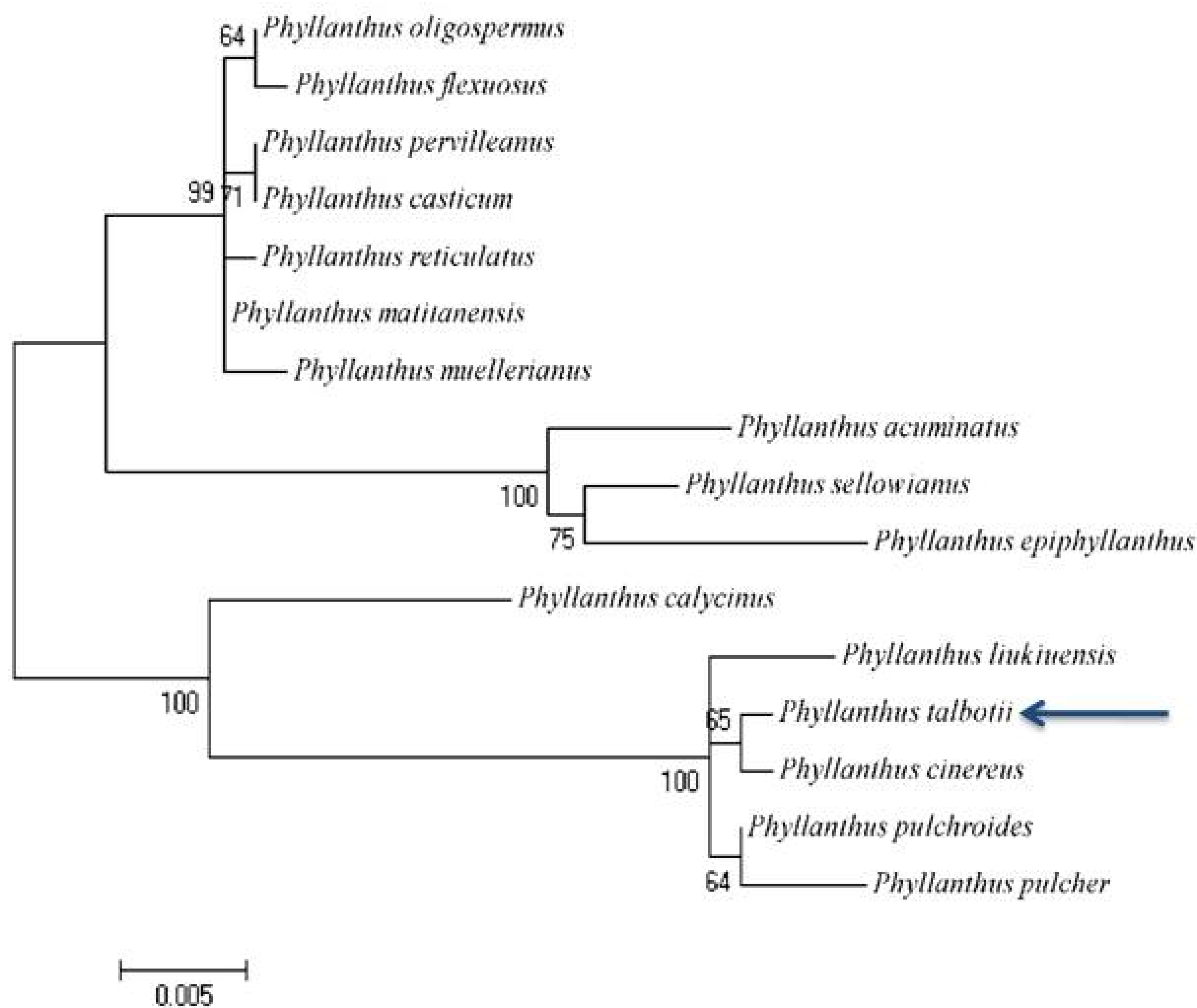


Fig. 7. **Maximum likelihood tree using *matK* gene sequences of *Phyllanthus talbotii* (Bootstrap percentage is indicated)**

sequences of 17 species for ITS, 25 species for *mat*K and 16 species for *rbc*L along with query sequence. The phylogenetic tree drawn with ITS sequence showed that *P. anamalayanum* is sister to *Phyllanthus columnaris* Muell. and *Phyllanthus oxyphyllus* Miq. While for *mat*K and *rbc*L sequence of *P. anamalayanum* showed that it is sister to *Phyllanthus polyphyllus* and *Phyllanthus emblica and P. oxyphyllus*. All the species forming clade with *P. anamalayanum* are tree species having phyllanthoid branching pattern, which strongly support that *P. anamalayanum* is the sister to *P. oxyphyllus*, *P. columnaris*, *P. pollyphyllus* and *P. emblica.* The phylogenetic tree generated showed that *P. anamalayanum* nested amidst *Phyllanthus* species (Figs. 8-10) thus supporting the position of *P. anamalayanum* under genus *Phyllanthus* as proposed by Webster (1994). Thus it is proved that *P. anamalayanum* cannot be a distinct genus and hence treated here as a species of *Phyllanthus viz. P. anamalayanum* (Gamble) G.L. Webster. Phylogenetically *P. anamalayanum* comes closer to *P. oxyphyllus*, which is distributed from Myanmar to Sumatra.

4.8.1.3. Gene Sequences Submitted to GenBank at NCBI

The ITS and *mat*K sequences obtained for *Phyllanthus talbotii* and ITS, *mat*K and *rbc*L sequences obtained for *Pseudoglochidion anamalayanum* were submitted to GenBank at NCBI and received the accession number. The details of sequences deposited including the accession number are given below:

Phyllanthus talbotii

ITS: KC414630

Bases 1 to 641

Base count 165 a 167 c 173 g 136 t

Origin

1 tttccgtaag gtgaacctgc ggaaggatca ttgtcgaaac ctgcaactgc agaatgaccc

61 gagaacaagt ttattcaccg cggagggtgc cttgtgctcc tgacgcaggg ccccgtgtgg

121 cgtaatgctc cattcggtgg ccacaagaac aaacccccggc gcggaaagcg ccaaggaaaa

181 taacgaagaa gagagatcgg cccaattagt tgggaaacgt tctccttaaa aaacgactct

241 cggcaacgga tatctcggct ctcgcatcga tgaagaacgt agcgaaatgc gatacttggt

301 gtgaattgca gaatcccgtg aaccatcgag tttttgaacg caagttgcgc ccgaagcctt

361 cgggccgagg gcacgtctgc ctgggtgtca cgcaacgtcg ctccctccaa ccccatgctg

421 gggtattttg aaggttgggc ggaaattggc ctcccgtgag catacatgtt tgcggttggc

481 ccaaaaaatg agaccaagtc ggcgaacgat gcgacattcg gtggtttaaa aattaccctc

541 acaatgtcgt tgtccgagtg ccaatcgaac aaggatctct caacgaccct cataactttt

601 gacgcgaccc caggtcaggc gggattaccc gctgagttta a

Phyllanthus talbotii

***mat*K   KC514101**

Bases 1 to 808

Base count   246 a   127 c   125 g   310 t

Origin

1 aatccttcgc taccgggtta aagatacttc ttctttgcat ttattacgat tttttcttca

61 cgagtattgg aattggaaca gttttatttt tcaaaaaaaa aaatttactt tttttgcaaa

121 aagtaatcca cgattattct tgtttctata taattctcat gtatatgaat atgaatccat

181 tttctttttt ctccgtaagc aatcctttca tttacgatca acattttttc gggtccttct

241 tgagcgaatc tatttttatg gaaaaataga acattttgca gaagtctttt ctaatgattt

301 tcaggctatt ttatggttgt tcaaagatcc tttcatgcat tatgttagat accaaggaaa

361 gtcaatcttg gctttaaagg atacccctct tctaataaaa aaatggaaaa gttaccttgt

421 caatttatgt caatgtcatt tttctgtgtg gtttcaatca gcaaagattt gtataaaccc

481 attatcgaag cagtctctca actttttggg ctatttttca agtctacgac tcaatttttc

541 agttgtacgg agtcaaatgc tagaaaatag ctttttaata gataatgcta tgacgaaagt

601 tgatacaaga attccaattt ttcctttgat tggatcattg gcaaaagcga aattttgtaa

661 cgcagtaggg catcctatta gtaaacctat ttggtctggt tcatccgatt ctgatattat

721 caaccgattt gtaggcatat ggagaaatct ttctcattgt tatagtgggt cttcaaaaaa

781 aaagagtttg tatcgaataa aatatata

Pseudoglochidion anamalayanum

ITS KC414629

Bases 1 to 658

Base count 164 a 177 c 170 g 147 t

Origin

1 tttccgtagg tgaacctgcg gaaggatcat tgtcaaaacc ttatactggt atgacccgcg

61 aacaagttta gtcactgcgg atggtgcccc gtgcacctga agcaaggcca cgtggggtgc

121 tatgctcctt gcgaaggcca cgtaatccaa ccccggcgcg gaatgcgcca aggaaaacga

181 atctaaatga gagaactcta cattcacctc ggaaacgatg tgtgcatggt agttgcttct

241 cctttcataa ccaaaacgac tctcggcaac ggatatctcg gctctcgcat cgatgaagaa

301 cgtagcgaaa tgcgatactt ggtgtgaatt gcagaatccc gtgaaccatc gagtctttga

361 acgcaagttg cgcccaaagc cttcgggtcg agggcacgtc tgcctgggtg tcacgcaacg

421 tcgctccctc acttccctca tgtagggctc gtgaatttgg ggcggaaaat ggcttcccat

481 gaacctcaag attgtggttg gcccaaacat gagaccaagt cggtcagtgc cgtggcattc

541 ggtggttgaa aataccctaa aaacgcctcg ttcatttggc cgaaccaaca aggatctcaa

601 cgaccctcta tgtatccgac gcgaccccag gtcaggcggg attacccgct gagtttaa

Pseudoglochidion anamalayanum

***mat*K   KC514100**

Bases 1 to 783

Base count 230 a   133 c   122 g   298 t

Origin

1 ttcttctttg catttattac ggttttttct tcacgagtat tggaattgga acagtcttat

61 ttttccaaat aattttcttt ctttttttgc aaaaggtaat ccacgattat tcctgtttct

121 atataattct catgtatatg aatatgaatc cattttcttt tttctccgta agcaatcctt

181 tcatttacga tcgacatttt ttcgggtcct tcttgagcga atatattttt ttggaaaaag

241 agaacatttt gcagaagtct ttgctaataa ttttcaggcc attctatggt tgttcaaaga

301 tcctttcatg cattatgtta gatatcaagg aaagtcaatc ttggcttcaa aggatacccc

361 tcttctatta aaaaaatgga aatactacct tgtcaattta tgtcaatgtc atttttctgt

421 gtggtttcaa ccagcaaaga tctgtataaa cccattatcg aagcagtctc tcgacttttt

481 gggctatctt tcaagtctac gactcaatct ttcagtggta cggagtcaaa tgctagaaaa

541 tgcattttta atagataatg ctatgaagaa agttgataca agaattccaa ttattccttt

601 gattcgatca ttggcaaaaa cgaaattttg taacgtagca ggacatccta ttagtcaacc

661 tatttgggct ggttcatcgg attctgatat tatcaaccga tttgtgcgca tatgcagaaa

721 tctttctcat tattatagtg ggtcttcaaa aaaaaagagt ttgtatcgaa taaaatatat

781 act

Pseudoglochidion anamalayanum

***rbc*L   KC514096**

Bases 1 to 655

Base count 181 a 134 c 147 g 193 t

Origin

1 gctggtgtta aagagtataa attgacttat tatactcctg actatgaaac caaagatact

61 gatatcttag cagcattccg agtaactcct caacctggag ttccgcctga ggaagcgggg

121 gctgcggtag cagctgaatc ttctactggt acatggacaa ctgtgtggac cgacggactt

181 accagtcttg atcgttataa aggacgatgc taccacatcg agcccgttgc tggagaagaa

241 aatcaatata ttgcttatgt agcttatcct ttagaccttt ttgaagaagg ttctgttact

301 aatatgttta cttccattgt gggtaatgta tttgggttca aagccttacg cgctctgcgt

361 ctggaagatt tgcgaatccc tcctgcttat tcgaaaactt tccaaggccc gcctcatggc

421 atccaagttg agagagataa attgaacaag tatggccgcc ctctattagg ctgtactatt

481 aaaccgaaat tggggttatc cgctaagaat tacggtagag ctgtttatga atgtcttcgc

541 ggtggacttg attttaccaa agacgatgaa aacgtaaact cccaaccatt tatgcgttgg

601 agagaccgtt tcttattttg tgctgaagca atttttaaag cacaagctga aacag

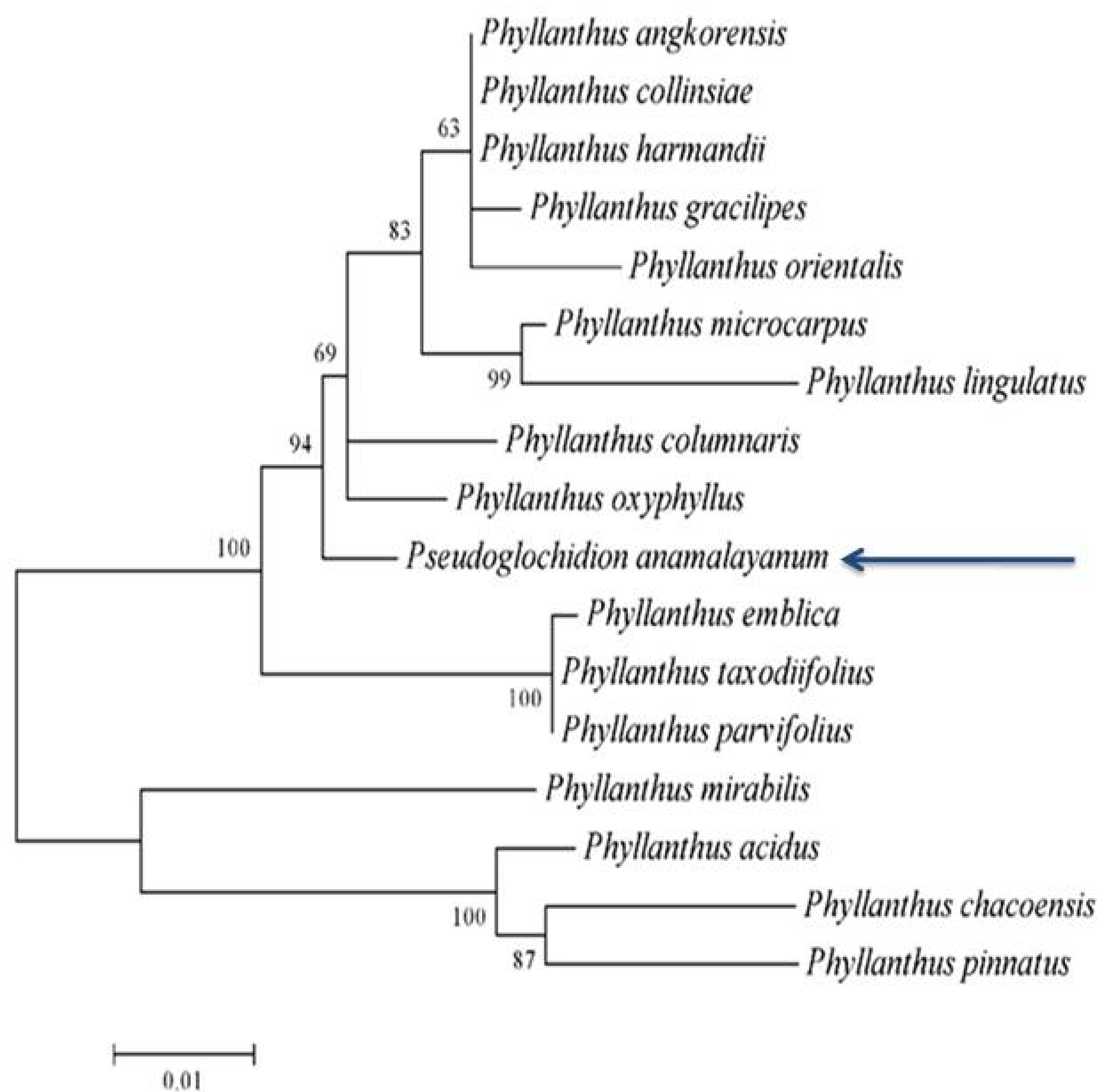


Fig. 8. Maximum likelihood tree using ITS sequences of *Pseudoglochidion anamalayanum* (Bootstrap percentage is indicated).

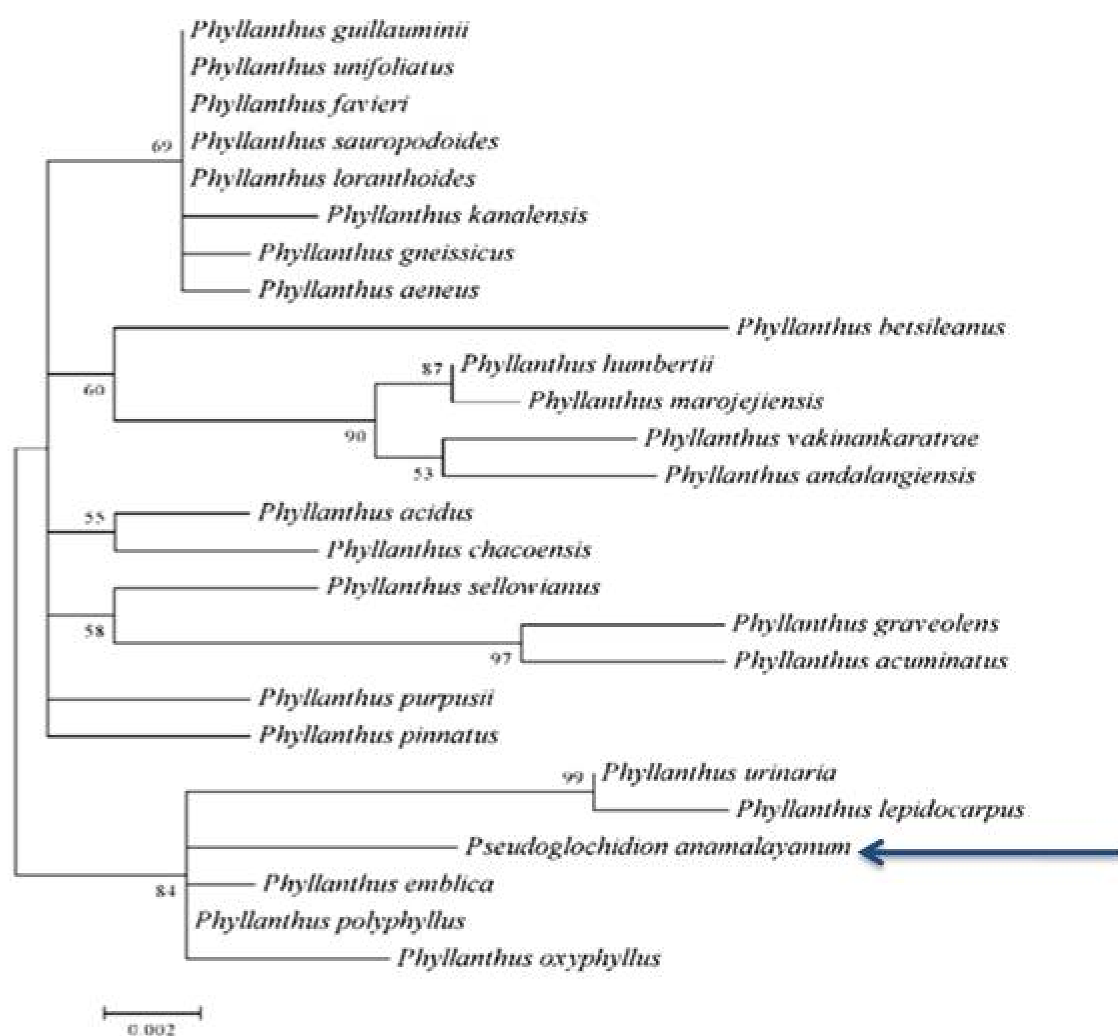


Fig. 9. **Maximum likelihood tree using *matK* gene sequences of *Pseudoglochidion anamalayanum* (Bootstrap percentage is indicated).**

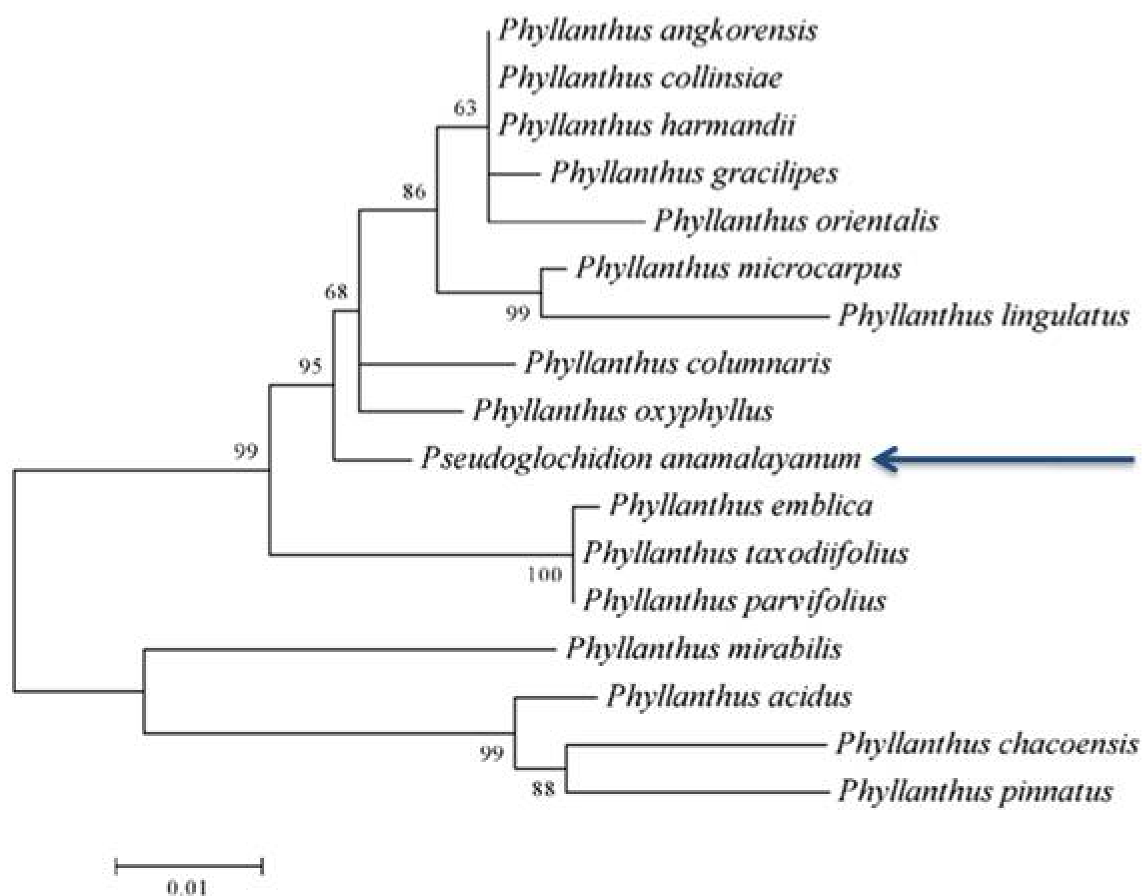


Fig. 10. Maximum likelihood tree using *rbc*L gene sequences of *Pseudoglochidion anamalayanum* (Bootstrap percentage is indicated).

DISCUSSION

5. DISCUSSION

5.1. Survey and Estimation of Populations and Ecology

5.1.1. *Phyllanthus talbotii*

Phyllanthus talbotii Sedg. is endemic to Western Ghats of Goa and Karnataka belongs to genus *Phyllanthus*, first described by Sedgwick in 1921 based on the collections made in 1918 and 1919 from North Kanara, it was known by its type collection till it was collected from Agumbe in Shimoga district (Raghavan, 1969). Singh and Kulkarni (1990) described it as 'rare' in spite of it being represented by a single collection after the type.

During this study, thirteen natural populations were located from the Western Ghats region of Goa and Karnataka. It is found growing in sandy loam soil and in between the rocks along the fresh water streams and river beds. Out of 13 populations, five are present in Goa at the altitudinal range of 40 to 60 m except Dudhsagar population at 205 m. In Karnataka, eight populations are found along the river beds at different altitude (20 to 636 m). All the Karnataka populations are restricted to Dakshin Kannada, Uttar Kannada, Udupi, Shimoga districts. In this study, *P. talbotii* specimens were re-examined, detailed taxonomic characteristics and systematic position has been studied (Naik *et al.*, 2013).

The population structure, species composition, ecological and environmental data of *P. talbotii* has been studied. During this study it is estimated that the *P. talbotii* occupies an area of about 16,150 m^2 and individual population size varied from 250–700 m^2 except the Jog Falls populations alone covers an area of 10,000 m^2 but with only 157 individuals. Total number of plants in all the populations is 2,518. Number of plants in the population

is varied from 32 (Salaulim dam, Goa) the smallest population to 327 (Collem, Goa) the largest population. The number of ramets per plant varied from 2 to 45. The populations of *P. talbotii* are found growing in the altitude ranging from 20 to 636 m. The altitudinal variation might be due to variation in temperature, relative humidity, radiation values, wind movements and edaphic factors (Nakashizukg *et al*., 1992). Swamy *et al*. (2000) studied the vegetation structure and species composition of 244 species belongs to 183 genera and 76 families at Veerapuli and Kalamalai reserve forests in the Western Ghats of Tamil Nadu, India.

5.1.2. *Pseudoglochidion anamalayanum*

Pseudoglochidion anamalayanum Gamble [=*Phyllanthus anamalayanus* (Gamble) G.L. Webster]: due to its intermediate characters between *Phyllanthus* and *Glochidion* J. R. Forst. &. Forst. (Euphorbiaceae), this species has been assigned to a new genus *Pseudoglochidion* by Gamble. *Pseudoglochidion* is monotypic genus (one of the six endemic tree genera of the Western Ghats) and distribution is restricted to above 1400 m in Anamalais and known from few populations (Rajkumar, 2001; Nayar, 2010). These areas are nearer to tea estates, roadsides and some populations were located in Sholayar dam reported to be submerged under water and experiencing greater threat. Due to this, it may be lost forever if not recovered and conserved. However, Webster considered *Pseudoglochidion* congeneric to *Phyllanthus* and within that genus it is somewhat related to *Phyllanthus baeobotryoides* (Balakrishnan and Chakrabarty, 2007) which distributed in Eastern Himalayas up to Myanmar. Subjectivity of the taxonomic status (Kruckberg and Rabinowitz, 1985) is a concern when it comes to conservation, and for this taxon rarity at the generic level is lost if it is considered as a species of *Phyllanthus*. Ahmedullah and Nayar (1990) could not find collections in herbaria (there are some collections at MH

collected from 1963; all from the same narrow geographical range of Anamalais) and assigned indeterminate status to this. However, IUCN Red List assigns 'CR' status [B1+2C (ver 2.3; 2015)] to this species (World Conservation Monitoring Centre 1998).

Pseudoglochidion anamalayanum is restricted only to Anamalai Tiger reserve, Valparai, Tamil Nadu and it experiencing greater threat. Hence, for purpose conservation elaborate study has been carried out. During this study, eight populations were located at different places in Anamalai Tiger reserve and recorded that the populations are found growing at an altitude ranging from 1,118 to 1,354 m. The plants found growing in the periphery of the forest receives good sunlight and showed luxuriant growth**.** In all the populations, a total of 1,495 matured plants and 2,163 saplings of *P. anamalayanum* were recorded. Muthuramkumar *et al*. (2006) recorded a small population of only 13 plants from Akkamalai forest in Anamalai Tiger reserve. However, during this study even after two days of through field survey the population in Akkamalai forest could not be relocated.

5.2. Vegetative Propagation

5.2.1. *Phyllanthus talbotii*

In the present study, various concentrations of IBA and IAA were used for vegetative propagation using stem cuttings. The strong fibrous rooting was observed in all treatments of IBA, IAA including Rootex powder in comparison with control. Experiment was carried out in poly house condition with humidity for the induction of roots in the stem cuttings. Majada *et al*. (2011) reported in *Pinus pinaster* Ait. that IBA treatments significantly influenced the rooting at 25°C and not at 4°C. Danu *et al*. (2015) standardized the vegetative propagation protocol for *Paris polyphylla* an important medicinal plant of Himalayan region and 100 mg/L IBA and 100 mg/L GA_3 showed maximum rooting.

Chandra *et al*. (2006) developed vegetative propagation methods for *Picrorhiza kurrooa* using root runner cuttings treated with IBA and NAA. Auxin induced effect on rooting of cuttings is presumed to be mediated through its effect in mobilising the reserve food material by enhancing the activity of hydrolytic enzymes. The benefit of stem cuttings over the other kinds of vegetative propagation methods is that it restores the genetic homogeneity among propagules (Nanda *et al*., 1968).

5.2.2. *Pseudoglochidion anamalayanum*

During this study, vegetative stem cuttings of *Pseudoglochidion anamalayanum* showed very low percentage of survival. This may be due to non-availability of poly-house facility at Valparai, Tamil Nadu, as temperature and humidity control are crucial factors responsible for induction of rooting in stem cuttings. However, few stem cutting of *P. anamalayanum* was survived and shown luxuriant growth. Eganathan *et al*. (2000) reported that the environmental condition and seasonality play an important role in the growth of the cuttings and survivability.

Air-layering of *P. anamalayanum* was successful. In the present study, a total of 70 branches were air-layered and 55 shown profuse thick rooting. Rooted air-layered branches were transferred to Research Nursery, Nature Conservation Foundation (NCF), Old Valparai, Tamil Nadu. This revealed that the *P. anamalayanum* favors air-layering method of propagation. Also, it shows an advantage of using this technique over the stem cutting since *P. anamalayanum* is a tree species. Rooting occurs faster in such marcotting than stem cuttings which do not root easily. When compared to other methods of vegetative propagation, branch rooting is most convenient method and factors such as

growth of stem and other physiological process greatly influence the success of air layering (Eganathan *et al*., 2000).

5.3. *In Vitro* Regeneration of *Phyllanthus talbotii*

5.3.1. Explants

During this study, shoot tips, leaf, nodal segments and seeds were used as explants for *in-vitro* regeneration of *P. talbotii*. Nodal segments and seeds responded well and produced substantial quantity of callus, however, these callus were not regenerated to plants even after several sub-cultures. Danaee *et al*. (2015) reported the use of leaf and inter-nodes as explants in *Phyllanthus pulcher* Wall. ex Mll. Arg. for callogenesis and secondary metabolites activities. Catapan *et al*. (2001) used nodal segments as explants which gave rise to axillary shoot proliferation in *Phyllanthus stipulates.* Chitra *et al*. (2009) carried out *in vitro* regeneration of *Phyllanthus amarus* using leaf discs and internodes as explants and derived whole plantlets from the callus. Sen and Batra (2013) reported the formation of high amount of callus by using internodal segments as explants and studied its phenolic contents and antioxidant activity in *Phyllanthus amarus.*

5.3.2. Induction of Callus

In present study, callus formation was observed in nodal segments and seed explants when cultured on MS medium supplemented with auxin (2, 4-D) and cytokinins (BAP and KIN) individually. The callus growth was obtained on media supplemented with BA and Kinetin alone. The callus developed was morphologically compact, greenish and not showing any further regeneration in to plantlets. The callus appeared loose and whitish when MS media supplemented with 2, 4-D. Seeds formed callus after 6 to 8 days of *in vitro* culture. Among the treatments extensive callus formation was observed in MS

medium supplemented with 2, 4-D (1 and 2 mg/L) and for nodal segment 2, 4-D (0.5 to 3 mg/L) after one month of *in vitro* culture. Danaee *et al*. (2015) observed the formation of callus on leaf explants of *Phyllanthus pulcher* when cultured on MS medium supplemented with four different concentrations of 2,4 -D (5, 10, 15 and 20 mg/L). The highest callus induction (90%), callus fresh weight (109.5 ± 4.5 mg) and dry weight (36.58 ± 3.8 mg) were seen at 15 mg/ L of 2, 4-D.

Pagare *et al*. (2009) reported the extensive callus formation in *Phyllanthus amarus* when leaf explants cultured on MS medium supplemented with combination BA (5 µM) + 2, 4-D (5 µM) and Kin (2.5 µM) + 2, 4-D (5.0 µM). Similarly Unander *et al*., (1995) studied the callus formation from stem or branch pieces of *Phyllanthus amarus* by using several media combinations. Optimum induction and growth of friable, undifferentiated calli occurred on MS medium with either BAP 0.5 mg/L or BAP 1 mg/L and 2, 4-D 1 mg/L. Jeyakumar *et al*. (2014) investigated that the callus growth was more in explants taken from apical bud and nodal segment nearer to the apical bud in medicinally important *Phyllanthus niruri*.

Catapan *et al*. (2001) showed the high frequency callus development *Phyllanthus stipulates* when nodal segments was cultured on MS medium supplemented with 5.0 µM NAA or 1.25 to 5.0 µM BA or 2iP on MS medium. Chitra *et al*. (2009) reported significant profuse callus growth in *Phyllanthus amarus* when leaf discs and intermodal segments cultured on MS medium supplemented with 4 mg/L NAA along with 4 mg/L 2, 4–D.

During this investigation, large quantity of anthocyanin pigment in the callus was observed, in almost all the treatments callus turned to purple-pink due to the accumulation of anthocyanin pigments and never regenerated to plantlets even after several sub-culture

with various cytokinin treatments. In woody plants, phenolic exudates are a frequently encountered problem while establishing explants in culture. These phenolic compounds oxidize to form a brown material in the medium and inhibit shoot formation (Ahmad *et al*., 2013). Santiago *et al*. (2000) have shown histochemically in *Phyllanthus tenellus* that the mature leaves with spongy cells are the main source of phenolic compounds and these substances accumulate randomly in the cells.

5.3.3. Direct Shoot Regeneration

Explants such as shoot tips and nodal segments were evaluated for direct multiple shoot formation in MS or WPM medium supplemented with various concentrations of cytokinins alone and in combinations with auxin. Among the individual concentrations and in cytokinin and auxin combinations, only BAP 0.5 mg/L and KIN 1 mg/L showed some response, but no multiple shoot development was observed in any of the treatments used during this investigation.

Bhattacharyya and Bhattacharya (2001) carried out *in vitro* culture of *Phyllanthus amarus* and shown that shoot tip explants when cultured on MS medium supplemented with KIN, BAP singly or in combination with IAA produced direct shoot regeneration. Catapan *et al*. (2000) used nodal segments of *Phyllanthus caroliniensis* and developed efficient micropropagation protocol for the production of axillary shoot proliferation. Maximum number of shoots of 21–23 per explants was recorded when cultured on MS or Anderson Rhododendron (AR) medium containing 5 µM BA or 1.25-5.0 µM kinetin or 2.5-5 µM 2-iP.

Shekhawat and Dixit, (2007) used nodal shoot segments as explants in *Phyllanthus amarus* and developed protocol for multiple shoot formation on modified MS medium

supplemented with 0.1 mg/L IAA + 0.5 mg/L BAP or 0.1 mg/L IAA + 0.5 mg/L KIN. Sen *et al*. (2009) developed protocol for multiple shoot formation in *Phyllanthus amarus* using nodal segments. MS medium supplemented with BAP 0.5 mg/L induced maximum number multiple shoots of 15.275±0.96 after 3 to 4 weeks of culture.

In the present study, interference of phenolic compounds may probably the major reasons for no multiple shoot formation even after several sub-culturing on MS medium supplemented with various auxin and cytokinin combinations. Bairu and Kane (2011) reported that the woody plant species shows recalcitrant characteristics with the presence of phenolic compounds which prevents them from regeneration to shoot/root or any other organized structures which may eventually get developed into new plantlets.

5.4. *In-Situ* Conservation

The effective conservation strategies must be developed to avoid further loss of essential species and ecosystem (Havens, 2006). *In situ* conservation of species is carried out in the field, so that it maintains and recovers the populations in the surrounding where they have developed their distinctive properties. This process helps to adapt the plant in the location site and thus its survivability. It is one of the traditional methods to improve and maintain the homogeineity of the plant.

In the present study, based on the survey, 21 new suitable locations were identified for re-introduction of *Phyllanthus talbotii.* Soil condition parameter was taken as one of the criteria for selection of new sites for reintroduction. Total of 12,083 cuttings propagated through vegetative method were re-introduced to twenty five sites in Goa (four existing populations for the enrichment, 21 sites). Among them 7,260 (60.08%) plants survived. Dhuner and Zambaulim the survival percentage of the cuttings was very low

(22.88 and 09.39%) because they were washed off due to torrential rain and fast water flow in the stream. The sites such as Ganjem, Nanus and Bhutpal also showed very less survival rate (35, 20.62 and 24.16%) the reason behind these is that the streams remained flooded for a longer time after the offset of the monsoon and the plants were submerged for longer time under the water which resulted in death of plants. However, wherever cuttings survived, it produced 6–8 ramets, height of the plant also increased and establishing well. Thus the populations of *P. talbotii* have been established at different locations in the State of Goa.

In situ conservation of *Pseudoglochidion anamalayanum*, total of 555 root suckers was re-introduced at six different locations of Anamalai Tiger Reserve. Re-introduced plants were periodically monitored for successful establishment and survival in new locations. It was found that re-introduced root suckers established well and showed survival of 252 (45.40%) root suckers after eight months of re-introduction. Based on this survival an additional 796 root suckers were reintroduced in two locations of Anamalai Tiger Reserve for establishment. Yadav *et al*. (2009) re-introduced a critically endangered and endemic grass of *Hubbardia heptaneuron* Bor, in 16 different ghat regions of Western Ghats. Re-introduced plants showed good growth and seed setting. This was the first successful reintroduction in the natural habitats for *in situ* conservation. Chandore *et al*. (2010) propagated *Ceropegia fantastica* a critically endangered and endemic to Western Ghats through *in vitro* culture. About 250 hardened tissue cultured plants were reintroduced in to 18 different locations of natural habitats for *in situ* conservation.

5.5. Genetic Diversity Studies of *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum*

For developing appropriate conservation and management strategies, understanding the level of genetic diversity within and among the natural populations is very important (Frankham *et al*., 2002). Loss of genetic diversity may lead to an extinction of particular species and it can also stop a species to respond to evolutionary process of natural selection (Qamaruz-Zaman *et al*., 1998). The plants species which are widely distributed to larger geographical areas have good genetic diversity (Hamrick and Godt, 1990). In nature, high genetic variation among the natural populations of same species is a sign of positive breeding and evolutionary prospective of the species (Falk and Holsinger, 1991).

In recent years, molecular markers are used as novel tools to differentiate the relationships among the plants. Several studies were carried out for the understanding of genetic diversity in various plant species (Luo *et al*., 2007). The different methods are used to measure the genetic variation among and within the population of a species. Presently, inter-simple sequence repeat (ISSR) markers are most commonly used method (Yang *et al*., 1996; Nagaoka and Ogihara, 1997; Parab *et al*., 2008; Parab and Krishnan, 2009). This technique is also shown to be useful in understanding the population genetic studies and detecting genetic variation in rare endangered and endemic plant species (Zietkiewicz *et al*., 1994; Jarne and Lagoda, 1996; Fang and Roose, 1997; Esselman *et al*., 1999; Raina *et al*., 2001; Qiu *et al*., 2004).

Li and Zhao (2007) used 12 ISSR primers to investigate the genetic diversity in populations of *Phyllanthus emblica* from dry-hot valleys of Yunnan, China. The banding pattern revealed the polymorphism of 85.19% among the studied populations without

linking to their geographical locations. Palaniappan and Marappa (2008) studied the genetic diversity in 54 genotypes of *Phyllanthus amarus* and each three genotypes of *Phyllanthus virgatus* and *Phyllanthus debilis* respectively using ISSR primers. Analysis showed 68.2% polymorphism in all 54 genotypes of *P. amarus*, however genotypes of other two related species are added, the polymorphism was increased to 96.5% showing that all three are distinct species of *Phyllanthus*.

During this investigation, genetic diversity and variation was studied among and within the populations of *Phyllanthus talbotii* and *Pseudoglochidion anamalayanum* using ISSR markers. A total of 10 ISSR primers were used for understanding the genetic diversity among eight different populations of *P. talbotii* from Goa and Karnataka. Average polymorphism of 71.85% was recorded among the eight populations and 75.12% polymorphism was recorded in within single population at Waddem, Sanguem, Goa. This is the first report showing the use of ISSR markers for analyzing the genetic variation and determination of genetic relationship in *P. talbotii*. Based on the cluster analysis, the populations of Karnataka did not form any cluster and each population stayed separately indicating the distant geographical locations of each population. Whereas, the populations of Goa, Saccordem and Waddem, Dudhsagar and Collem formed clusters by exhibiting the closer geographical locations and similar altitudinal ranges.

In case of *Pseudoglochidion anamalayanum*, eleven ISSR primers were employed for understanding the genetic diversity and variations. In the present study, an average of 65.73% polymorphism was seen among six populations of *Pseudoglochidion anamalayanum*. This revealed that the populations have good genetic diversity and they are separated based on the geographical distribution patterns of the populations. The

populations of 37th and 39th hairpin bend stood together, this may be due to their closer geographical locations. While, other populations stood independently may be due to their geographical separations mainly by tea estates which makes them as distinct populations.

ISSR analysis of eight randomly selected individual plants of single population of *Pseudoglochidion anamalayanum* located in PKT tea estate of Anamalai Tiger Reserve exhibited 70.67% polymorphism with good genetic diversity. This indicated the presence of higher genetic variability within the small region taken up during this study. In the cluster analysis, single population formed two major clusters and sub-clades. This revealed that there is a good genetic diversity and variation within the small population thus indicating their strong existence and survival of the population. Besides the geographical distribution, the population genetic structure is affected by various other factors viz. evolutionary history, mutation, gene flow, seed dispersal and natural selection (Hamrick and Godt, 1990).

5.6. Phylogenetic Studies

5.6.1. *Phyllanthus talbotii*

Phylogenetic analysis of family Phyllanthaceae has been studied by several researchers using different gene sequences (Wurdack *et al*, 2004; Kathriarachchi *et. al*, 2005, 2006; Samuel *et al*, 2005; Hoffmann *et al*, 2006; Vorontsova *et al*, 2007; Awomukwu *et al*, 2015). The systematic position of *Phyllanthus reticulatus* was confirmed by Luo *et al.* (2011) using nuclear ITS sequences in Africa. However, all these earlier workers have not studied the systematic position of *P. talbotii*. In the present study ITS and *mat*K genes were sequenced for *P. talbotii* and the sequences obtained were used for the construction of phylogenetic tree. The maximum likelihood tree revealed that the *P.*

talbotii nested in the same clade of *Phyllanthus cinereus*, which belong to subgenus *Eriococcus* supporting the treatment given by Balakrishnan and Chakrabarty (2007).

5.6.2. *Pseudoglochidion anamalayanum*

The systematic position of *Pseudoglochidion anamalayanum* remained a point of deliberate discussion as Webster (1994) transferred it to *Phyllanthus* and made new combination as *Phyllanthus anamalayanus* (Gamble) G.L. Webster. Webster (1994) transferred the genus *Pseudoglochidion* to *Phyllanthus* based on morphological similarities. However, over the years the plant name *Pseudoglochidion anamalayanum* has been treated as accepted name by various indices like www.plantlist.org and http://www.tropicos.org.

In present study, ITS, *mat*K and *rbc*L genes were sequenced for *Pseudoglochidion anamalayanum*. Phylogenetic tree generated using maximum likelihood method showed that *Pseudoglochidion anamalayanum* nested in *Phyllanthus* clade, thus supporting the position of *Pseudoglochidion anamalayanum* under genus *Phyllanthus* as proposed by Webster (1994). Thus, the present study confirmed that *Pseudoglochidion* cannot be considered as a distinct genus and hence treated here as a species of *Phyllanthus viz. Phyllanthus anamalayanus* (Gamble) G.L. Webster. *Phyllanthus oxyphyllus*, confined in Myanmar to Sumatera, while *P. columnaris*, *P. polyphyllus* and *P. emblica* are in peninsular India.

CONCLUSION

CONCLUSION

A total of 13 natural populations with 2,518 individuals of *Phyllanthus talbotii* have been located in the States of Goa and Karnataka all in river beds occupying an estimated area of 16,150 m^2. Individual population size varies from 250 to 700 m^2 except one population which is about 10,000 m^2 area but only with 157 individuals. *Pseudoglochidion anamalayanum* is distributed in eight wild populations (1495 matured plants and 2163 saplings) all located in Anamalai Tiger reserve, Valparai, Coimbatore district, Tamil Nadu. It was observed that the sand quarrying, construction of check dams and pollution of streams are the major threats for *P. talbotii.* Indian Gaur has been seen grazing on *P. anamalayanum* and threat is also in the form of trampling by elephants and Indian Gaur. The vegetative propagation using stem cuttings is successful method of propagation of *P. talbotii*. Hormonal treatment IBA 150 ppm found to be very effective in inducing roots in stem cuttings. Root induction through air layering method is successful for *P. anamalayanum.* IBA and IAA treatments produced profuse rooting in air-layering. For *in situ* conservation of *P. talbotii*, a total of 12,083 plants propagated through vegetative method have been reintroduced in 25 localities of which 21 are new locations and 4 are existing populations. Among them, 7,260 (60.08%) cuttings survived and established. In situ conservation of *P. anamalayanum*, a total of 555 root suckers have been introduced in 6 localities of which 252 (45%) plants survived and established in the forest. An additional 796 root suckers are also reintroduced into two locations of Anamalai Tiger reserve. ISSR primers revealed the existence of good genetic diversity among and within the populations of both *P. talbotii* and *P. anamalayanum.* Molecular phylogenetic studies using ITS and *mat*K gene sequences of *P. talbotii* showed that it belongs to subgenus *Eriococus.* ITS, *mat*K, and *rbc*L gene sequences of *Pseudoglochidion*

anamalayanum nested it in *Phyllanthus* under subgenus *Isocladus.* Thus the study confirms that *Pseudoglochidion* cannot be considered as distinct genus and it to be treated as a species of *Phyllanthus viz. Phyllanthus anamalayanus* (Gamble) G.L. Webster.

SUMMARY

SUMMARY

Phyllanthus talbotii and *Pseudoglochidion anamalayanum* are critically endangered plants which are endemic to Western Ghats of India.

Population Study, Environmental and Phenological Data

A total of 13 natural populations with 2,518 individuals of *Phyllanthus talbotii* have been located in the State of Goa and Karnataka, found growing in river beds of rocky to sandy loam areas, occupying a total area of about 16,150 m^2 with individual populations covering an area of 250-700 m^2 except a populations in Jog Falls area which occupies about 10,000 m^2 but with only 157 individuals. The highest numbers of plants are recorded in population found at Netravati River near to Halady village in Karnataka with 307 individuals and lowest at Salualim dam site in Goa with 32 plants. Flowering and fruiting is from August to November and new seedlings appear in the month of November. It is associated with other plants such as *Homonoia riparia*, *Rotula aquatica*, *Syzygium heyneanum* and *Osmunda regalis*.

For *Pseudoglochidion anamalayanum,* a total of eight wild populations (1,495 matured plants and 2,163 saplings) which are very narrow in their distribution, being restricted to only a small pocket of Anamalai Tiger Reserve, Valparai, Coimbatore district, Tamil Nadu. The highest numbers of plants are recorded in population found at Behind Nature Conservation Foundation Center, Iyerpady, with 469 numbers of individual and lowest numbers of 9 individuals was found in between 39th and 40th hair pin bend. The populations are found in protective area under Section 20 of Indian Forest Act 1927 of Anamalai Tiger Reserve and also in private forests under tea estates given for lease. Flowering and fruiting is observed throughout the year. The soil is loamy with pH from 5-

6. It is associated with other plants such as *Clerodendrum viscosum*, *Cullenia exarillata*, *Elaeocarpus munronii*, *E. serratus*, *Mallotus tetracoccus*, *Mesua ferrea* and *Nageia wallichiana*.

Vegetative Propagation

***Phyllanthus talbotii*:** The vegetative stem cuttings were treated with plant growth regulators such as indole-3-butric acid (IBA), indole-3-acetic acid (IAA) and commercially available rootex powder with the concentrations of 50, 100, 150, 200, 250 and 300 ppm of IAA and IBA for 24 hours. After planting in poly-house condition, cuttings sprouted in all treatments of IBA and IAA about 15 to 20 days. Among thirteen treatments, IBA 150 ppm was more effective in induction of roots in stem cuttings.

***Pseudoglochidion anamalayanum*:** Total of 400 stem cuttings were treated with various concentrations of IBA and IAA such as 100, 250, 500, 750 and 1000 ppm for induction of roots. Further, air layering was carried out using IBA, IAA and Rootex powder. Air-layered portion was opened after 75 days and observed with profuse thick rooting. A total of 70 branches were air-layered, of which 55 were rooted and survived. Among the treatment, rootex showed highest percentage of rooting and survival, followed by IBA and IAA respectively.

Tissue Culture of *P. talbotii*

For *in vitro* regeneration seeds, shoot tip, nodal segments and leaf explants were used. The various plant growth regulators such as auxin and cytokinins were tried individually or in combinations. Seed and nodal explants when cultured on MS medium supplemented with 2, 4-D (0.5–5 mg/L) showed callus formation after 7–14 days. Extensive callus development was observed in 1 and 2 mg/L 2, 4-D after 30 days of

culture. Later the callus developed anthocyanin pigments hence no further regeneration was observed. For direct multiple shoot formation explants such as shoot tips and nodal segments were tried using auxins and cytokinins individually and in combinations. Among the concentrations 0.5 and 1 mg/L BAP and KIN showed some response. However, no multiple shoot formations were observed in any of the treatments. In the present study, interference of phenolic compounds were seen in the cultures which may be the major reason for no multiple shoot formation and no organogenesis from the callus even after several sub-culturing with various auxin and cytokinin combinations.

***In-Situ* Conservation**

***Phyllanthus talbotii*:** During this investigation, a total of 12,083 cuttings were reintroduced to twenty five sites in Goa in which four were existing populations for enrichment and 21 sites were new locations. Among the reintroduced plants 7,260 (60.08%) cuttings survived. Re-introduction among existing populations, Waddem showed the maximum survival of 68.53% and among the newly introduced site Condimar showed the highest survival of plants of 85.95%. At Dhuner and Zambaulim the survival percentage of reintroduced plants was very low (22.88 and 09.39%). Reintroduced sites such as Ganjem, Nanus and Bhutpal also showed very less survival of 35, 20.62 and 24.16% respectively. However, wherever cuttings survived, height increased over 50% to 90–110 cm, produced 6-8 ramets and establishing well.

***Pseudoglochidion anamalayanum*:** First set of reintroduction was carried out using direct stem cuttings of 200 numbers treated with plant growth regulators in two different sites viz. near PKT tea estate and adjacent to 39th hair-pin bend of existing populations of Anamalai Tiger Reserve, Valparai, Coimbatore district, Tamil Nadu, for their enrichment. However, reintroduced cuttings were failed to establish. In the second set

of reintroduction, total of 555 root suckers were planted in six different sites to establish as new populations in Anamalai Tiger Reserve. Transplanted suckers were monitored regularly and found that the transplanted suckers established well with the overall survival of 252 (45.40%) root suckers after eight months of reintroduction.

Assessment of Genetic Diversity Using ISSR Markers

***Phyllanthus talbotii*:** Total of 22 ISSR primers were screened, among them 10 showed clear and reproducible banding pattern were used during this study. ISSR analysis was carried out with samples collected from eight populations covering the State of Goa and Karnataka. Average polymorphism across all samples of eight populations was 71.85% indicating good genetic diversity. Dendrogram separated the populations based on the geographic locations. Genetic diversity within a single population at Waddem showed average polymorphism of 75.12% with good genetic diversity.

***Pseudoglochidion anamalayanum*:** Out of 22 ISSR primers screened 11 showed the reproducible banding patterns. Eight populations were studied to understand the genetic diversity. A total of 88 ISSR bands were obtained, of which 58 bands were polymorphic. An average polymorphism across all the populations was 65.73% indicating good genetic diversity among the populations. Dendrogram separated the populations based on the geographical locations. For the understanding of genetic diversity within a single population, leaf samples from eight individual plants were collected from population near PKT tea estate area of Anamalai Tiger Reserve. Average polymorphism across all individual plants was 70.67% and showed with the existence of good genetic diversity.

Systematic Positions Using Gene Sequencing

For understanding the systematic positions ITS and *mat*K genes of *Phyllanthus talbotii* and ITS, *mat*K and *rbc*L genes of *Pseudoglochidion anamalayanum* were sequenced and the sequences of the same were submitted to GenBank at NCBI. The phylogenetic tree constructed for *Phyllanthus talbotii* using ITS and *mat*K gene sequences revealed the position of *P. talbotii* as close relative of *P. cinereus*, a plant found in Sri Lanka. It also confirms that the *P. talbotii* belong to the subgenus *Eriococcus*. For *Pseudoglochidion anamalayanum*, ITS, *mat*K and *rbc*L sequences were used for the construction of phylogenetic tree which supported the position of *Pseudoglochidion anamalayanum* under genus *Phyllanthus* as proposed by Webster (1994). Thus it is proved that *Pseudoglochidion anamalayanum* cannot be a distinct genus hence treated here as a species of *Phyllanthus viz. Phyllanthus anamalayanus* (Gamble) G.L. Webster. Phylogenetically *Pseudoglochidion anamalayanum* closer to *Phyllanthus oxyphyllus* that is found distributed from Myanmar to Sumatra.

REFERENCES

REFERENCES

Ahmad, I., T. Hussain, I. Ashraf, M. Nafees, Maryam, M. Rafay and M. Iqbal. 2013. Lethal effects of secondary metabolites on plant tissue culture. *American-Eurasian Journal of Agricultural and Environmental Sciences*, **13**: 539–547.

Ahmedullah, M. and Nayar, M. P. 1986. Endemic plants of the Indian region, Peninsular India. *Botanical Survey of India*, Kolkata.

Ahmedullah, M. and Nayar, M. P. 1990. *Pseudoglochidion anamalayanum* Gamble. In: Nayar, M. P. and Sastry, A. R. K. (eds.) *Red Data Book of Indian plants*, **3**: 126. Botanical Survey of India, Kolkata.

Akwatulira, F., Gwali, S., Ssegawa, P., Okullo, J. B. L., Tumwebaze, S. B., Mbwambo, J. R. and Muchug, A. 2011. Vegetative propagation of *Warburgia ugandensis* Sprague: An important medicinal tree species in Eastern Africa. *Journal of Medicinal Plants Research*, **5**(30): 6615–6621.

Albertson, R. C., Markert, J. A., Danley, P. D. and Kocher, T. D. 1999. Phylogeny of a rapidly evolving clade: The cichlid fishes of lake Malawi, East Africa. *Proceedings of the National Academy of Science*, **96**: 5107–5110.

Alvarez, I. and Wendel, J. F. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution*, **29**: 417–434.

Awomukwu1, D. A., Nyananyo, B. L., Spies, P. and Sizani, B. L. 2015. Identification, validation and classification of the genus *Phyllanthus* in Nigeria using ITS genetic marker and the taxonomic implication. *International Journal of Genetics and Genomics*, **3**(1): 1–7.

Bairu, M. W. and Kane, M. E. 2011. Physiological and developmental problems encountered by in vitro cultured plants. *Plant Growth Regulation*, **63**:101–103.

Bakkeren, G., Kronstad, J. W. and Levesque, C. A. 2000. Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in *Ustilaginomycetes*. *Mycologia*, **92**: 510–521.

Balakrishnan, N. P. and Chakrabarty, T. 2007. *The Family Euphorbiaceae in India*. Bishen Singh Mahendra Pal Singh, Dehra Dun. 361–391.

Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S. and Donoghue, M. J. 1995. The ITS region of nuclear ribosomal DNA- A valuable source of evidence on Angiosperm phylogeny. *Annals of the Missouri Botanic Garden*, **82**: 247–277.

Bancilhon, L. 1971. Contribution a´ l'e´tude taxonomique du genre *Phyllanthus* (Euphorbiace´es). *Boissiera*, **18**: 9–81.

Bandyopadhyay, S. and Raychaudhuri, S. S. 2013. Development and comparison of RAPD, SCAR and AFLP markers for distinguishing some medicinally important species of the genus *Phyllanthus*. *Plant Biosystems*, **147**(1): 12–20.

Bandyopadhyay, S. and Raychaudhuriy, S. 2010. Development of ITS based SCAR markers for some medicinally important species of *Phyllanthus*. *Asian Journal of Plant Sciences*, **9**(5): 264–270.

Bangar, A. R. and G. K. Zende (1978). Soil testing: A new basis for efficient fertilizer use. *Journal of Maharashtra Agricultural Universities*, **3**(2): 81–84.

Banu, S. and Handique, P. J. 2003. *In vitro* propagation of *Phyllanthus fraternus*. *Journal of Tropical Medicinal Plants*, **4**(1): 109–113.

Barrett, C. B., Brandon, K., Gibson, C. and Gjertsen, H. 2001. Conserving tropical biodiversity amid weak institutions. *Bioscience*, **451**: 497–502.

Barton, N. H. and Keightley, P. D. 2001. Multifactorial genetics: Understanding quantitative genetic variation. *Nature Reviews Genetics*, **3**: 11–21.

Bastin, M. and Jeyachandran, R. 2015. *Ex-situ* conservation of *Trichoglottis tenera* (Lindl.) A threatened, and endangered orchid of Western Ghats using asymbiotic seed germination technique. *International Journal of Recent Scientific Research*, **6**(4): 3488–3496.

Bhattacharyya, R. and Bhattacharya, S. 2001. High frequency *in vitro* propagation of *Phyllanthus amarus* Schum. and Thonn. by shoot tip culture. *Indian Journal of Experimental Biology*, **39**(11): 1184–1187.

Billotte, N., Risterucci, A. M., Barcelos, E., Noyer, J. L., Amblard, P. and Baurens, F. C. 2001. Development, characterisation, and acrosstaxa utility of oil palm (*Elaeis guineensis* Jacq.) microsatellite markers. *Genome*, **44**: 413–425.

Bormann, B. T., Haynes, R. W and Martin, J. R. 2007. Adaptive management of forest ecosystems: did some rubber hit the road?. *BioScience*, **57**: 186–191.

Caruso, T., Pigino, G., Bernini, F., Bargagli, R. and Migliorini, M. 2007. The Berger–Parker index as an effective tool for monitoring the biodiversity of disturbed soils: a case study on Mediterranean Oribatid (Acari: Oribatida) assemblages. *Biodiversity and Conservation*, **16**: 3277–3285.

Catapan, E., Moreno, F. N., Luís Busi da Silva, M., Otuki, M. F., Niero, R., Filho, V. C., Augusto Yunes, R. and Viana, A. M. 2002. Micropropagation, callus and root culture of *Phyllanthus urinaria* (Euphorbiaceae). *Plant Cell, Tissue and Organ Culture*, **70**: 301–309.

Catapan, E., Otuki, M. F. and Viana. A. M. 2000. *In vitro* culture of *Phyllanthus caroliniensis* (Euphorbiaceae). *Plant Cell Tissue and Organ Culture*, **62**(3): 195–202.

Catapan, E., Otuki, M. F. and Viana. A. M. 2001. *In vitro* culture of *Phyllanthus stipulatus* (Euphorbiaceae). *Brazilian Journal of Botany*, **24**(1): 25–34.

Champion, H.G. and Seth, S. K. 1968. A revised survey of the forest types of India. *Government of India Press*, Nasik, India.

Chandore, A. N., Nimbalkar, M. S., Gurav, R. V., Bapat, V. A. and Yadav, S. R. 2010. A protocol for multiplication and restoration of *Ceropegia fantastica* Sedgw.: a critically endangered plant species. *Current Science*, **99**(11): 1593.

Chandra, B., Palni, L. M. S. and Nandi, S. K. 2006. Propagation and conservation of *Picrorhiza kurrooa* Royle ex Benth.: an endangered Himalayan medicinal herb of high commercial value. *Biodiversity and Conservation*, **15**: 2325–2338.

Charlesworth, D. 2003. Effects of inbreeding on the genetic diversity of populations. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **358**(1434): 1051–1070.

Charlesworth, D. and Willis, J. H. 2009. The genetics of inbreeding depression. *Nature Reviews Genetics*, **10**: 783–796.

Chase, M. W., *et al*. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. *Annals of the Missouri Botanic Garden*, **80**: 528–580.

Chattopadhyay S. 1985. Deforestation in part of Western Ghats (Kerala). *Indian Journal of Environmental Management*, **20**(3): 219–230.

Chauhan, S. S. 2014. Status of biodiversity in India: issues and challenges. *Indian Journal of Plant Sciences*, **3**(3): 52–63.

Chaurasia, A. K., Subramaniam, V. R., Krishna, B. and Sane, P. V. 2009. RAPD based genetic variability among cultivated varieties of aonla (Indian gooseberry, *Phyllanthus emblica* L.). *Physiology and Molecular Biology of Plants*, **15**(2): 169–173.

Chen, C. W., Huang, Y. M., Kuo, L. Y., Nguyen, Q. D., Luu, H. T., Callado, J. R., Farrar, D. R., and Chiou, W. L. 2013. *trn*L-F is a powerful marker for DNA identification of field vittarioid gametophytes (Pteridaceae). *Annals of Botany*, **111**(4): 663–673.

Chitra, R., Rajamani, K. and Vadivel, E. 2009. Regeneration of plantlets from leaf and internode explants of *Phyllanthus amarus* Schum. and Thonn. *African Journal of Biotechnology*, **8**(10): 2209–2211.

Chung, M. Y. and Park, C. W. 2008. Fixation of alleles and depleted levels of genetic variation within populations of the endangered lithophytic orchid *Amitostigma gracile* (Orchidaceae) in South Korea: implications for conservation. *Plant Systematics and Evolution*, **272**: 119–130.

CIMMYT. 2005. Quantification and quality control of DNA. In: Laboratory Protocols (3rd Edition), 11-12. CIMMYT Applied Molecular Genetics Laboratory, Mexico.

Clark, L. G., Zhang, W. and Wendel, J. F. 1995. A phylogeny of the grass family (Poaceae) based on *ndh*F sequence data. *Systematic Botany*, **20**: 436–460.

Clegg, M. T. and Zurawski, G. 1991. Chloroplast DNA and the study of plant phylogeny. *In* P. S. Soltis, D. E. Soltis, and J. J. Doyle (eds). *Molecular systematics of plants*, 1–13. Chapman and Hall, New York, USA.

Clegg, M. T., Gaut, B. S., Learn, G. H. and Morton, B. R. 1994. Rates and patterns of chloroplast DNA evolution. *Proceedings of the National Academy of Science*, USA, **91**: 6795–6801.

Coates, D. J. 2000. Defining conservation units in a rich and fragmented flora: implications for the management of genetic resources and evolutionary processes in South-West Australian plants. *Australian Journal of Botany*, **48**: 329–339.

Coates, D. J. and Dixon, K. W. 2007. Current perspectives in plant conservation biology. *Australian Journal of Botany*, **55**: 187–193.

Daily, G. C., Soderqvist, T., Aniyar, S., Arrow, K., Dasgupta, P., Ehrlich, P. R., Folke, C., Jansson, A., Jansson, B., Kautsky, N., Levin, S., Lubchenco, J., Maler, K., Simpson, D., Starrett, D., Tilman, D. and Walker, B. 2000. The value of nature and the nature of value. *Science*, **289**: 395–396.

Danaee, M., Farzinebrahimi, R., Kadir, M. A., Sinniah, U. R., Mohamad, R. and Taha, R. M. 2015. *In vitro* sterilization and enhancement of callus induction from leaf and internode explants of *Phyllanthus pulcher* Wall. ex Mll. Arg. *The Philippine Agriculture Scientist*, **98**(1): 47–51.

Daniel, A. A., Bio, L. N., Paula, S. and Bulelani, L. Z. 2015. Identification, validation and classification of the genus *Phyllanthus* in Nigeria using ITS Genetic marker and the taxonomic implication. *International Journal of Genetics and Genomics*, **3**(1): 1–7.

Daniels, R. J. R. 2001. National biodiversity strategy and action plan: Western Ghats eco-region. *Report submitted to ministry of environment and forests*, Government of India.

Danu, K., Adhikari, R. S., Pande,V., Kumar, M. and Rawal, P. 2015. Vegetative propagation of an endangered medicinal plant of Himalayan region, Paris polyphylla Smith. *International Journal of Current Microbiology* and *Applied* Sciences, 4(6): 660–665.

Dayanandan, S., Bawa, K. S. and Kesseli, R. 1997. Conservation of microsatellites among tropical trees (Leguminosae). *American Journal of Botany*, **84**: 1658–1663.

De Groot, G. A., During, H. J., Maas, J. W., Schneider, H., Vogel, J. C., and Erkens, R. H. J. 2011. Use of *rbc*L and *trn*L-F as a Two-Locus DNA Barcode for Identification of NW-European ferns: An ecological perspective. *PLoS One*, **6**(1): e16371.

Decruse, W. S., Gangaprasad, A., Seeni, S. and Menon, S. V. 2003. Micropropagation and ecorestoration of *Vanda spathulatha*, an exquisite orchid. *Plant Cell, Tissue and Organ Culture*, **72**: 199–202.

Donoghue, M. J., Olmstead, R. G., Smith, J. F. and Palmer, J. D. 1992. Phylogenetic relationships of Dipsacales based on *rbc*L sequences. *Annals of the Missouri Botanic Garden*, **79**: 333–345.

Doyle, J .J. and Doyle, J. L. 1990**.** A rapid total DNA preparation procedure for fresh plant tissue. *Focus*, **12**:13–15.

Duvall, M. R., Clegg, M. T., Chase, M. W., Clark, W. D., Kress, J., W., Hills, H. G., Eguiarte, L. E., Smith, J. F., Gaut, B. S., Zimmer, E. A., and Learn, G. H. 1993. Phylogenetic hypotheses for the monocotyledons constructed from *rbc*L sequence data. *Annals of the Missouri Botanic Garden*, **80**: 607–619.

Edwards, R., Johnstone, C. and Thompson, C. A. 1991. Simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research*, **19**: 1349.

Eganathan, P., Rao, C. S. and Anand, A. 2000. Vegetative propagation of three mangrove tree species by cuttings and air layering. *Wetlands Ecology and Management*, 8: 281–286.

Esselman, E. J., Jianqiang, L., Crawford, D. J., Winduss, J. L. and Wolfe, A. D. 1999. Clonal diversity in the rare *Calamagrostis porteri* ssp. *insperata* (Poaceae): comparative results for allozymes and random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. *Molecular Ecology*, **8**:443-451.

Faisal, M. and Anis, M. 2003. Rapid mass propagation of *Tylophora indica* via leaf callus culture. *Plant Cell Tissue and Organ Culture*, **75**(2): 125–129.

Falk, D. A. 1990. Integrated strategies for conserving plant genetic diversity. *Annals of the Missouri Botanical Garden*, **77**: 38–47.

Falk, D. A. and Holsinger, K. E. 1991. *Genetics and conservation of rare plants*. Oxford University Press, New York, USA.

Fang, D. Q. and Roose, M. L. 1997. Identification of closely related citrus cultivars with inter-simple sequence repeats markers. *Theoretical and Applied Genetics*, **95**: 408–417.

Felsenstein, J. 1985. Confidence limits on phylogenetics: an approach using bootstrap. *Evolution*, **39**: 783.

Francisco-Ortega, J., Santos-Guerra, A., Kim, S. C. and Crawford, D. J. 2000. Plant genetic diversity in the Canary islands: a conservation perspective. *American Journal of Botany*, **87**(7): 909–919.

Frankham, R., Ballou J. D. and Briscoe D. A. 2002. *Introduction to conservation genetics. Cambridge*, UK: Cambridge University Press. 617.

Gahlaut, A., Gothwal1, A., Hooda, V. and Dabur, R. 2013. RAPD patterns of some important medicinal plants and their substitutes used in ayurveda to identify the genetic variations. *International Journal of Pharmacy and Pharmaceutical Sciences*, **5**(1): 239–241.

Gaikwad, S., Gore, R., Garad, K. and Gaikwad, S. 2014. Endemic flowering plants of Northern Western Ghats (Sahyadri ranges) of India: A checklist. *Journal of species lists and distribution*, **10**(3): 461–472.

Gajbhiye. 1985. Common extractants and critical limits for available Fe, Mn, Zn and Cu in soil. *Journal of Maharashtra Agricultural Universities*, **10**(3): 239–241.

Gangopadhyay, M., Chakrabarty, T. and Balakrishnan, N. P. 2007. *Phyllanthus*. In: N.P. Balakrishnan and T. Chakrabarty, The Family Euphorbiaceae in India: A synopsis of its profile, taxonomy and bibliography. Bishen Singh Mahendra Pal Singh, Dehra Dun, 361–391.

Ge, S., Oliveira, G. C. X., Schaal, B. A., Gao, L. Z. and Hong, D. Y. 1999. RAPD variation within and between natural populations of the wild rice *Oryza rufipogon* from China and Brazil. *Heredity*, **82**: 638–644.

Geburek, T. 1997. Isozymes and DNA marker in gene conservation of forest trees. *Biodiversity and Conservation*, **6**: 1639–1654.

Govaerts, R., Frodin, D. G. and Radcliffe-Smith, A. 2000. World checklist and bibliography of Euphorbiaceae (and Pandanaceae). Kew: The Royal Botanical Gardens.

Gunawardene, N. R., Daniels, A. E. D., Gunatilleke, I. A. U. N., Gunatilleke, C. V. S., Karunakaran, P. V., Nayak, K., G., Prasad, S., Puyravaud., P., Ramesh, B. R., Subramanian, K. A. and Vasanthy, G. 2007. A brief overview of Western Ghats–Sri Lanka biodiversity hotspot. *Current Science*, **93**: 1567–1572.

Hamrick, J. L. and Godt, M. J. W. 1990. Allozyme diversity in the grasses. *In* G. P. Cheplick (eds), *Population biology of grasss*, 11–29. Cambridge University Press, Cambridge, UK.

Hamrick, J. L. and Godt, M. J. W. 1996. Conservation genetics of endemic plant species. In: J. C. Avise and J. L. Hamrick (eds.), *Conservation genetics*; *case histories from nature*, 281–304. Chapman and Hall, New York, USA.

Havens, K., Vitt, P., Maunder, M., Guerrant Jr., E. O. and Dixon, K. 2006. *Ex-situ* plant conservation and beyond. *BioScience*, **56** (6): 525–531.

Hoffmann, P., Kathriarachchi, H. and Wurdack, K. J. 2006. A phylogenetic classification of Phyllanthaceae (Malpighiales; Euphorbiaceae sensu lato). *Kew Bulletin*, **61**(1): 37–53.

Hooker, J. D. 1887. In: *Flora of British India*. L. Reeve and Co, London, **5**: 285–305.

Hopper, S. D. 2000. How well do phylogenetic studies inform the conservation of Australian plants? *Australian Journal of Botany*, **48**: 321–328.

Hsiao, C., Chatterton, N. J., Asay, K. H., and Jensen, K. B. 1994. Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of internal transcribed spacers region in nuclear ribosomal DNA in monocots. *Genome*, **37**: 112–120.

Htun, N. Z., Mizoue, N., Kajisa, T. and Yoshida, S. 2010. Deforestation and forest degradation as measures of Popa mountain park (Myanmar) effectiveness. *Environment and Conservation*, **36**: 218–224.

Htun, N. Z., Mizoue, N. and Yoshida, S. 2011. Tree species composition and diversity at different levels of disturbance in Popa mountain park, Myanmar. *Biotropica*, **43**(5): 597–603.

Huff, D. R., Peakall, R. and Smouse, P. E. 1993. RAPD variation within and among natural populations of outcrossing buffalo grass [Buchloe dactyloides (Nutt.)]. *Theoretical and Applied Genetics*, **86**: 927–934.

Izawa, T., Kawahara, T. and Takahashi, H. 2007. Genetic diversity of an endangered plant, *Cypripedium macranthos* var. *rebunense* (Orchidaceae): background genetic research for future conservation. *Conservation Genetics*, **8**: 1369–1376.

Jain, N., Shasany, A. K., Sundaresan, V., Rajkumar, S., Darokar, M. P., Bagchi, G. D., Gupta, A. K., Kumar, S. and Khanuja, S. P. S. 2003. Molecular diversity in *Phyllanthus amarus* assessed through RAPD analysis. *Current science*, **85**(10): 1454–1458.

Jain, S. K. and Rao, R. R. 1977. *A handbook of field and herbarium methods*. Today and tomorrow's printers and publishers, New Delhi. 157.

James, D. and Schmidt, A. M. 2004. Use of an intron region of a chloroplast tRNA gene (*trn*L) as a target for PCR identification of specific food crops including sources of potential allergens. *Food Research International*, **3**(4): 395–402.

Jarne, P. and Lagoda, P. J. L. 1996. Microsatellites from molecules to populations and back. *Trends in Ecology and Evolution*, **11**: 424–429.

Jarrell, D. C. and M. T. Clegg. 1995. Systematic implications of the chloroplast-encoded *mat*K gene on the tribe Vandeae (Orchidaceae). *American Journal of Botany*, **82**: 137.

Jeyakumar, J. J., Kamaraj, M., Srinivasan, S., Anburaja, V. and Prema, D. 2014. *In vitro* callus regeneration and biochemical analysis in the medicinal plant *Phyllanthus niruri* L. *British Biomedical Bulletin*, (**2**)2: 437–446.

Johnsingh, A. J. T. 2001. The Kalakad-Mundanthurai Tiger Reserve: A global heritage of biological diversity. *Current Science*, **80**(3): 378–388.

Johnson, L .A. and Soltis, D. E. 1995. Phylogenetic inference using *mat*K sequences. *Annals of the Missouri Botanical Garden*, **82**: 149–175.

Johnson, L. A. and Soltis, D. E. 1994. *mat*K DNA sequences and phylogenetic reconstruction in Saxifragaceae s. str. Systematic Botany, **19**: 143–156.

Juthatip, M., Suchada, S., Nijsiri, R. and Mizukami, H. 2010. Molecular phylogenetic analysis of *Phyllanthus* species in Thailand and the application of polymerase chain reaction-Restriction Fragment Length Polymorphism for *Phyllanthus amarus* identification. *Biological and Pharmaceutical Bulletin*, **33**(10): 1723–1727.

Kathriarachchi, H., Hoffmann, P., Samuel, R., Wurdack, K. J. and Chase, M. W. 2005. Molecular phylogenetics of Phyllanthaceae inferred from five genes (plastid *atp*B, *mat*K, 3' *ndh*F, *rbc*L and nuclear PHYC). *Molecular Phylogenetics and Evolution*, **36**: 112–134.

Kathriarachchi, H., Samuel. R., Hoffmann, P., Mlinarec, J., Wurdack, K. J., Ralimanana, H., Stuessy, T. F. and Chase, M. W. 2006. Phylogenetics of the tribe Phyllantheae (Phyllanthaceae; Euphorbiaceae sensu lato) based on nrITS and plastid *mat*K DNA sequence data. *American Journal of Botany*, **93**: 637–655.

Kessler, W. B., Salwasser, H., Cartwright Jr., C. W. and Caplan, J. A. 1992. New perspectives for sustainable natural resources management. *Ecological Applications*, **2**: 221–225.

Kingston, N., Waldren, S. and Smyth, N. 2004. Conservation genetics and ecology of *Angiopteris chauliodonta* Copel. (Marattiaceae), a critically endangered fern from Pitcairn Island, South Central Pacific Ocean. *Biological Conservation*, **117**: 309–319.

Kong, Q., Chen, J., Liu, Y., Ma, Y., Liu, P., Wu, S., Huang, Y., and Bie, Z. 2014. Genetic diversity of Cucurbita rootstock germplasm as assessed using simple sequence repeat markers. *Scientia Horticulturae*, **175**: 150–155.

Kruckeberg, A. R. and Rabinowitz, D. 1985. Biological aspect of endemism in higher Landraces by RAPD markers. *American Journal of Agricultural and Biological Sciences*, **2**(2): 49–53.

Kumar, A., Mishra, P., Singh, S. C. and Sundaresan, V. 2014. Efficiency of ISSR and RAPD markers in genetic divergence analysis and conservation management of *Justicia adhatoda* L., a medicinal plant. *Plant Systematics and Evolution*, **300**: 1409–1420.

Kumar, K. M. P., Sreeraj, V., Thomas, B., Manudev, K. M. and Rajendran, A. 2012. Validation and documentation of rare endemic and threatened (RET) plants from Nilgiri, Kanuvai and Madukkarai forests of Southern Western Ghats, India. *Journal of Threatened Taxa*, **4**(15): 3436–3442.

Kumar, T. P., Hermon, R. R., Rao, V. M., Rao, P. K., Shankar, S. S. R. and Prasad, N. S. R. 2012. Monitoring of environmental change using geoinformatics technology: A case study of Attapady block, Wayanad district, Kerala. *International Journal of Life Sciences Biotechnology and Pharma Research*, **1**(2): 2250–3137.

Lam, W. Y., Leung, K. T., Law, P. T. W., Lee, S. M., Chan, H. L., Fung, K. P., Ooi., V. E. and Waye, M. M. (2005). Antiviral effect of *Phyllanthus nanus* ethanolic extract against hepatitis B virus (HBV) by expression microarray analysis, *Journal of Cellular Biochemistry*, **97**(4): 795–812.

Leakey, R. R. B., and Newton, A. C. (eds.). 1994. *Tropical Trees: The potential for domestication and the rebuilding of forest resources*. HMSO, London.

Lee, S. K., Li, P. T., Lau, D. T., Yung, P. P., Kong, R. Y. and Fong, W. F. 2006. Phylogeny of medicinal *Phyllanthus* species in China based on nuclear ITS and chloroplast *atp*B-*rbc*L sequences and multiplex PCR detection assay analysis. *Planta Medica*, **72**(8): 721–726.

Li, Q. and Zhao, J. 2007. Genetic diversity of *Phyllanthus emblica* populations in dry-hot valleys in Yunnan. *Biodiversity Science*, **15**(1): 84–91.

Liang, H., and Hilu, K. W. 1996. Application of the *mat*K gene sequences to grass systematics. *Canadian Journal of Botany*, **4**: 125–134.

Lienert, J. 2004. Habitat fragmentation effects on fitness of plant populations - a review. *Journal for Nature Conservation*, **12**(1): 53–72.

Linos, A., Nikoloudakis, N., Katsiotis, A. and Hagidimitriou, M. 2014. Genetic structure of the Greek olive germplasm revealed by RAPD, ISSR and SSR markers. *Scientia Horticulturae*, **175**: 33–43.

Lloyd, G. and McCown, B. 1981. Commercially feasible micropropagation of Mountain Laurel, *Kalmia latifolia*, by use of shoot tip culture. Combined Proceedings - *International Plant Propagator's Society*, **30**: 421–427.

Luo Xiao-ying., Zhuang Xue-ying and Yang Yue-sheng. (2007). Genetic diversity of *Camellia changii* Ye (Theaceae) using ISSR Markers. *Journal of Tropical and Subtropical Botany*, **15**(2): 93–100.

Luo, S. X., Esser, H. J., Zhang, D. and Renner, S. S. 2011. Nuclear ITS sequences help disentangle *Phyllanthus reticulates* (Phyllanthaceae), an Asian species not occurring in Africa, but introduced to Jamaica. *Systematic Botany*, **36**(1): 99–104.

Mabberley, D. J. 2008. *Mabberley's Plant-book: A portable dictionary of plants*, *their classification and uses*. Cambridge University Press, UK.

Maggini, F., Marrocco, R., Gelati, M. T. and De Dominicls, R. I. 1998. Lengths and nucleotide sequences of the internal spacers of nuclear ribosomal DNA in gymnosperms and pteridophytes. *Plant Systematics and Evolution*, **213**: 199–205.

Majada, J., Martı´nez-Alonso, C., Feito, I., Kidelman, A., Aranda, I. and Alı´a, R. 2011. Mini-cuttings: an effective technique for the propagation of *Pinus pinaster* Ait. *New Forests*, **41**: 399–412.

Malayaman, V., Ghouse, B. M. and Kolar, A. B. 2014. An efficient callus induction from *Phyllanthus debilis* Klein ex Willd.- A wild medicinal plant of Eastern Ghats, India. *International Journal of Pure and Applied Bioscience*, **2**(2): 181–186.

Manissorn, J., Sukrong, S., Ruangrungsi, N. and Mizukami, H. 2010. Molecular phylogenetic analysis of *Phyllanthus* species in Thailand and the application of polymerase chain reaction-Restriction Fragment Length Polymorphism for *Phyllanthus amarus* identification. *Biological and Pharmaceutical Bulletin*, **33**(10): 1723–1727.

Martin, K. P. and Pradeep, A. K. 2003. Simple strategy for the *in vitro* conservation of *Ipsea malabarica* an endemic and endangered orchid of the Western Ghats of Kerala, India. *Plant Cell*, *Tissue and Organ Culture*, **74**: 197–200.

Mathews, S., Tsai, R. C. and Kellogg, E. A. 2000. Phylogenetic structure in the grass family (Poaceae): Evidence from the nuclear gene phytochrome B. *American Journal of Botany*, **87**: 96–107.

McNeely, J. A., Miller, K. R., Reid, W. V., Mittermeier, R. A. and Werner, T. B. 1990. *Conserving the world's biological diversity*, IUCN publication services, Gland, Switzerland.

Mehrotra, S., Khwaja, O., Kukreja, A. K. and Rahman, L. 2012. ISSR and RAPD based evaluation of genetic stability of encapsulated micro shoots of *Glycyrrhiza glabra* following 6 months of storage. *Molecular Biotechnology*, **52**(3): 262–268.

Mudge, K. W. and Brennan, E. B. 1999. Clonal propagation of multipurpose and fruit trees used in agroforestry. In: Buck, L. E., Lassoie, J. P. and Fernandes, E. C. M. (eds.), *Agroforestry in Sustainable Agricultural Systems*, 157–190, CRC and Lewis Publishers, New York, USA.

Murashige, T. and Skoog, F. A. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*. **15**: 473–97.

Muthuramkumar, S., Ayyappan, N., Parthasarathy, N., Mudappa, D., Raman, T. R. S., Selwyn, M. A. and Pragasan, L. A. 2006. Plant community structure in tropical rainforest fragments of the Western Ghats, India. *Biotropica*, **38**: 143–160.

Muthusamy, S., Kanagarajan, S. and Ponnusam, S. 2008. Efficiency of RAPD and ISSR markers system in accessing genetic variation of rice bean (*Vigna umbellata*) landraces. *Electronic Journal of Biotechnology*, **11**(3): 1–10.

Myers, N. 1988. Threatened biotas: "hotspots" in tropical forests. *Environmentalist*, **8**(3): 1–20.

Myers, N., Mittermeier, R. A., Mittermeie,r C. G., da Fonseca, G. A. B, and Kent, J. 2000. Biodiversity hotspots for conservation priorities. *Nature*, **403**: 853–858.

Nadeem, M., Palni, L. M. S., Purohit, A. N., Pandey, H. and Nandi, S. K. 2000. Propagation and conservation of *Podophyllum hexandrum* Royle: an important medicinal herb. *Biological Conservation*, **92**(1): 121–129.

Nagaoka, T. and Ogihara, Y. 1997. Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. *Theoretical and Applied Genetics*, **94**:597–602.

Naik, A. D. and Juvekar, A. R. 2003. Effects of alkaloidal extract of *Phyllanthus niruri* on HIV replication. *Indian Journal of Medicinal Science*, **57**(9): 387–93.

Naik, S. S., Pagare, R. S., Krishnan, S. and Janarthanam, M. K. 2013. Systematic position of *Phyllanthus talbotii* (Phyllanthaceae), a critically endangered species of Western Ghats, India. *Rheedea*, **23**(1): 13–18.

Nair, N. C. and Daniel P. 1986. The floristic diversity of Western Ghats and its conservation: A review. *Proceedings of the National Academy of Sciences* (*Animal Sciences / Plant Sciences Supplement*), 127–163.

Nair, S. C. 1991. *The Southern Ghats: A Biodiversity Conservation Plan.* INTACH, New Delhi.

Nakashizuka, T., Yusop, Z. and Nik, A. R. 1992. Altitudinal zonation of forest communities in Selangor, peninsular Malaysia. *Journal of Tropical Forest Science*, **4**: 233–244.

Nanda, K. K., Purohit, A. N., Anand, V. K. 1968. Seasonal rooting response of stem cuttings of some forest tree species to auxins. *Indian Forest*, **94**(2): 154-162.

Nayar, M. P. and Sastry, A. R. K. (eds.). 1987. *Red Data Book on Indian Plants*, Vol. I. Botanical Survey of India, Kolkata.

Nayar, M. P. and Sastry, A. R. K. (eds.). 1988. *Red Data Book on Indian Plants*, Vol. II. Botanical Survey of India, Kolkata.

Nayar, M. P. and Sastry, A. R. K. (eds.). 1990. *Red Data Book on Indian Plants*, Vol. III. Botanical Survey of India, Kolkata.

Nayar, M. P. 2010. 1996. Hot spots of endemic plants in India, Nepal and Bhuthan, Tropical Botanical Garden and Research Institute, Thiruvananthapuram, Kerala, 252.

Nayar, M. P. 2010. *Plant endemism in Western Ghats and identification of critical species and habitats*. First Indian Biodiversity Congress (Abstract book), SS1. Held at Kerala University campus, Kariavattom, Kerala.

Nepal, M. P. and Ferguson, C. J. 2012. Phylogenetics of Morus (Moraceae) inferred from ITS and *trn*L-*trn*F sequence data. *Systematic Botany*, **37**(2): 442–450.

Nevo, E. 1998. Genetic diversity in wild cereals: regional and local studies and their bearing on conservation *ex situ* and *in situ*. *Genetic Resources and Crop Evolution*, **45**: 355–370.

Nybom, H. and Bartish, I. V. 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plant. *Perspectives in Plant Ecology*, *Evolution and Systematics*, **3**: 93–114.

Okumus, A. 2007. Genetic Variation and relationship between Turkish flint maize plants. *The Annual Review of Ecology*, *Evolution and Systematics*, **16**: 447–479.

Olsen, K. M. and Schaal, B. A. 1999. Evidence on the origin of cassava: Phylogeography of *Manihot esculenta*. *Proceedings of the National Academy of Science*, USA. **96**: 5586–5591.

Pacheco, G., Gagliardi, R. F., Valls, J. F. M. and Mansur, E. 2009. Micropropagation and *in vitro* conservation of wild *Arachis* species. *Plant Cell*, *Tissue and Organ Culture*, **99**(3): 239–249.

Pagare, R. S., Auti, S. G., Aglave, B. A. and Nikam, T. D. 2009. Protocol for callus culture of *Phyllanthus amarus* Schum and Thonn. and possibility of use of callus culture in production of phyllanthin and hypophyllanthin. *Advances in Plant Sciences*, **22**(2): 433–436.

Palaniappan, M. and Marappa, M. 2008. RAPD and ISSR analyses reveal low levels of genetic diversity in *Phyllanthus amarus*. *Acta Horticulturae*, **765**: 179–187.

Palmer, J. D., Jansen, R. K., Michaels, H. J., Chase, M. W. and Manhart, J. R. 1988. Chloroplast DNA variation and plant phylogeny. *Annals of the Missouri Botanic Garden*, **75**: 1180–1206.

Parab, G. V. and Krishnan, S. 2009. Assessment of genetic variation among populations of *Rhynchostylis retusa*, an epiphytic orchid from Goa, India using ISSR and RAPD markers. *Indian Journal of Biotechnology*, **7**(3): 313–319.

Parab, G. V., Krishnan, S., Janarthanam, M. K., Sivaprakash, K. R. and Parida, A. 2008. ISSR and RAPD markers assessed genetic variation of *Aerides maculosum*-an epiphytic orchid from Goa, India. *Journal of Plant Biochemistry and Biotechnology*, **17**(1): 107-109.

Patel, H. K., Fougat, R. S., Kumar, S., Mistry, J. G., and Kumar, M. 2014. Detection of genetic variation in *Ocimum* species using RAPD and ISSR markers. *3 Biotech*, DOI 10.1007/s13205-014-0269-y.

Pavendan, P., Gideon, V. A. and Rajasekaran, C. S. 2012. Cost effective, *ex situ* conservation (air layering) of *Elaeocarpus blascoi* - an endangered medicinal tree in Western Ghats of Palni hills, Tamil Nadu, South India. *The Indian Forester*, **138**(11): 1056–1058.

Puyravaud, J. P., Davidar, P., Pascal, J. P. and Ramesh, B. R. 2003. Analysis of threatened endemic trees of the Western Ghats of India sheds new light on the Red Data Book of Indian plants. *Biodiversity and Conservation*, **12**: 2091–2106.

Qamaruz-Zaman, F., Fay, M. F., Parker, J. S. and Chase, M. W. 1998. The use of AFLP fingerprinting in conservation genetics: a case study of *Orchis simia* (Orchidaceae). *Lindleyana*, **13**: 125–133.

Qiu, Q.S., Guo, Y., Quintero, F.J., Pardo, J.M.,Schumaker, K.S., Zhu, J.K. 2004. Regulation of vascular Na+/ H+ exchange in *Arabidopsis thaliana* by the salt-overly-sensitive (SOS) pathway. *Journal of Biological Chemistry*, **279**:207–215.

Raghavan, R. S. 1969. Flora of Agumbe and Tirthahalli area, Shimoga district. Ph. D. thesis, University of Pune.

Raina, S. N., Rani, V., Kojima, T., Ogihara, Y., Singh, K. P. and Devarumath, R. M. 2001. RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity varietals identification and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. *Genome*, **44**: 763–772.

Rajkumar, S. 2001. Thesis: *"Studies on Systematics and Biology of Endemic Tree Genera of Western Ghats India*. Goa University.

Ramage, C. M., Borda, A. M., Hamill, S. D. and Smith, M. K. 2004. A simplified PCR test for early detection of dwarf off-types in micropropagated Cavendish banana (*Musa* spp. AAA). *Scientia Horticulturae*, **103**: 145–151.

Ramesh, B. R., and Pascal J. P. 1997. Atlas of endemics of the Western Ghats (India): *Distribution of tree species in the evergreen and semi-evergreen forest*. Institute Franc¸ais de Pondichery, Pondicherry, India, **38**: 403.

Rani, D. and Dantu, P. K. 2012. Direct shoot regeneration from nodal, inter nodal and petiolar segments of *Piper longum* L. and *in vitro* conservation of indexed plantlets. *Plant Cell*, *Tissue and Organ Culture*, **109**: 9–17.

Rao, V. R. and Hodgkin, T. 2002. Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell*, *Tissue and Organ Culture*, **68**: 1–19.

Reddy, C. S., Dutta, K. and Jha, C. S. 2013. Analysing the gross and net deforestation rates in India. *Current Science*, **105**(11): 1492–1500.

Reed, B. M., Sarasan, V., Kane, M., Bunn, E. and Pence, V. C. 2011. Biodiversity conservation and conservation biotechnology tools. *In Vitro Cellular* and *Developmental Biology*, **47**: 110–122.

Richardson, J. E., Fay, M. F., Cronk, Q. C. B. and Chase, M. W. 2000. A revision of the tribal classification of Rhamnaceae. *Kew Bulletin*, **55**: 311–340.

Richardson, J. E., Fay, M. F., Cronk, Q. C., Bowman, D. and Chase, M. W. 2000. A phylogenetic analysis of Rhamnaceae using *rbc*L and *trn*L-F plastid DNA sequences. *American Journal of Botany*, **87**(9): 1309–1324.

Rout, G. R. and Aparajita, S. 2010. Phylogenic study of twelve species of *Phyllanthus* originated from India through molecular markers for conservation. *American Journal of Plant Sciences*, **1**: 32–37.

Roxburgh, W. (W. Carey, eds.). 1832. *Flora Indica or Description of Indian plants.* Mission Press, Serampore.

Sahu, P. K., Sagar, R. and Singh, J. S. 2008. Tropical forest structure and diversity in relation to altitude and disturbance in a Biosphere reserve in Central India. *Applied Vegetation Science*, **11**: 461–470.

Samali, A., Florence, D. T., Odeniran, O. A. and Cordelia, O. N. 2012. Evaluation of chemical constituents of *Phyllanthus niruri*. *African Journal of Pharmacy and Pharmacology,* **6**(3): 125–128.

Samuel, R., Kathriarachchi, H., Hoffmann, P., Barfuss, M. H. J., Wurdack, K. J. C., Davis, C. and Chase, M. W. 2005. Molecular phylogenetics of Phyllanthaceae: evidence from plastid *mat*K and nuclear PHYC sequences. *American Journal of Botany*, **92**: 132–141.

Sanjappa, M. 2005. Plant diversity in India-status, conservation and challenges (P. Maheshwari medal award lecture). In: *XXVIII Conference of Indian Botanical Society*, **24**(26): 5–6.

Santiago, L. J. M., Louro, R. P. and De Ol Ive Ira, D. E. 2000. Compartmentation of phenolic compounds and Phenylalanine Ammonia-Lyase in leaves of *Phyllanthus tenellus* Roxb. and their induction by Copper Sulphate. *Annals of Botany*, **86**: 1023–1032.

Saw, L. G., Chua, L. S. L., Suhaida, M., Yong, W. S. Y. and Hamidah, M. 2010. Conservation of some rare and endangered plants from Peninsular Malaysia. *Kew Bulletin*, **65**: 681–689.

Schaal, B. A., Hayworth, D. A., Olsen, K. M., Rauscher, J. T. and Smith, W. A. 1998. Phylogeographic studies in plants: problems and prospects. *Molecular Ecology*, **7**: 465–474.

Seeni, S. and Latha, P. G. 2000. *In vitro* multiplication and eco-rehabilitation of the endangered Blue Vanda. *Plant Cell, Tissue and Organ Culture*, **61**(1): 1–8.

Sen, A. and Batra, A. 2013. The study of *in vitro* and *in vivo* antioxidant activity and total phenolic content of *phyllanthus amarus* schum. & thonn.: A medicinally important plant. *International Journal of Pharmacy and Pharmaceutical Sciences*, **5**(3): 942–947.

Sen, A., Sharma, M. M., Grover, D. and Batra, A. 2009. *In vitro* regeneration of *Phyllanthus amarus* Schum. and Thonn.: An important medicinal plant. *Our Nature*, **7**:110–115.

Senapati, S. K., Aparajita, S. and Rout, G. R. 2011. Identification of species-diagnostic inter simple sequence repeat markers for ten *Phyllanthus* species. *Zeitschrift Für Naturforschung C*, **66**(3-4):167–72.

Sharma, S., Rathi, N., Kamal, B., Pundir, D., Kaur, B. and Arya, S. 2010. Conservation of biodiversity of highly important medicinal plants of India through tissue culture technology - a review. *Agriculture and Biology Journal of North America*, **1**(5): 827–833.

Shaw, J., Lickey, E. B., Schilling, E. E. and Small, R. L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany*, **94**(3): 275–288.

Shekhawat, M. S. and Dixit, A. K. 2007. Micropropagation of *Phyllanthus amarus* (Schum and Thonn) through nodal shoot segment culture. *International Journal of Plant Science*, **2**(2): 79–84.

Sheng Liu, S.,Wei, W., Shi, K., Cao, X., Zhou, M. and Liu, Z. 2014. *In vitro* and *in vivo* anti-hepatitis B virus activities of the lignan niranthin isolated from *Phyllanthus niruri* L. *Journal of Ethnopharmacology*, **55**(2): 1061–1067.

Shilpa, J., Silambarasan, T., Pandian, S. K. and Ramesh, M. 2013. Assessment of genetic diversity in *Solanum trilobatum* L., an important medicinal plant from South India using RAPD and ISSR markers. *Genetic Resources and Crop Evolution*, 2013. **60**: 807–818.

Sica, M., Gamba, G., Montieri, S., Gaudio, L. and Aceto, S. 2005. ISSR markers show differentiation among Italian populations of *Asparagus acutifolius* L. *BMC Genetics*, **6**: 17.

Sinclair, A. R. E., Hik, D. S., Schmitz, O. J., Scudder, G. G. E., Turpin, D. H. and Larter, N. C. 1995. Biodiversity and the need for habitat renewal. *Ecological Applications*, **5**: 579–587.

Singh, N. P and B. G. Kulkarni. 1990. *Phyllanthus talbotii* Sedg. In: Nayar, M. P. and A. R. K. Sastry (Eds.) *Red Data Book of Indian Plants*, **3**: 124–125.

Singh, R. and Tekale, N. S. 2009. The conservation strategies for endangered medicinal plants used by tribals in Central India. *Acta Horticulturae*, **826**: 129–134.

Singh, S. K., Meghwal, P. R., and Pathak, R. 2014. Molecular characterization of commercial varieties of *Phyllanthus emblica* using RAPD and nuclear rDNA SNPs. In: *Proceedings of the National Academy of Sciences*, *India Section B: Biological Sciences*, DOI: 10.1007/s40011-014-0453-1.

Slik, J. W. F, Arroyo-Rodríguez, V., Aiba, S-I., Alvarez-Loayza, P., Alves, L. F. and Ashton, P. *et al.* 2015. An estimate of the number of tropical tree species. *Proceedings of the National Academy of Sciences*, **112**(24): 7472–7477.

Sodhi, N. S., Koh, L. P., Brook, B. W. and Ng, P. K. L. 2014. Southeast Asian biodiversity: an impending disaster. *Trends in Ecology and Evolution*, **19**(12): 654–660.

Steele, K. P. and Vilgalys, R. 1994. Phylogenetic analyses of Polemoniaceae using nucleotide sequences of the plastid gene *mat*K. *Systematic Botany*, **19**: 126–142.

Straub, S. C. K., Parks, M., Weitemier, K., Fishbein, M., Cronn, R. C. and Liston, A. 2012. Navigating the tip of the genomic iceberg: next-generation sequencing for plant systematics. *American Journal of Botany*, **99**(2): 349–364.

Subbaiyan, B., Samydurai, P. M., Prabu, M. K., Ramakrishnan, R. and Thangapandian, V. 2014. Inventory of Rare, Endangered and Threatened (RET) plant species in Maruthamalai hills, Western Ghats of Tamil Nadu, South India. *Our Nature*, **12**(1): 37–43.

Swamy, P. S., Sundarapandian, S. M., Chandrasekar, P., Chandrasekaran, S. 2000. Plant species diversity and tree population structure of a humid tropical forest in Tamil Nadu, India. *Biodiversity and Conservation*, **9**: 1643–1669.

Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J. 1991. Universal primers for amplification of 3 noncoding regions of chloroplast DNA. *Plant Molecular Biology*, **17**: 1105–1109.

Tamura, K., Nei, M. and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbour-joining method. *Proceedings of the National Academy of Sciences* (*USA*), **101**: 11030–11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, **28**(10): 2731–2739.

Thapa, S. and Chapman, D. S. 2010. Impacts of resource extraction on forest structure and diversity in Bardia national park, Nepal. *Forest Ecology and Management*, **259**: 641–649.

Theerakulpisut, P., Kanawapee, N., Maensiri, D., Bunnag, S. and Chantaranothai, P. 2008. Development of species specific SCAR markers for identification of three medicinal species of *Phyllanthus*. *Journal of Systematics and Evolution*, **46**: 614–621.

Thilaga, S., Largia, M. J. V., Parameswari1, A., Rahul Raveendran Nair, R. R. and Ganesh, D. 2013. High frequency somatic embryogenesis from leaf tissue of *Emblica officinalis* Gaertn. - A high valued tree for non-timber forest products. *Australian Journal of Crop Science*, **7**(10): 1480–1487.

Thomas, S. C. 2004. Ecological correlates of tree species persistence in tropical forest fragments. *In* E. C. Losos and E. G. Leigh. (eds.). *Forest diversity and dynamism: Findings from a large-scale plot network*, 279–313. University of Chicago Press, Chicago, Illinois.

Tsumura, Y., Ohba, K. and Strauss, S. H. 1996. Diversity and inheritance of inter-simple sequence repeat polymorphism in douglass-fir (*Pseudotsuga menziesii*) and sugi (*Crytomeria japonica*). *Theoretical and Applied Genetics*, **92**: 40–45.

Uma Shaanker, R. and Ganeshaiah, K. N. 1997. Mapping genetic diversity of *Phyllanthus emblica*: forest gene banks as a new approach for in situ conservation of genetic resources. *Current Science*, **73**(2): 163–168.

Unander, D. W., Webster, G. L. and Blumberg, B. S. 1995, "Usage and bioassays in *Phyllanthus* (Euphorbiaceae)-IV: clustering of antiviral uses and other effects,". *Journal of Ethnopharmacology*, **45**(1): 1–18.

Upadhyay, R., Kashyap, S. P., Singh, C. S., Tiwari, K. N., Singh, K. and Singh, M. 2014. *Ex situ* conservation of *Phyllanthus fraternus* Webster and evaluation of genetic fidelity in regenerates using DNA-based molecular marker. *Applied Biochemistry and Biotechnology*, **174**(6): 2195–2208.

Vashistha, R. K., Chaturvedi, A. K., Nautiyal, B. P. and Nautiyal, M. C. 2009. Vegetative propagation of *Angelica glauca* Edgew. and *Angelica archangelica* Linn.: Two high value medicinal and aromatic herbs of the Himalaya. *Nature and Science*, **7**(8): 76–82.

Venkateswaran, P. S., Millman, I. and Blumberg, B. S. 1987. Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: *in vitro* and *in vivo* studies. *Proceedings of the National Academy of Sciences*, **84**(1): 274–278.

Victório, C. P., Henriques, A. B., Tavares, E. S., Esquibel, M. A. and Lage, C. L. S. 2010. Standardized production of *Phyllanthus tenellus* Roxb. by plant tissue culture. *Revista Ciência Agronomica*, **41**(2): 272–278.

Vogel, E. F. de 1987. *Manual of herbarium taxonomy: Theory and practice.* Rijksherbarium, Leiden, The Netherlands.

Volis, S. and Blecher, M. 2010. Quasi *in situ*: a bridge between *ex situ* and *in situ* conservation of plants. *Biodiversity Conservation*, **19**: 2441–2454.

Vorontsova, M. S., Hoffmann, P., Maurin, O. and Chase, M. W. 2007. Molecular phylogenetics of tribe poranthereae (phyllanthaceae; euphorbiaceae sensu lato). *American Journal of Botany*, **94** (12): 2026–2040.

Vorontsova, M. S., Hoffmann, P., Kathriarachchi, H., Kolterman, D. A. and Chase, M. W. 2007. *Andrachne cuneifolia* (Phyllanthaceae; Euphorbiaceae *s.l.*) is a *Phyllanthus*. *Botanical Journal of the Linnean Society*, **155**(4): 519–525.

Webster, G. L. 1956. A monographic study of the West Indian species of *Phyllanthus. Journal of the Arnold Arboretum*, **7:** 9–122, 217–268, 340–359.

Webster, G. L. 1957. A monographic study of the West Indian species of *Phyllanthus. Journal of the Arnold Arboretum*, **38:** 51–80, 170–198, 295–373.

Webster, G. L. 1958. A monographic study of the West Indian species of *Phyllanthus*. *Journal of the Arnold Arboretum*, **39**: 49–100.

Webster, G. L. 1994. Synopsis of the genera and suprageneric taxa of Euphorbiaceae. *Annals of the Missouri Botanical Garden*, **81**: 33–144.

Webster, G. L., and Carpenter, K. J. 2002. Pollen morphology and phylogenetic relationships in Neotropical *Phyllanthus* (Euphorbiaceae). *Botanical Journal of the Linnean Society*, **138**(3): 325–338.

Whitlock, B. A. and Baum, D. A. 1999. Phylogenetic relationships of *Theobroma* and *Herrania* (Sterculiaceae) based on sequences of the nuclear gene vicilin. *Systematic Botany*, **24**: 128–138.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Raflaski, J. A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**: 6531–6535.

Williamson, E. M. (eds.). 2002. *Major herbs of ayurveda*, Churchill Livingstone, New York, 210–219.

Withers, L. A. 1990. Prospects and problems of *in vitro* gene banks. *In* Proceedings of workshop, *Musa*: conservation and documentation. Leuven, Belgium, 21–24.

Wolfe, A. D. and Randle, C. P. 2001. Relationships within and among species of the holoparasitic genus *Hyobanche* (Orobanchaceae) inferred from ISSR banding patterns and nucleotide sequences. *Systematic Botany*, **26**: 120–130.

World conservation monitoring centre 1998. *Pseudoglochidion anamalayanum*. The IUCN red list of threatened species. Version 2015.2. <www.iucnredlist.org>. Downloaded on 09 July 2015.

Wu, K. R., Jones, R., Danneberg, L. and Scolink, P. A. 1994. Detection of microsatellite polymorphism without cloning. *Nucleic acid Research*, **22**: 3257–3258.

Wurdack, K. J., Hoffmann, P. and Chase, M. W. 2005. Molecular phylogenetic analysis of uniovulate Euphorbiaceae (Euphorbiaceae Sensu Stricto) using plastid *rbc*L and *trn*L-F DNA sequences. *American Journal of Botany*, **92**(8):1397–1420.

Wurdack, K. J., Hoffmann, P., Samuel, R., De Bruijn, A., Bank, M. V. D. and Chase, M. W. 2004. Molecular phylogenetic analysis of phyllanthaceae (Phyllanthoideae Pro Parte, Euphorbiaceae Sensu Lato) using plastid *rbc*L DNA sequences. *American Journal of Botany*, **91**(11): 1882–1900.

Xiaohong, Y., Li, G. and Yang, B. 2007. Genetic diversity of wild *Cymbidium goeringii* (Orchidaceae) populations from Hubei based on inter-simple sequence repeats analysis. *Frontiers of Biology in China,* **2**(4): 419–424.

Xiao-ying, L., Xue-ying, Z., Yue-sheng, Y. 2007. Genetic diversity of *Camellia changii* Ye (Theaceae) using ISSR markers. *Journal of Tropical and Sub tropical Botany*, **15**(2): 93-100.

Yadav, S. R., Chandore, A. N., Nimbalkar, M. S. and Gurav, R. V. 2009. Reintroduction of *Hubbardia heptaneuron* Bor, a critically endangered endemic grass in Western Ghats. *Current science*, **96**(7): 880.

Yang, W., de Oliveira A.C., Godwin I, *et al.*1996. Comparison of DNA markers technologies in characterizing plant genome diversity:Variability in Chinese sorghums. *Crop Science*, **36**: 1669-1676.

Zietkiewicz, E., Rafalski, A. and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*, **20**(2)**:** 176-183.

Zimmer, E. A. and Wen, J. 2012. Using nuclear gene data for plant phylogenetics: Progress and prospects. *Molecular Phylogenetics and Evolution*, **65**: 774–785.

Zonneveld, M. v., Scheldeman, X., Escribano, P., Viruel, M. A., Van, Damme, P., Garcia, W., Tapia, C., Romero, J., Sigueñas, M., and Hormaza, J. I. 2012. Mapping genetic diversity of cherimoya (*Annona cherimola* Mill.): Application of spatial analysis for conservation and use of plant genetic resources. *PLoS One*, **7**(1): e29845.

PUBLICATIONS

PAPERS PUBLISHED

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Systematic position of *Phyllanthus talbotii* (Phyllanthaceae), a critically endangered species of Western Ghats, India

S.S. Naik, R.S. Pagare, S. Krishnan and M.K. Janarthanam*
Department of Botany, Goa University, Goa-403 206, India
*E-mail: jana@unigoa.ac.in

Abstract

Phyllanthus talbotii Sedgw. (Phyllanthaceae) is endemic to Western Ghats regions of Goa and Karnataka, India and is critically endangered. For a long time, it was known only from the type collection and a subsequent untraceable collection and hence taxonomically not fully understood, though a recent treatment has placed it under subgenus *Eriococcus*. In the present study, attempts were made to confirm its position using morphological characters and sequences of nuclear internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) and chloroplast *mat* K genes. Results from ITS and *mat* K phylogenetic analyses supported its placement in subgenus *Eriococcus*.

Keywords: CR plant, ITS, matK, Phyllanthaceae, *Phyllanthus talbotii*, phylogeny, systematic.

Introducion

Phyllanthus dominates tribe *Phyllantheae*, being the largest genus in the family Phyllanthaceae. The genus has a remarkable diversity of growth forms and floral morphology (Bancilhon, 1971). Webster (1956, 1957, 1958) divided the genus into eight subgenera and over 30 sections based on vegetative architecture and pollen morphology in addition to floral characters.

Phyllanthus talbotii Sedgw. (Phyllanthaceae) is a rare, critically endangered (CR) and endemic species of Western Ghats region of Goa and Karnataka. It was described by Sedgwick (1921) based on collections made in 1918 and 1919 from North Kanara, presently in Karnataka State, India and later it was again collected from Agumbe in adjacent Shimoga district (Raghavan, 1969). Known by a single untraceable collection after type, Singh and Kulkarni (1990) reported it as rare species. Though poorly known taxonomically, Balakrishnan and Chakrabarty (2007) placed it in subgenus *Eriococcus.*

The internal transcribed spacers (ITS) nrDNA sequences contain potential informative sites and been used as a useful molecular marker in studying phylogeny of many taxa (Lee *et al.*, 2006; Maria *et al.*, 2007; Juthatip *et al.*, 2010; Shi-Xiao *et al.* , 2011). The *mat*K gene also potentially contributes to plant molecular systematic and evolutionary studies (Steele & Vilgalys, 1994; Liang & Hilu, 1996; Kathriarachchi *et al.*, 2006; Samuel *et al.*, 2005). The *mat*K gene with about 1500 base pairs (bp) is located within the intron of the chloroplast gene *trn*K. The present study is aimed at understanding the systematic position of *P. talbotii* using ITS and *mat*K gene sequences and also to provide an adequately detailed description which was not available in the literature.

Materials and Methods

Taxon sampling

Leaf samples were collected from naturally occurring healthy plant population from Saccordem (Longitude - E 074°09.647′ and Latitude - N 15° 24.822′; altitude 49 m), Goa [Goa University Herbarium 4011] and silica gel dried. Flowering twigs were collected and processed for herbarium and morphological study. Fresh and pickled specimens were studied and photographed under Leica EZ4D microscope with inbuilt camera.

Isolation of DNA and sequencing:

Silica gel dried leaf materials were used for the extraction of DNA. Total genomic DNA was isolated using CTAB method (Doyle & Doyle, 1990) with modification. Polymerase chain reaction was performed for the amplification of genes such as ITS and *mat*K by using universal random primers (Table 1). The PCR products were further purified using Exo-SAP-IT (GE Healthcare) treatment. The Exo-SAP treated PCR products were used for

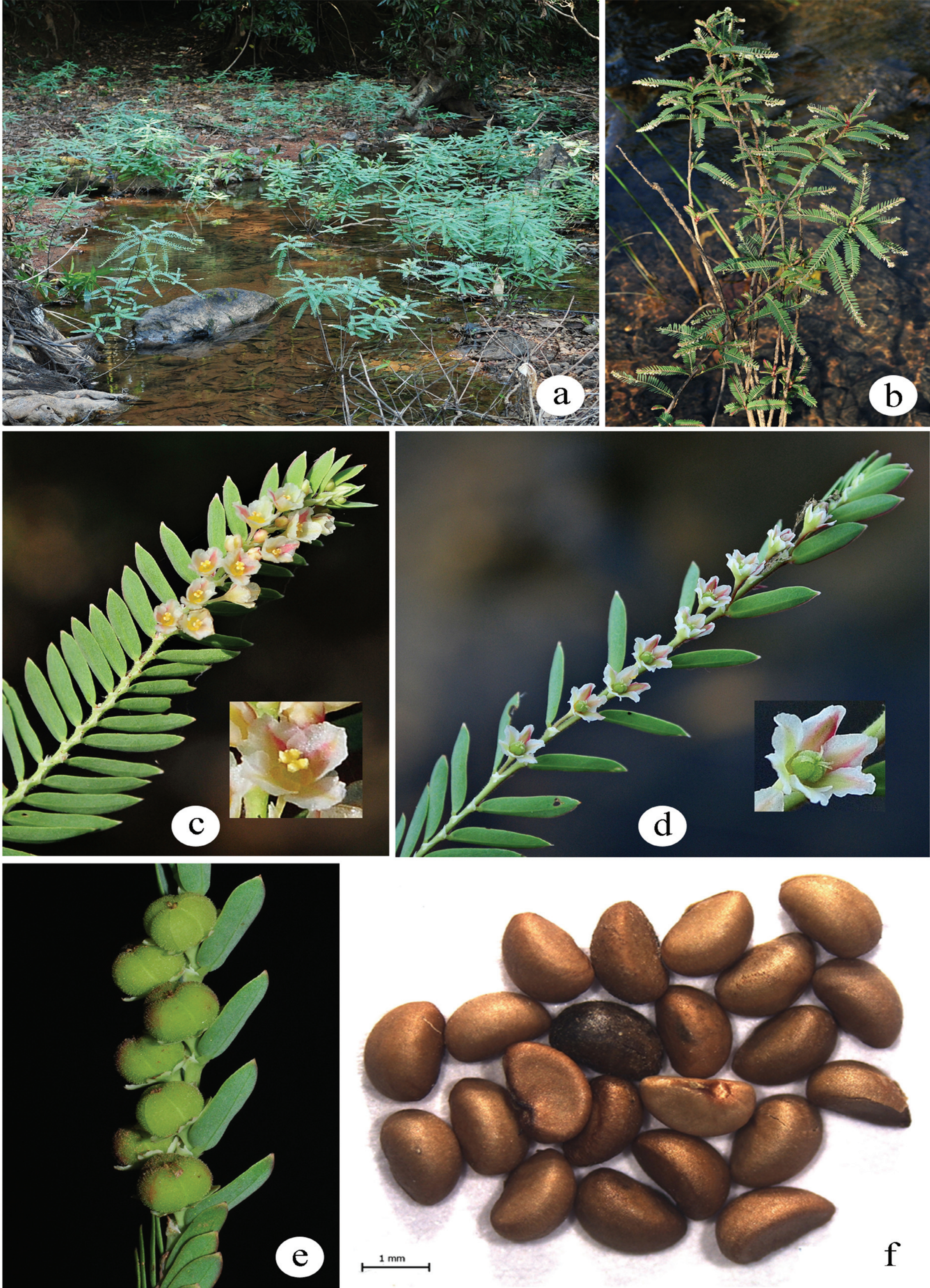

Fig.1. *Phyllanthus talbotii* Sedgw.: **a**. Habitat; **b.** Habit; **c.** Staminate flowers; **d.** Pistillate flowers; **e.** Capsule; **f.** Seeds.

Table 1. Primers used for sequencing of ITS and *mat*K genes.

Target gene	Primer name	Sequence (5'→ 3')	Reference
ITS	ITS5-F	GGAAGTAAAAGTCGTAACAAGG	White *et al.*, 1990
	ITS4-R	TCCTCCGCTTATTGATATGC	
mat **K**	3F KIM-F	CGTACAGTACTTTTGTGTTTACGAG	CBOL Plant Working Group (http://www.barcoding.si.edu/plant_working_group.html)
	1R KIM-R	ACCCAGTCCATCTGGAAATCTTGGTTC	

gene sequencing. Both the genes were sequenced using the Big Dye terminator v3.1 sequencing kit and ABI 3730 DNA Analyzer (Applied Biosystem, USA). DNA isolation, amplification and Gene sequencing was carried out at Rajiv Gandhi Center for Biotechnology, Regional Facility for DNA fingerprinting, Thiruvananthapuram, Kerala, India. The sequences are deposited at NCBI (ITS: Acc. No.KC414630; *mat*K: Acc. No. KC514101).

Sequence Analysis

The sequence analysis was carried out using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1. The ITS and *mat*K sequences were blast with highly similar sequences of related species. The maximum identical sequences to *P. talbotii* were retrieved from NCBI in FASTA format and aligned with the query sequences.

Sequence alignment and construction of phylogenetic tree

For ITS, indent value was taken as 83% and above and for *mat*K 95% and above. The sequence analysis involved *Phyllanthus* nucleotide sequences of 13 species for ITS and 15 species for *mat*K along with query sequences. The sequence alignment was carried out using clustalW (ver. 1.6) and improved by visual treatment (Kelchner, 2000). Further analysis was carried out using the Maximum Likelihood tree model (Tamura *et al.*, 2004, 2011). The confidence limit (bootstrap percentage) for clades was assessed by performing 1000 replicates of bootstrapping (Felsenstein, 1985). The individual Maximum likelihood analyses were performed using MEGA (version 5.05) software.

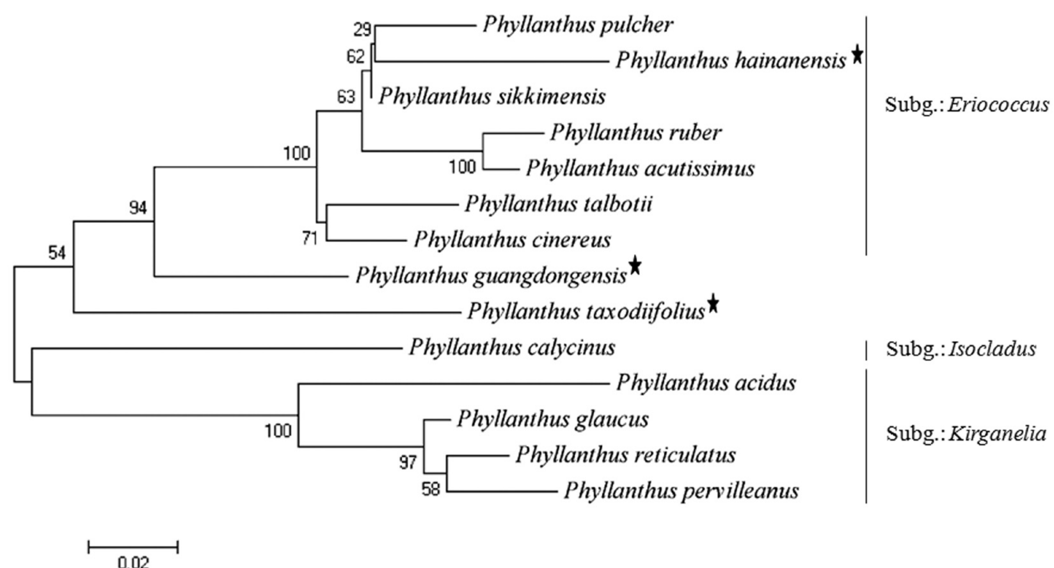

Fig 2. Maximum likelihood tree using ITS gene sequences of *Phyllanthus talbotii* (Bootstrap percentage indicated on side). Asterisks' indicate that the position of these species is not known.

Results and Discussion

Taxonomc description

Phyllanthus talbotii Sedgw., J. Indian Bot. Soc. 1: 124, f. 2. 1921 (as '*talboti*'); Singh & Kulkarni in Red Data Book of Indian Pl. 3: 124. 1990; Balakrishnan & Chakrabarty, Family Euphorbiaceae in India, 374. 2007. **Fig. 1**

A monoecious subshrub, up to 150 cm high; stem terete, suffrutescent, brown, often with vertical streaks with prominent nodes; branchlets up to 25 cm long, with up to 100 leaves, hirsute. Stipules triangular to deltoid, *c.* 1.5 × 1 mm, margin white or brown, acuminate at apex. Petiole *c.* 0.5 mm long; lamina oblong, up to 5.9 × 3 mm, rounded at base, entire, acute or slightly apiculate at apex, mid vein prominent, secondary veins 4 or 5-pairs, often invisible, sparsely hairy on both surfaces. Staminate flowers: axillary, solitary or in clusters of 2 or 3, *c.* 2.5 × 2 mm. Bracts ovate, shorter than stipule; bracteoles subulate. Pedicels capillary, *c.* 2 mm long, slightly dilated at apex. Sepals 4, obovate to elliptic, *c.* 2 × 1.3 mm, acute to rounded at apex, green in the middle with a broad white wavy margin, one-veined. Disc of 4 distinct units, yellow. Staminal column *c.* 0.7 mm long; anthers 4, in two pairs, *c.* 0.5 mm across, yellow. Pistillate flowers: solitary, axillary on the same branchlet as staminate flowers, *c.* 3 mm across when open. Bracteoles lanceolate to ovate, up to 1 mm long. Pedicel *c.* 2 mm long, dilated at apex. Sepals 6, ovate to elliptic, 1.5–2 × 1 mm, entire, acute to acuminate at apex, greenish in the middle and white to creamy on either side. Disk yellow, wavy, slightly 5-lobed. Ovary 3-lobed, *c.* 0.5 × 1 mm; styles 3, each distinctly forked, horizontal, adpressed to ovary. Fruiting pedicel *c.* 3 mm long, dilated at apex; fruiting calyx reflexed. Capsule subglobose, *c.* 3 × 4 mm, distinctly 3-lobed, hirsute; cocci 3; seeds 2 in each cocci, *c.* 1.8 × 1 mm, 3-angled, curved on dorsal side, smooth brown in colour.

Flowering & Fruiting: August – November.

Specimens examined: INDIA. **Goa**, North Goa District, Sanguem , 23.9.1997, *V. Joshi* & *S. Rajkumar* 1022, 1070; Selaulim dam site, 30.8.2010, *S.S. Naik* 4001, 4002, Collem stream, 11.9.2010, *S.S. Naik*, 4003, 4004, Waddem stream, 12.10.2010, *S.S. Naik*, 4005, 4006, 4007; Dudhsagar waterfalls, 11.9.2010, *S.S. Naik*, 4008, 4009, 4010. (All the specimens are deposited at Goa University Herbarium).

Morphologically, *P. talbotii* with the characters such as woody shrub, 4 sepals in staminate flowers, connate filaments and 6 sepals in pistillate flowers place it in subgenus *Eriococcus* of *Phyllanthus,* as proposed by Balakrishnan and Chakrabarty (2007).

Systematic position using molecular phylogeny

The ITS and *mat*K based phylogenetic studies

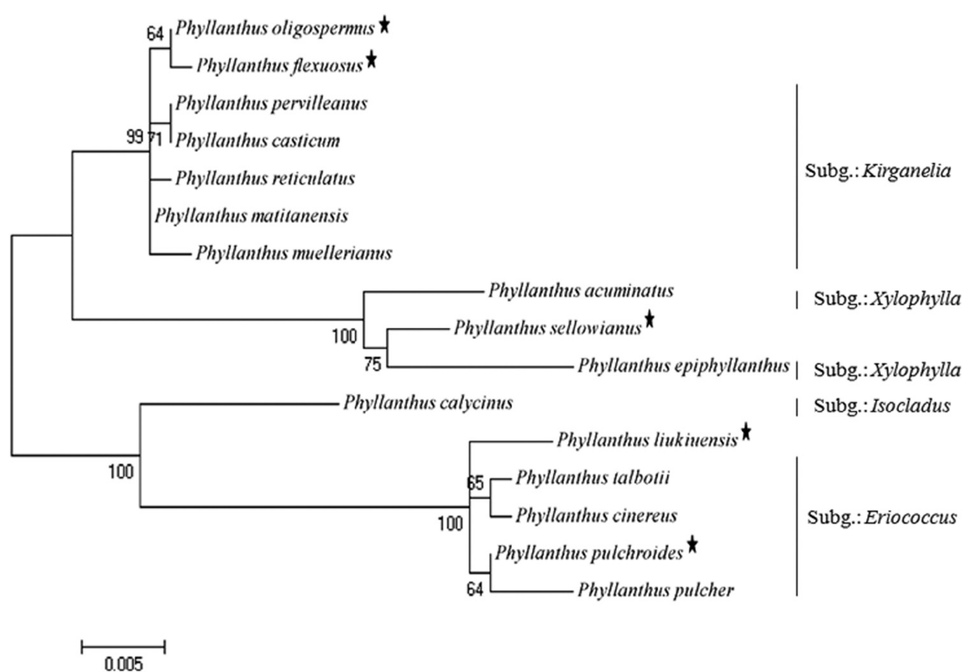

Fig 3. Maximum likelihood tree using *mat* K gene sequences of *Phyllanthus talbotii* (Bootstrap percentage indicated on side). Asterisks' indicate that the position of these species is not known.

have contributed largely for understanding the evolutionary and ancestral relationships within the family Phyllanthaceae and specifically within the genus *Phyllanthus*. Shi-Xiao *et al.* (2011) used nuclear ITS sequences to disentangle *Phyllanthus reticulatus* Poir. in Africa. Maria *et al.* (2007) reported the use of ITS and *mat*K gene sequences to solve the taxonomic position of *Andrachne cuneifolia* Britton. Kathriarachchi *et al.* (2006) studied the phylogenetic relationship within the tribe Phyllantheae using ITS and *mat*K sequences of 95 species.

Phyllanthus talbotii is taxonomically an insufficiently known species till Balakrishnan and Chakrabarty (2007) placed it in the subgenus *Eriococcus*. The earlier works such as Webster (1956, 1957, 1958) and Kathriarachchi (2006) never dealt this species due to its narrow endemic nature. The phylogenetic tree constructed (Fig. 2) using ITS gene sequences shows the species nesting in a distinct clade along with *P. cinereus* Müll. Arg., *P. pulcher* (Baill.) Wall. ex Müll. Arg., *P. sikkimensis* Müll. Arg., and *P. hainanensis* Merr. which are members of the subgenus *Eriococcus* (Kathriarachchi *et al.*, 2006; Balakrishnan & Chakrabarty, 2007; Juthatip *et al.*, 2010). This clade is supported by 100% boot strap value. The sub-clade formed by *P. cinereus* and *P. talbotii* is supported by 71% bootstrap value, though geographically these are the closely distributed species among the species studied. Tree constructed using *mat* K sequences (Fig. 3) also supported the grouping of *P. talbotii* with *P. cinereus* and *P. pulcher* along with three more species with 100% boot strap value thus providing additional support for placing *P. talbotii* in the subgenus *Eriococcus*. As in ITS sequence, within the clade, bootstrap value supporting the subclade formed by *P. talbotii* and *P. cinereus* is low (65%). However, the systematic position of *P. talbotii* in subgenus *Eriococcus* is well supported by both ITS and *mat* K sequences.

Conclusion

The position of *P. talbotii*, a less known endemic and Critically Endangered (CR) species, in subg. *Eriococcus* has been confirmed by present morphological and molecular studies. Analyses of its closely related endemic species in the Western Ghats will certainly strengthen the knowledge on the group.

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Literature Cited

Balakrishnan, N.P. & T. Chakrabarty 2007. *The Family Euphorbiaceae in India***.** Bishen Singh Mahendra Pal Singh, Dehra Dun.

Bancilhon, L. 1971. Contribution a′ l′e′tude taxonomique du genre *Phyllanthus* (Euphorbiace′es). *Boissiera* **18**: 9–81.

CBOL Plant Working Group (http://www.barcoding.si.edu/plant_working_group.html)

Doyle, J.J. & J.L. Doyle 1990. A rapid total DNA preparation procedure for fresh plant tissue. *Focus* **12**: 13–15.

Felsenstein, J. 1985. Confidence limits on phylogenetics: an approach using bootstrap. *Evolution* **39**: 783–791.

Juthatip, M., Suchada S., Nijsiri R. & H. Mizukami 2010. Molecular Phylogenetic Analysis of *Phyllanthus* Species in Thailand and the Application of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism for *Phyllanthus amarus* Identification. *Biol. Pharm. Bull.* **33(10)** 1723–1727.

Kathriarachchi, H., Samuel R., Hoffmann, P., Mlinarec, J., Wurdack, K.J., Ralimanana, H., Stuessy, T.F. & M.W. Chase 2006. Phylogenetics of tribe Phyllantheae (Phyllanthaceae; Euphorbiaceae *sensu lato*) based on nr ITS and plastid *mat*K sequence data. *Am. J. Bot.* **93 (4)**: 637–655.

Kelchner, S.A. 2000. The evolution of non-coding chloroplast DNA and its application in plant systematics. *Ann. Mo. Bot. Gard.* **87**: 482–498.

Lee, S.K., Li, P.T., Lau, D.T., Yung, P.P., Kong, R.Y. & W.F. Fong 2006. Phylogeny of medicinal *Phyllanthus* species in China based on nuclear ITS and chloroplast *atp*B- *rbc*L sequences and multiplex PCR detection analysis. *Planta Med.* **72**: 721–726.

Liang, H., & K.W. Hilu 1996. Application of the *mat*K gene sequences to grass systematics. *Can. J. Botany.* **4**: 125–134.

Maria, S.V., Petra, H., Hashendra, K., Duane, A.K. & W.C. Mark 2007. *Andrachne cuneifolia* (Phyllanthaceae; Euphorbiaceae *s.l.*) is a *Phyllanthus. Biol. J. Linn. Soc.*, **155**: 519–525.

Raghavan, R.S. 1969. *Flora of Agumbe and Tirthahalli*

area, Shimoga district. Ph. D. thesis, University of Pune, India.

Samuel, R., Kathriarachchi, H., Hoffmann, P., Barfuss, M.H.J., Wurdack, K.J.C., Davis, C. & M.W. Chase 2005. Molecular phylogenetics of Phyllanthaceae: evidence from plastid *mat*K and nuclear PHYC sequences. *American J. Bot.* **92**: 132–141.

Sedgwick, L.J. 1921. New Bombay species. *J. Indian Bot.* **2**: 124

Shi-Xiao, L., Hans-Joachim, E., Dianxiang, Z. & S.S. Renner 2011. Nuclear ITS Sequences Help Disentangle *Phyllanthus reticulatus* (Phyllanthaceae), an Asian Species not Occurring in Africa, but Introduced to Jamaica. *Syst. Bot.* **36(1)**: 99–104.

Singh, N.P. & B.G. Kulkarni 1990. *Phyllanthus talbotii* Sedg. In: Nayar MP, Sastry ARK (eds) *Red Data Book of Indian plants, Volume* **3**. Botanical Survey of India Calcutta, pp. 124–125.

Steele, K.P. & R. Vilgalys 1994. Phylogenetic analyses of Polemoniaceae using nucleotide sequences of the plastid gene *mat*K. *Syst. Bot.* **19**: 126–142.

Tamura, K., Nei, M. & S. Kumar 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (*USA*) **101**: 11030–11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & S. Kumar 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* (doi: 10.1093/molbev/msr121).

Webster, G.L. 1956. A monographic study of the West Indian species of *Phyllanthus J. Arnold Arbor.* **37**: 9–122, 217–268, 340–359.

Webster, G.L. 1957. A monographic study of the West Indian species of *Phyllanthus J. Arnold Arbor.* **38**: 51–80, 170–198, 295–373.

Webster, G.L. 1958. A monographic study of the West Indian species of *Phyllanthus J. Arnold Arbor.* **39**: 49–100, 111–212.

White, T.J., Bruns, T., Lee, S. & J.W. Taylor 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications, (Ed.), Innis, M.A., Gelfand, D.H., Sninsky, J.J. & T.J. White. *Academic Press, Inc., New York,* pp. 315–322.

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PLATE 14

THE TIMES OF INDIA, GOA
THURSDAY, FEBRUARY 3, 2011

TIMES

GU botanists on mission to save endangered plant

The Species Is So Rare That It Has Not Been Named By Locals

Gauree Malkarnekar | TNN

Panaji: The Save Tiger campaign may have hogged all the limelight, but three botanists from the Goa university (GU) are silently working towards saving from extinction Phyllanthus Talbotti, an endangered plant that is entwined with the sensitive ecology of the Western Ghats.

In 1997-98, the botanists found a small population of the species near a river belt, the natural habitat of the plant, in Netravali. But when they visited the place next, to their utter horror, they found that the shrubs had been cleared for construction of a check dam. The botanists, though, struck gold again, when nearly a decade of toiling led to the discovery of another population of the plant at a river bed in Mollem.

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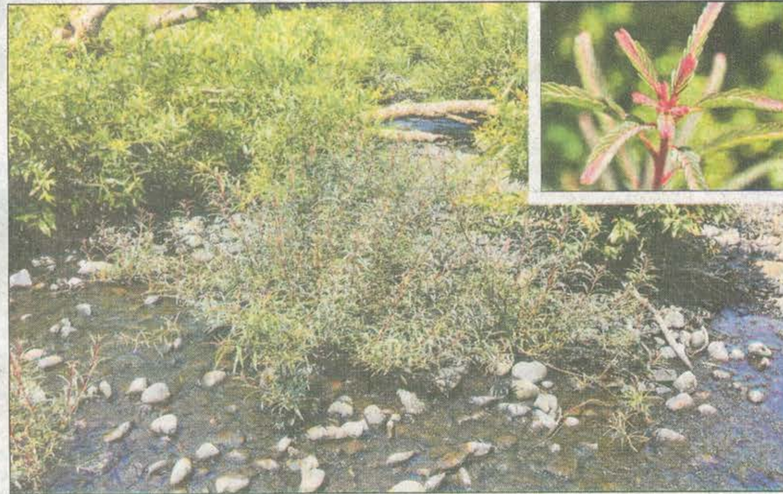

Phyllanthus Talbotti seen growing along a rivulet in the Western Ghats

The Phyllanthus Talbotti population at Mollem found by the team is the only identified growth of it in the world. The Phyllanthus Talbotti, a plant specific to the Western Ghats, has been entered as an endangered plant species by the International Union for Conservation of Nature (IUCN), a wing of the United Nations, in its red list category.

"It is an endemic species, which means that it is restricted only to smaller geographical areas. There too, the plant exists in rare pockets. If these pockets are destroyed the species will be eliminated forever," said M K Janarthanam, professor from the GU department of botany.

Before the GU began working on the project, funded by the Department of Bio Technology (DBT) in New Delhi, the existence of the Phyllanthus Talbotti was recorded only twice in human history – first by a group of British biologists in 1883 from near Jog falls and Supa in Karnataka and then by an Indian botanist in 1965.

However, the second collection is untraceable and only the first and only collection, until the Goa University project, in the world remains preserved at a laboratory in Mumbai. This first collection earned the plant its name after its collector Talbotti in 1921 when the plant was established as a species unknown to science until then.

The Western Ghats, according to Janarthanam, is one of 34 biodiversity hotspots around the world and is home to several endangered species, Phyllanthus Talbotti being one of them.

Under the project - with generous help from the state forest department - the botanists try to locate new population of the species and save them to reintroduce the species in new places where its natural conditions exist.

"The plant's extinction can adversely affect the sensitive ecology of the Western Ghats. We do not know how badly it can affect the adjoining life such as butterflies, insects etc as far as pollination is concerned. If you do not take measures to preserve it, it will be extinct soon," said associate professor S Krishnan. What the botanists do know is that this plant is one of the vital species that prevents soil erosion near river beds.

At the successful end of five years of the project in 2013, the DBT is hoping to replant the plantlets in its natural habitat throughout the Western Ghats to save it from extinction.

The botanists mainly use the tissue culture method which enables them to produce 1,000 of plantlets from a single shoot or seed. Till date, the botanists have planted 2,500 of the cuttings in two new habitats, which have to be first established to be the same as the plant's natural habitat.

"Sixty per cent of the replanted population is surviving so far. The shrubs have to survive the monsoons now. The plants are such that they get submerged completely in the rivers during monsoon with only twigs remaining. The shrub shoots grow again only once the monsoons end," junior research fellow, Sidhesh Naik, explained.

Janarthanam added, "The plant is so rare that it has not even been named by the local population living near its habitat and, therefore, has no local Konkani name. A particular thing has to either be a menace or should be widely used for it to be named. It does not fit in either category, so has remained anonymous."

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