

Conservation genetics of endemic *Indirana* frogs of the Western Ghats biodiversity hotspot

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Academic dissertation

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in Auditorium 2041, Biocenter 2 (Viikinkaari 5) on 26th October 2012 at 12 noon.

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ISBN 978-952-10-8348-8 (paperback)

ISBN 978-952-10-8349-5 (PDF)

<http://ethesis.helsinki.fi>

Yliopistopaino, Helsinki 2012

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This thesis is based on the following articles which are referred to in text by their Roman numerals:

- I** Nair, A., Gopalan, S. V., George, S., Kumar, K. S., Teacher, A. G. F., & Merilä, J. (2012) Endemic *Indirana* frogs of the Western Ghats biodiversity hotspot. *Annales Zoologici Fennici* 49: 257-286.
- II** Nair, A., Gopalan, S. V., George, S., Kumar, K. S., Teacher, A. G. F., & Merilä, J. (2012) High cryptic diversity of endemic *Indirana* frogs in the Western Ghats biodiversity hotspot. *Animal Conservation* 15: 489-498. doi:10.1111/j.1469-1795.2012.00539.x.
- III** Nair, A., Kumar, K. S., George, S., Gopalan, S. V., Li, M--H., Leder, E. H., & Merilä, J. (2011) Sixty-two new microsatellite markers for an endemic frog *Indirana beddomii* from the Western Ghats biodiversity hotspot. *Conservation Genetics Resources* 3: 167-171.
- IV** Nair, A., Gopalan, S. V., George, S., Kumar, K. S., & Merilä, J. (2012) Cross-species testing and utility of microsatellite loci in *Indirana* frogs. *BMC Research Notes* 5: 389. doi:10.1186/1756-0500-5-389.
- V** Nair, A., Gopalan, S. V., George, S., Kumar, K. S., Shikano, T., & Merilä, J. (2012) Genetic variation and differentiation in *Indirana beddomii* frogs endemic to the Western Ghats biodiversity hotspot. *Conservation Genetics*. doi:10.1007/s10592-012-0389-z.
- VI** Nair, A., Daniel O., Gopalan, S. V., George, S., Kumar, K. S., Merilä, J., & Teacher, A. G. F. (2011) Infectious disease screening of *Indirana* frogs from the Western Ghats biodiversity hotspot. *Herpetological Review* 42: 554-557.

Contributions

	I	II	III	IV	V	VI
Original idea	JM, AN	AN, JM	JM, AN	JM, AN	AN, JM	AN, AT
Field work	AN, SVG, SG, KSK	SVG, SG, KSK	AN, SVG, SG, KSK	SVG, SG, KSK	SVG, SG, KSK	SVG, SG, KSK
Lab work	-	AN, SVG	AN	AN	AN	AN, OD
Data analysis	AN, SVG	AN	AN, EL	AN	AN, TS	AN
Manuscript preparation	AN, JM	AN, JM	AN, JM	AN, JM	AN, TS, JM	AN, JM, AT

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Abstract

The Western Ghats-Sri Lanka biodiversity hotspot is one of the World's 34 recognized biodiversity hotspots. The current knowledge about amphibian fauna of the Western Ghats is limited, but this region is known to exhibit a high degree of diversity and endemism. Although many species of amphibians are yet to be described from India, about 40% of known amphibian species from this region are threatened by extinction. The *Indirana* frogs belong to an endemic family, Ranixalidae, and are comprised of ten known species. Studies of this Western Ghats amphibian group are rare, hence the evolutionary relationships, taxonomy and species-level diversity of *Indirana* frogs have remained unresolved. Furthermore, nothing is known about the extent of genetic variability and differentiation among local populations of a given species. Hence, there is a high degree of uncertainty about the taxonomic status (cf. cryptic species) and potential genetic problems that the *Indirana* populations are likely to be facing.

This study focused on phylogenetic relationships and population genetics of *Indirana* frogs. Phylogenetic analyses clarified the evolutionary relationships among extant taxa and identified five new cryptic candidate species within the genus. For one of the taxa, *Indirana beddomii*, detailed population genetic analyses based on novel microsatellite markers represent the first phylogeographic analysis of amphibian differentiation in the Western Ghats. Apart from developing a large number of novel microsatellite loci for *I. beddomii*, cross-species amplification tests performed with eight other taxa should provide useful genetic tools for studies of other species in this genus. Finally, the first infectious disease (cf. Chytrid and *Ranavirus* infections) screening of Indian amphibians was performed using samples collected from the Western Ghats. In general, the results of the studies included in this thesis should provide useful information, guidelines and resources for amphibian conservation and biodiversity research in the Western Ghats.

Introduction

Amphibians are facing a global decline, with 41% of known amphibian species threatened by the risk of extinction (Stuart et al. 2004; Hoffmann et al. 2010). Many causes are suggested to contribute to the observed declines, including increased levels of UV-B radiation and pollution, climate change, emerging infectious diseases, habitat loss and fragmentation (Collins and Storer 2003; Beebe and Griffiths 2005). On the other hand, recent studies have identified many new cryptic amphibian lineages and species from different biodiversity hotspots (e.g. Fouquet et al. 2007; Vieites et al. 2009), indicating that the amphibian diversity within these hotspots may be heavily underestimated. Biodiversity hotspots are regions rich in endemic species diversity, but at the same time, are also known to experience exceptional habitat losses (Myers et al. 2000; Brooks et al. 2002). An increasing number of reports about amphibian extinctions from biodiversity hotspots – even before their scientific description (e.g. Crawford et al. 2010) – have generated much concern regarding efforts of their conservation. Therefore, there is an urgent need to increase efforts towards studying diversity and identifying the potential genetic problems that local amphibian populations are likely to be facing in these hotspots.

At present, 34 biodiversity hotspots are recognised worldwide; these regions comprise only 2.3% of global land surface, and yet harbour 50% of all plant species and 42% of all vertebrate species (www.biodiversityhotspot.org). Growing human populations have inflicted substantial environmental and demographic changes in these regions, causing conservation concerns (Cincotta et al. 2000). However, current conservation efforts have remained insufficient in controlling the biodiversity and habitat loss (Hoffmann et al. 2010). In view of these facts, protection of these hotspots

should be an efficient way of preserving a large proportion of the world's biodiversity. Yet, our understanding of the local biodiversity remains poor in most of these hotspots (e.g. Western Ghats; Bossuyt et al. 2004; Krishnankutty and Chandrasekaran 2007). This hampers any rational approach towards conservation in biodiversity hotspots, and questions any strategy that protects these regions as a surrogate sample of total global biodiversity (Grenyer et al. 2006). It has also been suggested that the conservation programmes in these hotspots would be more effective if the characterization of the biodiversity heterogeneity is done on a finer local scale (Bossuyt et al. 2004). Therefore it is important to focus our efforts towards thorough assessments of the genetic diversities in these hotspots, and to deepen our understanding of the patterns and processes generating and maintaining such biodiversity.

Amphibian diversity and conservation status in the Western Ghats

The Western Ghats-Sri Lanka biodiversity hotspot is one of the world's recognized biodiversity hotspots (Myers et al. 2000). The Western Ghats are comprised of mountain chains running parallel to the west coast of India for over 1600 km (Fig. 1). Along its entire length, there is only one major discontinuity – the Palghat Gap of Kerala – which is a low mountain pass at an elevation of only 100 m asl and about 30 km in width. Another smaller (7.5 km) gap – the Shencottah Gap – is present at 9° N (Fig. 1). These mountain chains harbour diverse endemic flora and fauna (Bossuyt et al. 2004). The endemic diversity is particularly pronounced for amphibians, as many new families and genera have recently been discovered from these mountain ranges (e.g. Biju and Bossuyt 2003; Roelants et al. 2004). In general, the amphibian fauna of southern India is one

of the most diverse – and poorly known – in tropical Asia (Inger 1999). Presently, about 132 species are known to be endemic to this region (Dinesh et al. 2009).



Figure 1. Map showing the location and extent of the Western Ghats.

About 40% of these endemic amphibian species are threatened with extinction (Biju et al. 2008). These facts, combined with the realization that amphibians comprise a group of organisms facing particularly pronounced declines and extinction risks worldwide (Houlahan et al. 2000; Stuart et al. 2004), suggest that studies about the diversity and conservation biology of Western Ghats amphibians should be well motivated.

Current knowledge of the amphibian fauna of the Western Ghats is scant and fragmented, but it is known to be unique with a high degree of endemism (Inger 1999; Biju 2001). There are three families (*viz.* Micrixalidae,

Nasikabatrachidae and Ranixalidae) and 10 genera which are endemic to the Western Ghats. Most of the genetic studies on Western Ghats amphibians have been conducted at the interspecific level with focus on taxonomic questions (e.g. Biju and Bossuyt 2009), while detailed intraspecific studies are entirely lacking. Except for a few well studied taxa (e.g. Biju and Bossuyt 2009; Bocxlaer et al. 2012), the evolutionary relationships, taxonomy and species-level diversity of the Western Ghats amphibian fauna are poorly resolved, and nothing is known about the extent of genetic variability and differentiation among local populations of most species. Consequently, there is a high degree of uncertainty about the taxonomic status (*cf.* cryptic species) and potential genetic problems such as loss of genetic diversity, inbreeding, restrictions to gene flow due to habitat fragmentation, faced by local amphibian populations. From this, it follows that any plans for the conservation and management of amphibian biodiversity in this biodiversity hotspot currently has to be based on educated guesses, rather than on scientifically based knowledge. The major problem for conserving the amphibian fauna of the Western Ghats is the lack of detailed systematic and other biological information of the amphibian species from this region. About 35% of the amphibian species are categorised as data deficient (IUCN 2011) and have insufficient details available on taxonomic identity, distribution and potential threats, to determine their global conservation concern.

In focus: Indirana

The genus *Indirana* belongs to the endemic family Ranixalidae and is comprised of 10 known species (Biju 2001). Roelants et al. (2004) had identified *Indirana* as one of the ancient lineages endemic to the Western Ghats, which now represents a small relict clade that is remnant of a once much more diverse and

widespread anuran fauna. These frogs are also unique in that they have semi-terrestrial tadpoles which are adapted for life on moist, steep rocky surfaces (Roelants et al. 2004).

Two of the species within *Indirana* are classified as critically endangered (*I. gundia* and *I. phrynoderma*), three as endangered (*I. brachytarsus*, *I. diplosticta*, and *I. leptodactyla*), one as vulnerable (*I. leithii*), two as least concern (*I. beddomii* and *I. semipalmata*), whereas two (*I. longicrus* and *I. tenuilingua*) are classified as data deficient (IUCN 2011). The populations of all these species are small and isolated, owing to the destruction and fragmentation of their natural habitat that has resulted from various anthropogenic activities (Nair 1991). Consequently, these species may face extinction in the near future (Daniels 1992).

Aims of this thesis

The main aim of this thesis was to resolve the phylogenetic relationships between species within the endemic family Ranixalidae, including identification of yet unknown (i.e. cryptic) species in this family. The secondary aim was to study genetic variability and differentiation within and among different populations of *Indirana* frogs, with the aid of novel microsatellite markers developed specifically for this purpose. In addition, I also investigated the possible presence of amphibian diseases (cf. Chytrid and *Ranavirus* infections), known to have caused amphibian declines, in the Western Ghats biodiversity hotspot. The broader aim of this thesis was to contribute to the understanding of taxonomic and genetic biodiversity of the poorly studied amphibian fauna of the Western Ghats biodiversity hotspot, as well as to produce information useful for delimiting management and conservation units in *Indirana* frogs.

In Chapter **I**, I conducted a comprehensive and critical literature review of *Indirana* frogs to bring together the basic information on the morphology, ecology and biology of these frogs, in order to identify the knowledge gaps and future research needs. In Chapter **II**, I investigated the phylogenetic relationships among different *Indirana* species using mitochondrial and nuclear DNA sequence data. The primary goal was to identify the presence of possible cryptic lineages in this taxon – the existence of which has been earlier suggested in literature (Biju et al. 2004).

Another major objective of this thesis was to study genetic variability and differentiation among *Indirana* populations. As far as I am aware, there have not been any studies of population structure and genetic variability of the Western Ghats amphibians, since codominant molecular markers have thus far been unavailable. To this end, I developed a large number of microsatellite markers as a useful resource for genetic studies of *Indirana* frogs (Chapter **III**). I further tested their utility in a number of different *Indirana* species by means of cross-species amplification tests (Chapter **IV**), including the cryptic species identified in Chapter **II**. In Chapter **V**, I studied genetic diversity and population structuring of *Indirana cf. beddomii* from different parts of the Western Ghats to gain insights on the factors influencing its genetic diversity and population structuring. Finally, I also investigated the presence of Chytrid and *Ranavirus* infections in the Western Ghats (Chapter **VI**). The rationale for this was that both of these diseases have been recently spreading around the world (Schloegel et al. 2010) and causing local amphibian extinctions (Daszak et al. 1999). However, it is not known whether they are also present in India, as no screening of these diseases has been performed as of yet.

Materials and Methods

I hereby present briefly the methods used in the studies constituting this thesis. The detailed description of methods and analyses are given in the original papers (I-VI).

Study species and populations

The samples of *Indirana* frogs were collected from field surveys done in different regions of the Western Ghats between 2008 and 2011 (I-V). The sampling was done from localities in major National parks and Wildlife sanctuaries. The populations for the genetic differentiation study of *I. cf beddomii* (V) were sampled spanning a range of ~200km from the south of Shencottah Gap up to the Palghat Gap (Fig. 2).

The specimens were identified using the published information on morphology of species within *Indirana* (Günther 1876; Boulenger 1920; Inger et al. 1984; Daniel and Sekar 1989; Daniels 2005), as well as by comparison with the type specimens (I) deposited at National History Museum in London (NHM) and National Museum of Natural History in Paris (MNHN). Voucher specimens were not collected; instead, a tissue sample (toe-clip) was taken from each specimen and stored in 95% alcohol for the genetic analyses. *I. longicrus* and *I. tenuilingua* could not be included in this study as they are only known from the type specimens which are now lost (I). Additionally, swabs for Chytrid screening were taken for species *Micrixalus fuscus*, *Hylarana temporalis* and *Fejervarya keralensis* from Peppara Wildlife Sanctuary (VI).

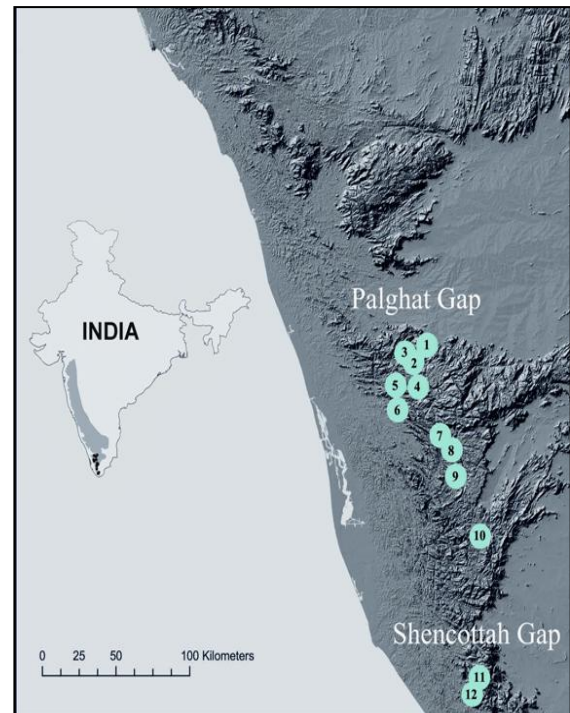


Figure 2. Map showing the collection localities of populations of *I. beddomii* for the population genetics study (V).

Molecular markers and genetic methods

I used sequence information from both mitochondrial (*16S rRNA*, *12S rRNA* and *COI*) and nuclear (*rag1*, *rhodopsin*) genes for the phylogenetic study of species within genus *Indirana* (II). These genes have been proven useful in studying amphibian phylogenies and diversity in earlier studies (Kosuch et al. 2001; Vences et al. 2007; Smith et al. 2008). The analyses were done using Bayesian inference, Maximum Likelihood and Maximum parsimony methods. Additionally, species tree estimation was done using a multi-species coalescent model to validate the results obtained by the phylogenetic methods.

I also developed 62 microsatellite markers from 454 pyrosequencing data of *I. beddomii*, and these markers were tested in a sample ($N=23$) of individuals from the

Ponmudi (08°45'59''N, 77°06'34''E) population in Kerala (III). These microsatellite markers were further used in cross-species amplification tests in eight other *Indirana* species, including new candidate species (IV). Fifteen polymorphic loci were also used to study the genetic diversity and differentiation among 12 populations of *I. cf beddomii* from the southern Western Ghats. The allelic richness (A_r), expected (H_E) and (H_O) observed genetic diversity of the populations, as well as the genetic differentiation between the populations (F_{ST}) were estimated. Isolation-by-distance (IBD) was also investigated for these populations (V).

Infectious disease screening

The screening for the presence of Chytrid fungus (*Batrachochytrium dendrobatidis*) was done by performing a Quantitative Real Time PCR (qPCR) using a Taqman assay following methods detailed in Boyle et al. (2004). For the screening of the Chytrid infections, I visited the Institute of Zoology (UK). The *Ranavirus* screening was done using PCR protocols utilizing *Ranavirus* specific primers as described in Teacher et al. (2010; VI).

Results and Discussion

The main study questions and results of all the chapters are summarised in Table 1. Below I discuss these results and their relevance for the conservation of *Indirana* frogs.

Basic information

In the literature review of *Indirana* in Chapter I, I brought together all the information available on *Indirana* frogs to provide a resource for researchers interested in this endemic genus. This resource is not only comprehensive, but accessible to all, in contrast to many of the

original publications which were scattered around in hard-to-find journals, books and booklets. In light of the existing taxonomic confusions, photographs of all the available type specimens are provided as a reference for the species with identifiable morphological features. In addition, photographs of some of the specimens collected in our own field surveys are also provided, as a means of illustrating the range of colour variation present in some of the species (e.g. Fig. 8 in Chapter I).

Previously, there have not been any attempts to map the distribution of the different *Indirana* species, and not in a particular format which would allow one to evaluate the reliability of the information behind the maps in an easy and accessible manner. Hence, apart from providing detailed distribution maps and a comprehensive compilation of available biological information on *Indirana* frogs, Chapter I should also be useful in identifying the knowledge gaps and future research needs of these frogs.

Cryptic lineages within Indirana

The *Indirana* frogs were first described in the late 19th century (Günther 1876; Boulenger 1888), and since then there has been no critical revision of the species diversity within this genus. As evident from Chapter I, very little is known about the ecology and biology of these frogs. This, together with the morphological similarity among the species (Chapter I), has led to many taxonomic confusions (e.g. Boulenger 1920; Chari and Daniel 1953; Abdulali and Daniel 1954; Abdulali and Daniel 1955). For example, Boulenger (1882; 1920) considered *I. brachytarsus* to be a synonym of *I. beddomii*, but later Inger et al. (1984) designated them to be separate species. Yet, the type series of *I. beddomii* is comprised of both *I. beddomii* and *I. brachytarsus* individuals, and the lectotype (i.e. specimen selected as the type of a species when a holotype has not been defined) of *I. brachytarsus* was

designated from the *I. beddomii* type series (Inger et al. 1984). Instead of the six expected groupings (species) from the region surveyed, I found eleven well-supported monophyletic clades in my samples (II). The phylogenetic trees obtained with Bayesian, maximum likelihood and maximum parsimony methods all yielded concurrent results: eleven distinct monophyletic clades with high posterior probabilities (= 1) and bootstrap support values (>97%, Fig. 3). *I. beddomii* was found to be polyphyletic with four strongly supported (100% bootstrap) monophyletic clades (clades a, c, d and i). These clades also showed high

interclade genetic divergence (4.2-12.5%), whereas the intraspecific divergence was low (0.2-2%). Similarly, *I. diplosticta* was divided into two distinct clades (clades e and k, Fig. 3) with 14.5% divergence among them. The individuals of ‘clade g’ were divergent from all other clades (5.7-14% divergence; Fig. 3). This suggests it to be a new – earlier unrecognized – candidate species within *Indirana*. However, the validation of all discovered candidate species as new species would require their formal comparison with all the type specimens of *Indirana* frogs (II).

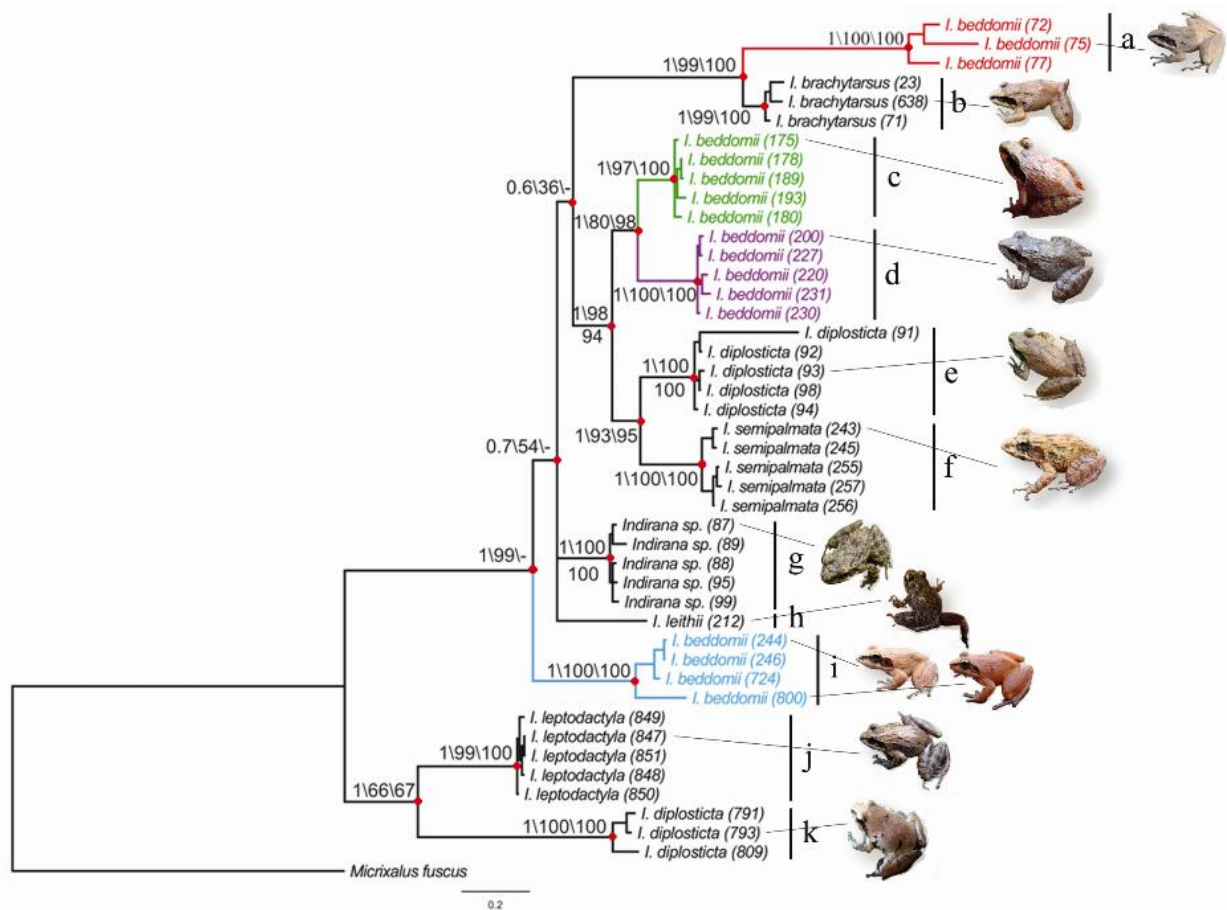


Figure 3. Bayesian consensus tree of *Indirana* species based on a combined dataset (mitochondrial + nuclear genes). Eleven monophyletic clades (a–k) are shown, and the distinct *Indirana beddomii* clades (a, c, d and i) are depicted in colour. The numbers at the nodes indicate the posterior probabilities and the bootstrap values obtained from maximum likelihood and maximum parsimony methods, respectively.

Table 1. Summary of the study questions and results in the articles included in this thesis.

Main study questions	Main results
<p>I What is known about <i>Indirana</i> frogs?</p> <p>What is the present distribution of the species within <i>Indirana</i>?</p>	<p>The published information on species within <i>Indirana</i> is summarised, which gives insight into the unique biology and conservation status of these frogs. New information on many species (e.g. <i>I. gundia</i>) is also provided.</p> <p>A detailed distribution map for each species was compiled based on published and newly collected information.</p>
<p>II Are there cryptic species present in the genus <i>Indirana</i>?</p> <p>Do <i>Indirana</i> frogs have the correct IUCN conservation status?</p>	<p>The analysis of mtDNA and nuclear genes support the existence of multiple cryptic lineages in <i>Indirana</i> which cannot be identified on the basis of morphology.</p> <p>The IUCN conservation criteria of <i>Indirana</i> frogs are likely to be incorrect for some species (e.g. <i>I. beddomii</i>). The species that are believed to be of least concern because of large distribution is in fact comprised of multiple cryptic species.</p>
<p>III Development of resources for genetic study of <i>Indirana</i> frogs.</p>	<p>62 polymorphic microsatellite markers were developed for <i>I. beddomii</i>.</p>
<p>IV Can the developed microsatellite markers be used to study other <i>Indirana</i> species?</p> <p>Does the microsatellite data support the existence of cryptic lineages in <i>Indirana</i>?</p>	<p>Part of the developed microsatellite markers amplify successfully and are polymorphic in other <i>Indirana</i> species closely related to <i>I. beddomii</i>. The amplification success and polymorphism in other species are reduced as compared to the source species.</p> <p>The reduction in microsatellite amplification success and polymorphism in <i>Indirana</i> in relation to increased genetic divergence from the source species support the presence of cryptic lineages in <i>Indirana</i>.</p>
<p>V What are the patterns of population structuring in the Western Ghats, and are there differences in the levels of genetic diversity between local <i>I. beddomii</i> populations?</p> <p>Do the geographic gaps in the Western Ghats influence the genetic structuring of <i>I. beddomii</i> frogs?</p>	<p>Clear genetic structuring was observed in <i>I. beddomii</i> frogs from the southern Western Ghats. The genetic diversity was not significantly different between localities.</p> <p>Genetic data support that the geographic gaps do influence the genetic structure of <i>I. beddomii</i> frogs from southern Western Ghats.</p>
<p>VI Is <i>Batrachochytrium dendrobatidis</i> (<i>Bd</i>) and <i>Ranavirus</i> infection present in Western Ghats biodiversity hotspot?</p>	<p><i>Ranavirus</i> infection was not detected in the analysed samples. <i>Batrachochytrium dendrobatidis</i> was detected to be positive, multiple times in one <i>I. brachytarsus</i> sample.</p>

Cross-species amplification test

In order to further study interspecific differentiation, I tested 62 microsatellites developed for *I. beddomii* (clade a, Fig. 3) in eight other species which included the putative species identified as *I. beddomii* and *I. diplosticta* in the previous study (IV). The cross-species amplification tests resulted in successful amplification of 7-18 loci (11.3-29.0%) depending on the species analysed; 2-14 of these loci were polymorphic in the target species. I also found that the extent of the cross-species amplification success ($r=-0.87$, $r^2=0.76$, $P<0.01$; Fig. 4a) and proportion of the polymorphic loci ($r=-0.94$, $r^2=0.88$, $P<0.01$; Fig. 4b) were strongly negatively correlated with genetic divergence (16S divergence) between the target and source species (IV; Fig. 4).

The cross-species amplification success rate of 11.3-29% (mean = 21.2%) observed in *Indirana* was comparable to the within-genus amplification rate of 21% observed in ranid frogs (Primmer and Merilä 2002). The low amplification success of the microsatellite markers was observed in *I. beddomii* individuals from Aralam (21%), Kudremukh (25.8%) and Periyar (25.8%) region of the Western Ghats (IV). These results, along with the previous study based on analysis of nuclear and mitochondrial genes (II), indicate that the Aralam, Kudremukh and Periyar populations of what was thought to be *I. beddomii* are indeed distinct species.

The cross-species amplification success in *Indirana* frogs depended on the degree of evolutionary divergence between the source and target species. However, given the relatively high levels of microsatellite polymorphism in some of the target species, these markers may provide useful genetic tools for future conservation genetic studies aiming to address taxonomic uncertainties, or to study genetic variability and differentiation in other *Indirana* species.

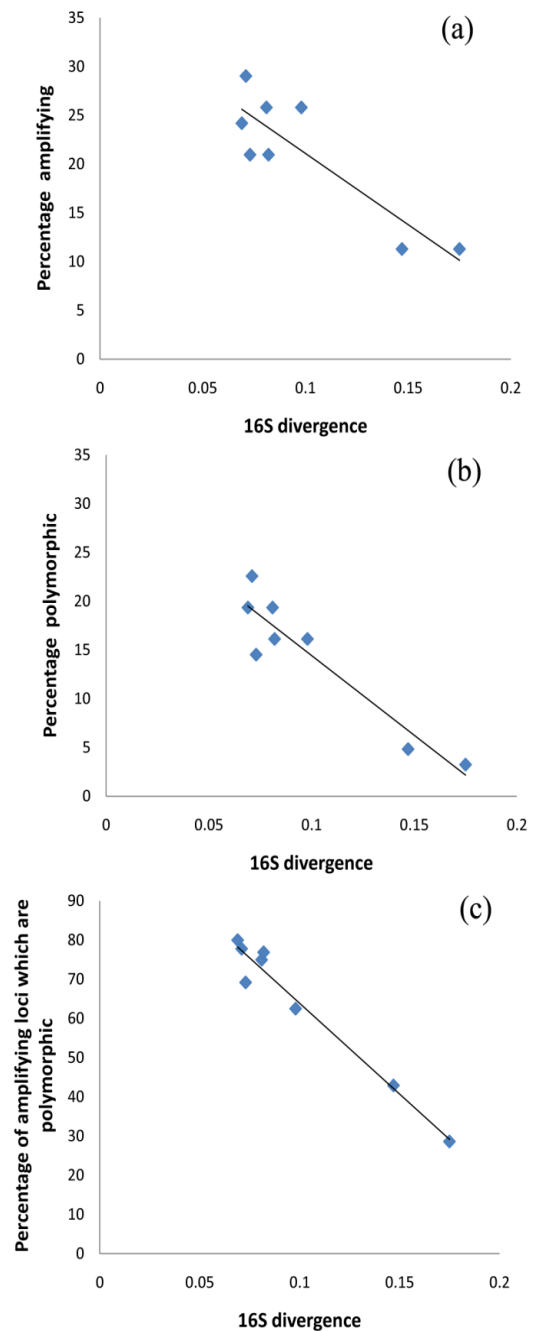


Figure 4. Relationship between genetic divergence (16S divergence) and cross species amplification success and polymorphism of the tested microsatellite loci.

Genetic structure of *Indirana cf beddomii*

The interspecific study revealed high levels of genetic diversity and differentiation, but whether this applies also at an intraspecific level remained unstudied (II). The geographical and ecological discontinuities in the Western Ghats have influenced the distribution and evolutionary history of biota (e.g. Deshpande et al. 2001; Bahulikar et al. 2004; Vidya et al. 2005; Robin et al. 2010), but their impact on population structuring of lower vertebrates has remained largely unstudied.

I found clear structuring in *I. cf beddomii* frogs and existence of three distinct genetic clusters, corresponding to northern, central and southern localities (V). The average F_{ST} estimate for all the populations/localities was 0.075 (S.E.=0.012; $P<0.001$). The pairwise F_{ST} between localities tended to be highest between the northernmost and the southernmost sites, and this was also reflected in strong isolation-by-distance (IBD) patterns ($r=0.85$, $r^2=0.72$, $P<0.001$; Fig. 5). No significant difference was observed in allelic richness among the localities (ANOVA: $F_{11,168}=0.656$, $P=0.778$). Both the levels of observed (mean $H_O=0.783$) and expected (mean $H_E=0.831$) heterozygosities were high and also not significantly different among the localities (ANOVA: $F_{11,168}=0.715$, $P=0.723$). The two southernmost localities were the most divergent, separated from the rest of the region by the 7.5 km wide Shencottah Gap (Fig. 2). The geographic trend in the pattern of genetic differentiation was also evident in the neighbour joining tree, with populations clustering together according to their geographic proximity (Fig. 2 and 6). The STRUCTURE analyses revealed three distinct genetic clusters corresponding to the northern, central and southern localities (Fig. 7). The three genetic clusters could

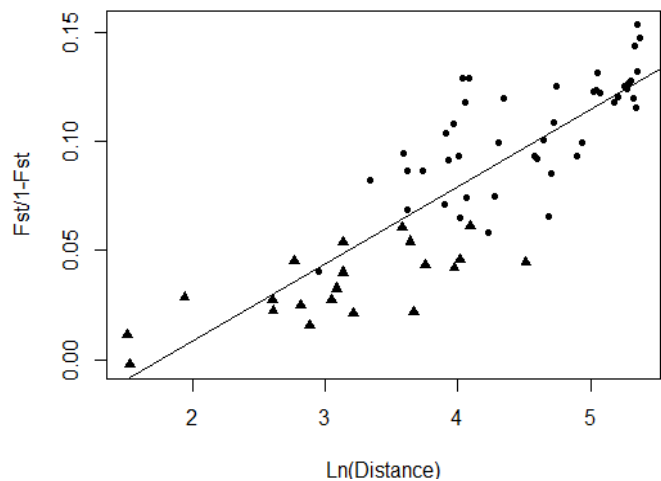


Figure 5. IBD in *Indirana. cf beddomii*. Correlation between genetic distance ($F_{ST}/1-F_{ST}$) and geographical distance (log). The pairwise genetic distances within group (northern, central and southern localities) are indicated by ▲ and those between groups by ●.

be attributed to (i) Anamalais group (Pop 1-6), (ii) Periyar group (Pop 7-10) and (iii) Agasthyamalai group (south of Shencottah Gap; Pop 11 & 12). The Anamalais group and Periyar group were only weakly divergent (Fig. 6, Pop 1–5 and Pop 6–10), whereas the populations south of the Shencottah Gap formed a genetically distinct group. This gap is known to act as a geographical barrier to gene flow and has been identified as a cause of population structuring in other species as well (e.g. Robin et al. 2010).

In a previous study, I found significant genetic differentiation in the morphologically similar *I. cf beddomii* frogs across the Palghat Gap (II), supporting the idea that *I. cf beddomii* has diverged into distinct species across this gap. Therefore it becomes evident that the genetic diversity of these frogs has been influenced by the geographic discontinuities in the Western Ghats. Additionally, the degree of genetic differentiation among localities was strongly correlated with geographical distance separating the localities. This isolation-by-distance pattern suggests the existence of some degree of gene flow among local populations (Fig. 5), but that the impact of this exchange dissipates as the geographic distance increases.

The geographic area covered in this study included some high mountain peaks, but these did not appear to exert any strong influence on population structuring in this species. The results of this study are in accordance with the contention that the low-lying geographical gaps in the Western Ghats might act as barriers for dispersal of amphibians due to the limited period of optimal conditions resulting in isolation across these gaps (Bocxlaer et al. 2012).

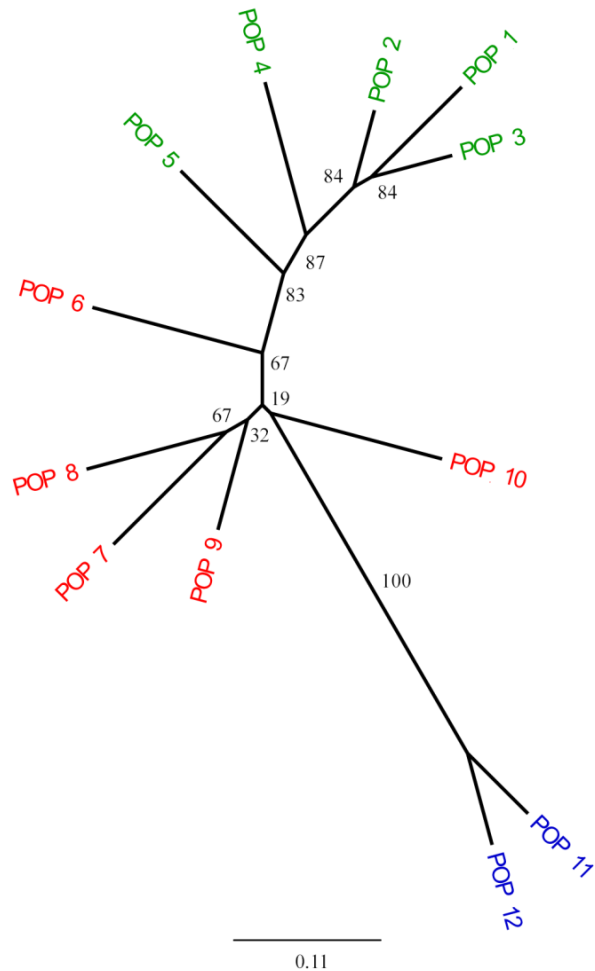


Figure 6. Unrooted neighbour joining tree based on Nei's D_A distances estimated from the microsatellite data. The nodes indicate the bootstrap values over loci (1,000 replicates). The population numbers correspond to localities in Fig 2. Colour of the population abbreviations correspond to Fig. 7.

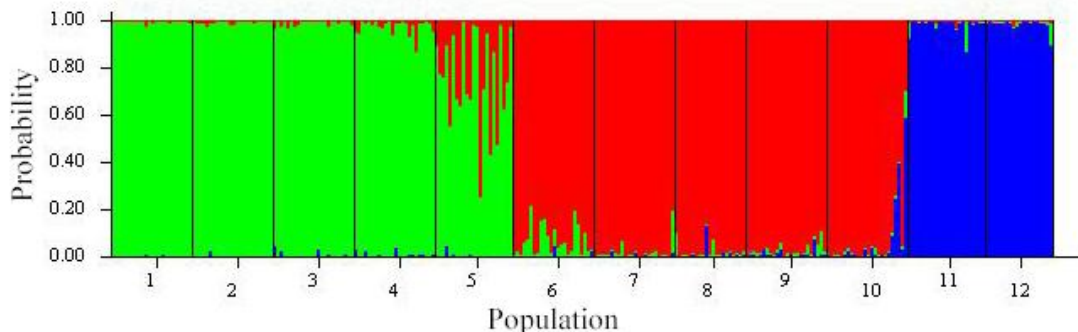


Figure 7. Population clustering as indicated by a structure analyses. The vertical column numbers correspond to populations in Fig 1. The populations are partitioned into three ($K = 3$) clusters.

Amphibian diseases

Batrachochytrium dendrobatidis (*Bd*) and *Ranavirus* are emerging pathogenic infections that have both been implicated in amphibian declines (e.g. Collins and Storfer 2003; Daszak et al. 1999; Schloegel et al. 2010). *Bd* infections have caused large-scale population declines and extinctions in amphibians. *Ranavirus* is also known to cause large-scale mortalities in amphibians in many parts of the world (Gray et al. 2009). To the best of my knowledge, there have been no reports of amphibian disease screening from anywhere in India. We screened for the presence of both *Bd* and *Ranavirus* in species within the endemic genus *Indirana*: *I. beddomii* (from Agumbe, Kudremukh, Aralam, Kanamvayal, Athirapalli), *I. brachytarsus* (Ponmudi, Periyar, Malakapara), *I. semipalmata* (Periyar), *I. diplosticta* (Periyar), *I. leptodactyla* (Munnar) and *Indirana* sp. (Vellarimala). Additionally, Chytrid screening was also done for *Hylarana temporalis*, *Fejervarya keralensis* and *Micrixalus fuscus* from Peppara Wildlife Sanctuary in the Western Ghats (Fig. 8).

Ranavirus was not detected in any of the samples, whereas *Bd* was detected from one specimen of *I. brachytarsus* (mean *Bd* zoospore Genomic Equivalent = 2.92) from Ponmudi in Kerala. This specimen was re-tested in another laboratory and was again found to be positive in both replicates (mean *Bd* zoospore Genomic Equivalent = 0.30). Similar low-level infections of *Bd* (≤ 1 zoospore equivalent) in wild populations of native amphibians have also been reported from Indonesia and China (Changming et al. 2010; Kusrini et al. 2008). The occurrence of *Bd* infection in individuals with no apparent physical abnormalities is also not unusual (e.g. Changming et al. 2010; Kielgast et al. 2010).

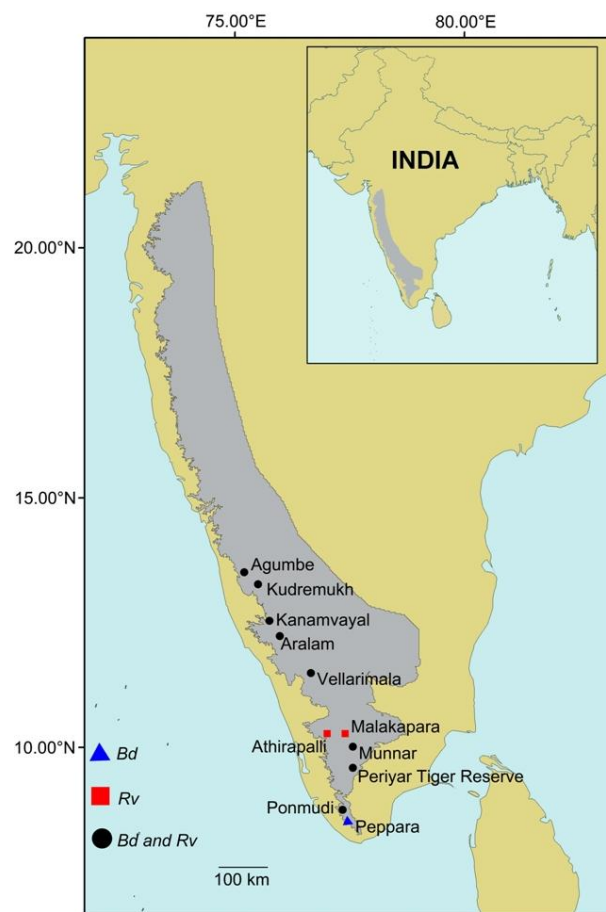


Figure 8. Locations of the sites in India screened for Chytrid (*Bd*) and *Ranavirus* (*Rv*).

Conclusions and future directions

In this thesis, I investigated the genetic diversity and broad patterns of population structuring in *Indirana* frogs. Based on the results in Chapters II and IV, *Indirana* appears to be a morphologically conserved taxa with hidden genetic diversity, as morphologically similar frogs can show a high degree of genetic divergence. I found both *I. beddomii* and *I. diplosticta* to be polyphyletic taxa with a high degree of genetic divergence between each clade, indicating that the species diversity within *Indirana* is currently underestimated due to the existence of cryptic lineages/species. The cryptic lineages within *I. beddomii* are not surprising, considering the fact that this species is thought to be very widely

distributed throughout the Western Ghats (Daniels 2005) and displays large variation both in size and in coloration (Inger et al. 1984; Daniel and Sekar 1989).

The results from these studies have important implications for the conservation of *Indirana* species. The need for conservation in a given species is often assessed on the basis of the IUCN conservation status attributed to it. The recent IUCN report (<http://www.iucn.org>) indicates that 35% of amphibian species from the Western Ghats are data deficient, and 37% of the species are not threatened and are considered of least concern. However, some of the taxa that are believed to be not threatened on the basis of their wide distribution and local abundance could in fact represent several cryptic species. For example, *I. beddomii* is classified as least concern by the IUCN on the basis of its wide geographic distribution. However, my results suggest that '*I. beddomii*' consists of a number of cryptic species, each of which will have smaller population sizes and distribution ranges than the currently recognized *I. beddomii*. Hence, the conservation status of the individual cryptic species should be re-evaluated, and this is also likely to be the situation not only for other species within *Indirana*, but also in other endemic genera in the area (e.g. *Nyctibatrachus*; Biju et al. 2011). This underestimation of the number of species has undoubtedly led to the incorrect assignment of IUCN categories, highlighting a problem with the current categorization system. The identification of species boundaries prior to assigning conservation statuses is clearly a crucial element that is currently compromised by a lack of detailed genetic information. Therefore, the taxonomy of *Indirana* as well as other endemic genera is in need of revision, and molecular methods should be incorporated for correct species delimitation. Detailed genetic studies of amphibians throughout understudied regions are needed in order to re-assess the true species numbers, their

abundance, distributions and conservation statuses.

In chapters **III** and **IV**, I developed genetic resources that could be useful for the genetic studies in this endemic genus as well as in other closely related taxa. In chapter **V**, the results show that the topographic gaps in the Western Ghats do influence the population structuring of montane amphibian species in the Western Ghats biodiversity hotspot. These results help in understanding the genetic diversity of endemic amphibian species unique to the Western Ghats in relation to the geography of the area, and could also provide insights into genetic structuring in other closely related endemic taxa in the area. The study in chapter **V** identified different genetic clusters – some of which should be perhaps treated as different management units. Fragmentation of natural habitats is one of the top threats to biodiversity (Fahrig 2003; Henle et al. 2004) and is known to affect the genetic diversity of populations due to decrease in effective population size and reduced inter-population connectivity (Johansson et al. 2007). The biodiversity of the Western Ghats is also under pressure from anthropogenic activities and has become severely fragmented (Jha et al. 2000; Davidar et al. 2007; Baskaran et al. 2012). Habitat fragmentation causes reduction in genetic diversity and connectivity among amphibian populations (e.g. Andersen et al. 2004; Dixo et al. 2009), therefore rational approaches to conservation and management of biodiversity can benefit from such genetic information. Hence, it is essential to generate information on genetic differentiation in other *Indirana* species covering their entire distribution range. The preliminary screening of *Batrachochytrium dendrobatidis* (*Bd*) in *Indirana* frogs suggests the presence of low level of *Bd* infections in the Western Ghats (**VI**). Therefore, it would also be worthwhile to implement wide-spread screening for *Bd* infections from the entire

range of the Western Ghats biodiversity hotspot.

In general, the information generated in this thesis should be helpful in identifying different management units, and in implementing conservation measures to protect one of the ancient amphibian lineages of the Western Ghats that has a unique evolutionary history (Roelants et al. 2004) and requires urgent protection measures.

Acknowledgements

First of all, I would like to thank my supervisor Prof. Juha Merilä for giving me the opportunity to do my PhD studies in Ecological Genetics Research Unit. I am grateful to you for your valuable suggestions and for guiding me through difficult times in my study period – especially in meetings and in getting things sorted out with the collaborators. I would also like to thank my second supervisor Takahito Shikano for guiding me in the final stages of my studies.

I thank Erica Leder for helping me get started with my work on the project. I am also grateful to Amber Teacher who guided me in the initial stages of my work and for putting me in contact with the Institute of Zoology, London.

I would like to thank Prof. Pekka Pamilo, Perttu Seppä and Meng-Hua Li for giving me valuable suggestions as my thesis advisory committee members.

I thank Marika Karjalainen and Kirsi Kähkönen for being good friends and company in the lab, and for helping me in initiating the genetic work in the MES lab. I would also like to thank MES lab personnel who were involved in processing of my samples: Leena Laaksonen, Minttu Ahjos and Heini Ali-Kovero.

EGRU members of past and present: Meng-Hua Li, Abigél Gonda, Gábor Herczeg, Jussi Alho, Yukinori Shimada, Graciela Sotelo, Cécile Patrelle,

Bineet Panda, John Loehr, Scott McCairns, Tuomas Leinonen, Jacquelin DeFaveri, Nurul Izza Ab Ghani, Kaisa Välimäki, Heini Natri, Niina Nurmi and Kristina Noreikiene – thank you all for your help in various things and for providing a nice working environment. I have pleasant memories of our group meetings and visits to Lammi, COE meetings in Turku and our trip to Estonia. Thank you Jacquelin for doing the English checks for me – usually at very short notice – and for being ready to help me whenever I required. I am also thankful to Scott for his useful suggestions in my studies, as well as for giving advices in dealing with problems pertaining to completion of PhD studies.

I also thank EGRU secretaries, Marika Hölttä, Maarit Haataja and Johanna Paananen, for helping me to deal with the administrative work at various stages of my studies. Marika, I am grateful to you for helping me in getting my immigration issues sorted out. Thank you for going through all the trouble.

I would also like to thank Prof. Veijo Kaitala and Jussi Alho for help with the administrative formalities. I am also thankful to LUOVA coordinator, Anni Tonteri, for helping me and advising me in my studies. You were always available whenever I was in need of help with various things during my studies.

I also thank my colleagues and friends from the Department, Martina, Anton, Christina, Petra and Alex, for being around and for making the department a lively place. I am grateful to my friends and colleagues from various other departments in the University: Pradeep, Kishor, Himanshu, Aniket, Nagabhooshan and Kiran for helping in keeping my mind off work and studies. Thank you for your companionship and support, especially for the cricket matches during short summers of Helsinki.

For funding I thank the Academy of Finland, Department of Biotechnology (Govt. of India), University of Helsinki

thesis finalisation grant and Otto A. Malm foundation. I also thank LUOVA for travel grants given to me for travels abroad for workshops.

My project was the result of collaboration between EGRU and Rajiv Gandhi Centre for Biotechnology in Thiruvananthapuram, Kerala. I am grateful to Dr. Sanil George, Dr. Santhosh Kumar, and Dr. Sathish Mundayoor from RGCB for extending their help and for allowing me to work in RGCB during my visits to Kerala. I am also grateful to Sujith Gopalan for doing extensive sampling in the Western Ghats for my studies – without which the work in this thesis would not have been possible. You were always great company in the field work that I have done in Western Ghats. I also thank here all the people who helped me in sample collections. I thank all my friends from RGCB, I cannot mention all of you here but I would like to specially mention Parvin, Meenakshi, Parvathy, Madhavi, Anil, Suresh, Preeti, Indu, Anish, Anoop, Divya and Sreeja for their help and support extended to me. I am also grateful to my friends from Indian Institute of Science, Bengaluru and University of Delhi: Geetha, Joyshree, Anirudh, Suman, Arvind, Sweety and Vaibhav. Thank you for being my friends and for the fun we had whenever we met.

I extend my gratitude to my teachers from University of Delhi, Prof. T. R. Rao and Prof. Duraiswami, for inspiring and persuading me to do a PhD. The discussions that I have had with both of you have been very useful in channelizing my thoughts. Thank you for motivating and inspiring me to pursue PhD studies.

Finally and most importantly, I want to thank my family for their support and love to me. I thank my father and mother for extending their support and encouragement during my stay away from home. I also thank my brother, Abhishek, for taking care of important matters during my absence. I am most grateful to my wife, Sreesha, for being understanding and

supportive for the period of this thesis when I have been away from you. Thank you for being there whenever I needed you the most, this work would not have been possible without the support and encouragement from you. I apologise for the time that I could not be there with you.

I dedicate this thesis to my father N. Chandran, it was his long standing wish to see me pursue science and complete my doctoral studies.

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