DOCTORAATSPROEFSCHRIFT

2009 | Faculteit Wetenschappen

Molecular phylogenetics and biogeography of the Old World true freshwater crabs, with emphasis on the fauna of Sri Lanka

Proefschrift voorgelegd tot het behalen van de graad van
Doctor in de Wetenschappen, richting biologie, te verdedigen door:

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Natalie Beenaerts

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22 December 2009

Acknowledgments....

First pages...last words...and probably the only chapter that everybody will read till the end...

Most of my family, friends and colleagues have always - somehow - believed in my capacities to finish my 'research' (as I called it for years, not having the guts to say 'PhD') successfully, often more than I did myself. I gladly admit they were right. Even at this moment, I have a hard time realizing ...I did it!!!

These same family members, friends and colleagues – some almost friends ☺, and some definitely a combination of the three categories - deserve at least a word of gratitude from me. I couldn't have done it without them and I wish to thank all of them at this stage without personalizing (this way I cannot forget anybody). Thank you all!!!!!!

There are however a couple of people that I need to thank in person. First, I would like to thank the co-authors of the publications in this work, but also my jury members for their valuable remarks and comments made to the earlier version of this work.

Then there is Franky, my co-promoter. Several years ago, after brainstorming a couple of days and over many coffees (no alcohol of course ☺), we reached a consensus. I decided to temporarily leave my 'marine research' ideas. Franky decided to put his toe in the water (and he hates water) and take me on in his brand new lab, so I could start working on the freshwater crabs of Sri Lanka. I'm sure he has often regretted the fact that he didn't put more effort in talking me into 'frogs', but he was still young and less determined, and I was not going to give in completely. Franky, you managed to get more out of me than I thought I had in me and made me reach higher. You know I admire your scientific know-how and, art to tell it (mainly on paper ☺). After all these years, we're still friends and I hope to keep it that way for a long, long time. Thanks, Miester!

Staying in the same lab, Kim (Kemstje) deserves and has as much of my devoted (scientific) admiration as Franky. Next to your endless knowledge and (luckily for me) willingness to share it, we also laughed, gossiped and made fun in our mutual years in the lab, didn't we. Growing up was never our thing. I'm sure we will always be (almost ☺) friends, but Montse and me will have to arrange the future family get-togethers, I fear. Kim, I owe you!

Of course, I wouldn't dare to forget An. We had lots of fun, supported each other and kept the boys with their visions on Earth. Meanwhile you have Ines and Vietmans (Wim) to replace me. Worthy replacements, I might add.

Traveling east, back to my roots, I also need to thank a couple of people. First of all, there is Tom. He convinced me to get my act together, take some time off from my regular job and take 'my research' all the way...to the end. He promised to help and motivate me, and so he did. Your door was always open to give me advice, to discuss, or just to listen to my uncertainty or new ideas. Tom you are a good mentor and friend.

I know by now that universities are filled with very intelligent people, but every now and then, some of them really astonish me. Geert Jan, is one of them. His interest in and knowledge of all aspects of sciences is just amazing. Many thanks for all the help with the Pearl-problems and 'cluster' assistance.

And then there is Jean. I don't owe you an immediate scientific word of gratitude, but I definitely owe you lots of gratitude. Without your belief in me, your patience - because the last months I was not that concentrated on the job, was I -, and your incredible optimism, I couldn't have managed to finish this work in time! Jean, I will make up for it in the future...we'll make an ever better tandem.

Most of my family, friends and colleagues only occasionally had to experience my doubts, nerves and moods (regarding my scientific endeavors, that is)...still they had the chance to walk away whenever they wanted. Luckily, I never had that feeling. However, there are a few people that stood by me throughout this entire episode of my life and also deserve a little personal attention at this stage. Those are old friends (as I promised no names!), my parents, my brother and sister-in-law, Kris(tof) and Anja, my sweet god-child and his sis, Robbe en Sam, and last but by no means least, my 'rock', my best friend, my love, the best father for my children, the ever optimistic Yves. He organized our lives for the past year(s) – he loves it though – and kept me from dreaming and forgetting the things in life that matter most. I will be more 'present' in the future and hope to do lots of fun, great stuff together. But above all, I already promised my two little, - oh so - patient, sweethearts, Jonas and Anke, to make up for the lost time, especially during the last year. I'll definitely keep this promise. We'll start immediately...

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given enough time, many things that seem unlikely can happen...

Darwin

SAMENVATTING

Met ongeveer 6800 beschreven soorten, zijn de Brachyura of de 'echte' krabben de meest soortenrijke groep van de Decapoda (kreeften, garnalen, krabben, ...). Hiervan zijn ongeveer één vijfde (~1280 soorten) *echte* zoetwaterkrabben. Ze behoren tot vier superfamilies: Gecarcinucoidea, Potamoidea, Pseudothelphusoidea en Trichodactyloidea (Cumberlidge et al., 2008).
Tijdens de laatste twintig jaren waren de taxonomie en de systematiek van de zoetwaterkrabben onderhevig aan grote veranderingen, met vele nieuwe soortbeschrijvingen. Diagnostische morfologische kenmerken worden herbekeken op alle niveaus (van superfamilie tot soortniveau). De zeer beperkte fossiele bestanden en de onbekende mariene zustergroep(en) en ancestrale taxa belemmeren de reconstructie van fylogenetische relaties tussen de zoetwaterkrabben. Moleculaire technieken en fylogenetische modellen zijn zeer belangrijk gebleken voor evolutionaire biologie, die van de zoetwaterkrabben inbegrepen. Met behulp van moleculaire technieken tracht ik de fylogenie van de *echte* zoetwaterkrabben uit de Oude Wereld, op te helderen en benader ik andere evolutionaire vraagstukken die verband houden met historische biogeografische gebeurtenissen, en meer specifiek voor Sri Lanka vraagstukken rond biodiversiteit en natuurbehoud. Alvorens de lezer onder te dompelen in deze wereld, laat ik hem eerst kennis maken met de diergroep in kwestie, haar verspreiding, de globale geologische historische gebeurtenissen, het biodiversiteit 'hotspot' concept (hoofstuk 1) en een aantal toegepaste methoden (hoofdstuk twee).

In hoofdstuk drie, stel ik een multi-locus dataset samen van 228 zoetwaterkrabbensoorten, hoofdzakelijk van de Oude Wereld. Na een eerste voorbereidende analyse, selecteer ik 107 soorten, waarvan 102 Oude Wereld soorten. Dit hoofdstuk biedt buiten een omvangrijk fylogenetisch kader van de zoetwaterkrabben van de Oude Wereld, ook een verklaring binnen een tijdskader voor de diversificatie en huidige distributie van de zoetwaterkrabben. Uit de fylogenetische resultaten van deze studie leid ik af dat de drie grote zoetwaterkrabbenfamilies van de Oude Wereld een monofyletische groep vormen. Bovendien, toont deze studie ook aan dat de huidige verspreiding van

de zoetwaterkrabben waarschijnlijk een gevolg is van post-Gondwanaanse diversificatie met frequente overzeese dispersie.

In hoofdstuk vier benadruk ik de ogenschijnlijke overeenkomsten tussen de fauna van Sri Lanka en die van de 'western Ghats' op het Indische schiereiland. Deze biologische overeenkomsten werden verklaard door het bestaan van een landbrug tussen Sri Lanka en het schiereiland meerdere keren in het Pleistoceen. Tijdens deze periodes van laag zeeniveau was er migratie naar en van het schiereiland mogelijk. Samen met collega's, gebruik ik de moleculaire fylogenieën van vier vertebratengroepen en twee invertebratengroepen om aan te tonen dat dispersie tussen India en Sri Lanka zeldzamer is dan voorheen aangenomen werd. Ondanks de verschillende periodes dat Sri Lanka door een landbrug verbonden was met India, bleef de fauna zeer verschillend van die van India. Omwille van de opvallende morfologische gelijkenissen tussen de fauna's van de twee landmassa's, is hun substantiële genetische differentiatie ontsnapt aan de aandacht van biologen en natuurbeheerders. Onze bevindingen belichten het belang van minder opvallende intrinsieke en extrinsieke factoren als significante drempels voor dierendispersie, en sporen aan tot de erkenning van Sri Lanka als een unieke biodiversiteits-'hotspot'.

In hoofdstuk vijf concentreer ik me op de zoetwaterkrabben als mogelijke indicatoren voor het behoud van de biodiversiteit van Sri Lanka. Ik onderzoek de biodiversiteit van deze krabben in verhouding tot de verschillende hoogtezones (laagland, tussenzone ('upland'), hoogland), gebaseerd op zowel soortenrijkdom als fylogenetische diversiteit. De uitgebreide radiatie van de Sri Lankese zoetwaterkrabben (d.w.z. 51 soorten, waarvan 50 endemische) heeft met succes de vochtige habitatten en alle klimaat- en hoogtezones van Sri Lanka gekoloniseerd. Drie verschillende lijnen hebben schijnbaar simultaan op de drie verschillende zones geradieerd, met weinig tot geen uitwisseling hierna. De hoogland en 'upland' zones vertonen een hogere soortenrijkdom, terwijl – onverwacht – de fylogenetische diversiteit het hoogst is in het laagland, wat het belang voor het overwegen van beide metingen in het natuurbehoud illustreert. In elk van de drie zones suggereren de diversiteitsindices voor de soorten in de verschillende IUCN Red List categorieën, dat het gevaar voor uitsterven verband

zou kunnen hebben met de hoogtezones. Deze resultaten tonen ook aan dat er meer dan 50% van de Sri Lankese zoetwaterkrabbensoorten (inclusief nog onbeschreven soorten), of ongeveer 72 miljoen jaar aan evolutionaire geschiedenis, bedreigd is met uitsterven.

Tijdens mijn inspanningen om bovenstaande vragen te beantwoorden, zijn er onvermijdelijk nieuwe vragen naar boven gekomen. Een aantal daarvan heb ik als potentiële toekomstige onderzoeksonderwerpen in een laatste hoofdstuk (hoofdstuk zes) kort omschreven.

SUMMARY

With about 6,800 species described , the Brachyura or true crabs are the most species-rich group of Decapoda, such as lobsters, crabs and shrimp (Ng et al. 2008; De Grave et al. 2009). An astonishing one fifth (~1280 species) of those crabs are *true* freshwater crabs, belonging to four superfamilies: Gecarcinucoidea, Potamoidea, Pseudothelphusoidea and Trichodactyloidea (Cumberlidge et al., 2008).

During the past twenty years, freshwater crab taxonomy and systematics have undergone major changes, with many new species described. Diagnostic morphological characters are re-weighted at all levels (from superfamily to species). The scant fossil record and the unresolved marine sistergroup(s) and ancestors hamper the reconstruction of freshwater crab relationships. The growth of molecular tools is of great importance for evolutionary biology, including freshwater crab evolutionary history. In this study, I use molecular tools to elucidate the phylogeny of the Old World *true* freshwater crabs, and address other evolutionary questions that pertain to past biogeographical events and with Sri Lanka in particular, questions concerning biodiversity and conservation issues. Before dropping the reader into that world, I briefly introduce the faunal group, its distribution, past global geological events, the concept of biodiversity hotspots (chapter one) and some methods used (chapter two).

In chapter three, I compile a multi-locus data set of 228 specimens of freshwater crabs mainly from the Old World. After a preliminary analysis on this data set, we selected a data set of 107 species, of which 102 were Old World freshwater crab species. This chapter provides a comprehensive phylogenetic framework and explains within a temporal framework the freshwater crab diversification and contemporary distribution. The phylogenetic results of this study infer the monophyly of the three major Old World freshwater crab families. Moreover, this study demonstrates that the contemporary freshwater crab distribution is a consequence of post-Gondwanan diversification with frequent oceanic dispersals.

In chapter four I focus on the apparent similarities between the Sri Lanka fauna and the fauna of the Western Ghats, Indian peninsula. The regular land connections between Sri Lanka and the Indian peninsula in the Pleistocene have been used to explain these biological similarities, because during periods of low stand, migration to and from the mainland was possible. I use molecular phylogenies of two vertebrate and four invertebrate groups[1], and demonstrate, that dispersal between mainland India and Sri Lanka has been much more limited than was assumed. Despite several periods in which Sri Lanka was connected by a land bridge to the Indian Subcontinent, it maintained a fauna that is largely distinct from that of the Indian mainland. Due to the striking morphological resemblance between the mainland and Sri Lankan faunas, the substantial genetic differentiation has escaped the attention of biologists and conservation managers. Our findings highlight the importance of less conspicuous intrinsic and extrinsic factors as significant barriers to animal dispersal and prompt recognition of Sri Lanka as a unique biodiversity hotspot.

In chapter five, I focus on the freshwater crabs as possible important indicators for biodiversity conservation on Sri Lanka. I assess the biodiversity of these crabs in relation to the different elevational zones (lowland, upland and highland) based on both species richness and phylogenetic diversity. The extensive radiation of freshwater crabs on Sri Lanka, i.e., 51 species (50 of them endemic), successfully colonized most moist habitats and all climatic and elevational zones in Sri Lanka. Three different lineages appear to have radiated simultaneously, each within a specific elevational zone, with little interchange thereafter. The lowland and upland zones show a higher species richness than the highland zone while—unexpectedly—phylogenetic diversity is highest in the lowland zone, illustrating the importance of considering both these measures in conservation planning. The diversity indices for the species in the various IUCN Red List categories in each of the three zones suggest that extinction risk may be related to elevational zone. Our results also show that overall more than 50% of Sri Lanka's freshwater crab species (including several as yet undescribed

[1] It was only possible to cover this large set of faunal groups through collaboration with other research groups. I covered the freshwater crabs and the freshwater shrimps.

ones), or approximately 72 million years of evolutionary history, are threatened with extinction.

In my efforts to unravel the above questions, I evidently stumbled on new questions, which would be interesting to tackle in the future. Some of those are mentioned at a glance in the last chapter (chapter six).

Chapter 01

Introduction

1. INTRODUCTION

There is no better year to finalize this study than the international Darwin year. Evolution is after all the keyword throughout this work! Between the start and 'end' of this project not only my knowledge on molecular systematics, biogeography, phylogenetic diversity and so much more increased spectacularly (at least in my own view, that is), but also a scientific world opened up that was previously poorly explored and unknown to me. Over these past years this research field also evolved continuously, with computationally faster and more complex methods, as there are: multiple sequence alignment methods, methods to estimate nucleotide evolution, statistical methods to infer phylogenetic relationships, etc.

When trying to unravel the past evolution using molecular phylogenies or to assess phylogenetic diversity, there are certainly easier groups to study than freshwater crabs. Moreover, the main actors in a major part of this study are living in a relatively understudied area[1], the beautiful island Sri Lanka. This study describes in three chapters how we addressed a number of evolutionary questions using molecular phylogenetics, (1) is the contemporary distribution of freshwater crabs a consequence of vicariance through continental drift or rather a consequence of oceanic dispersal; (2) is the fauna of Sri Lanka very similar to the fauna of the Western Ghats, Indian peninsula and were the Pleistocene land bridges indeed gateways for much faunal interchange; (3) can the different elevational zones be related to phylogenetic diversity and therefore the evolutionary history of the freshwater crabs? The choice of this study group stems from the initial research area of the 'amphibian evolution lab' at the VUB. At first the focus was on diversity, biogeography, etc of the amphibians of the Indian subcontinent. The collaboration between researchers from Sri Lanka and this lab was extended by expanding the faunal groups of interest to freshwater

[1] Compared to other regions the geological past, the morphological geography, etc. are less studied.

crabs[2]*. Later on, the crabs remained my study object and the field of interest expanded.*

In the overview below I gathered substantial information on the group and area of interest. Also a brief introduction on the tectonic drift theory and biodiversity hotspot concept is added to clarify the further chapters. In a separate chapter, a concise description of various materials and methods is provided.

1.1. The group under study: freshwater crabs

1.1.1. Introduction

Currently 1.9 million animal and plant species have been described worldwide (Chapman, 2009)[3]. Crustaceans are a group with an enormous morphological diversity (disparity) and consist of at least 68,000 species (Brusca & Brusca, 2002). Within this group, the Decapoda (such as the lobsters, crabs and shrimps) are probably the most studied group, also because of their economic importance. Consisting of about 6,800 species, the Brachyura (Ng et al. 2008) or true crabs are the most species-rich group of Decapoda (De Grave et al. 2009). An astonishing one fifth (~1280 species) of those crabs are *true* freshwater crabs, belonging to four superfamilies: Gecarcinucoidea, Potamoidea, Pseudothelphusoidea and Trichodactyloidea (Cumberlidge et al., 2009).

In this study we use the term freshwater crab solely for the *true* freshwater crabs, which are entirely restricted to freshwater habitats. The reproduction is adapted to freshwater: they produce a small numbers of large, yolky eggs, have direct development (no larval stages) and have (mostly) brood-care (Sternberg and Cumberlidge, 1999, Yeo et al., 2008, see Cumberlidge & Ng, 2009 for more

[2] Chapter 4 includes more faunal groups. I performed also the analyses on the freshwater shrimps.

[3] Estimates are that between 5 and 50 million different species exist, depending on the authors.

references). Adult males use two pairs of modified abdominal appendages, the gonopods, as copulatory structures to pass the sperm from the penises to the female sexual opening (Cumberlidge, 1999; Yeo et al., 2008). This structure is used as an important taxonomic feature (see later). Other groups called 'freshwater crabs', such as Aeglidae (Anomura-Bond-Buckup, 2008) and Varunidae (Ng, 1988, 2004), need to return to the marine environment at some stage in their life cycle to reproduce. There are two exceptions – the genus *Geosesarma* from South-East Asia (Ng, 1988) and various endemic Sesarmidae (genera *Sesarma* and *Metopaulias)* from Jamaica (Schubart & Koller, 2005) that are completely undependable from the marine environment. Some of these sesarmids even show direct development. The body plan of freshwater crabs is similar to that of other brachyuran crabs, with a head and thorax covered by a carapace, and a reduced abdomen, folded under the thorax. Most freshwater crabs have nine pairs of gills underneath the carapace, just as most marine crabs.

True freshwater crabs are found in rivers and lakes of rainforests, savanna, mountain streams, and even in caves throughout the (sub)tropics worldwide (Sternberg et al. 1999, Yeo et al., 2008; Cumberlidge & Ng, 2009). They are present on the mainland, but also on many islands (see more in section '*Island biogeography and colonization*'). Many true freshwater crabs are semi-terrestrial to terrestrial (i.e., in the latter case they take their water from drinking or air humidity). Although they are highly restricted to freshwater conditions, some lowland species are known to withstand partial desiccation or tolerate salt water for a short period (Bott, 1970a, Morris and Van Aardt, 1998; Esser, 2007). Most freshwater crabs are thought to be nocturnal and scavengers (Ng, 1988).

It is only during the last decades that the importance of freshwater crabs as possible bio-indicators is acknowledged (Ng & Rodriguez, 1995). Almost all require pristine water to survive and thus excellent indicators of water pollution (Yeo et al., 2008). In addition, their importance in the food chain (from the main component of the diet for some species of otters to part of the diet of lizards, eels, birds and humans), and nutrient recycling has also been highlighted

recently (see Cumberlidge et al., 2008; Dobson et al., 2002).

1.1.2 Freshwater crab taxonomy

The last 20 years have been dynamic decades for freshwater crab taxonomy at all levels. Early in the twentieth century over 300 species of freshwater crabs were described, including 41 species described by Alcock and 99 species described by Rathbun (e.g. Alcock, 1909, 1910, Rathbun, 1904-1908). In addition, Rathbun's (1904-1906) 'Les crabes d'eau douce' may have been the first worldwide work. Bott (1950, 1969, 1970a, b) compiled the most recent comprehensive work including more than 400 species worldwide.

The renewed interest in freshwater crabs has lead to an enormous increase in the collection and description of new taxa, followed by many taxonomical revisions. Moreover, the recent molecular phylogenies and improved morphological techniques have resulted in major re-assessments of diagnostic morphological characters. In freshwater crabs, only a small number of morphological characters have been used as taxonomic criteria, especially at the family-level (Bott, 1970a; Ng, 1988). These are mainly the structures of the mandibular palp, the frontal median triangle and the external male reproductive organs (gonopod one and two). The number of segments and the shape of the terminal segment were for instance used as criteria for the mandibular palp disparity at family level, but within the Potamidae these are highly variable (Cumberlidge & Ng, 2009). The presence/absence and the shape of the frontal median triangle has been used to differentiate between Gecarcinucidae and Parathelphusidae. However, Parathelphusidae have now been synonymized with Gecarcinucidae (Klaus et al., 2009). The utility of the frontal median triangle at family level was already questioned thirty years ago by Holthuis (1979), a doubt shared by others (e.g., Ng, 1988, Ng & Stuebing, 1989, Ng & Sket, 1996). The use of features pertaining to the gonopods as a higher-level diagnostic character remains valid. For the gonopods, especially the number of segments and form of the distal segment are used as diagnostic characters. For instance, the Potamidae, Potamonautidae and Gecarcinucidae share a four-segmented G1

compared to the three-segmented G1 of Pseudothelphusidae (Rodriguez, 1992). The variability of G1 is adequate enough to distinguish between the first three families (Cumberlidge & Ng, 2009). A careful use of the gonopod structures, especially gonopod 2, also allows for taxonomy in some cases (Klaus et al., 2006, 2009). Alternative synapomorphic characters for the Potamoidea and Trichodactylidae have been reviewed in Cumberlidge and Ng (2009).

However, the validity of the above characters as diagnostic characters, and other characters at the genus- and species-level have been heavily disputed[4], resulting in considerable taxonomical instability. Table 1 presents an overview of the taxonomical changes at the family and superfamily level from Bott, 1970 till now (Cumberlidge and Ng, 2009). The number of families recognized has been reduced from as high as twelve (Bott, 1970) to five (Cumberlidge and Ng, 2009; Klaus et al., 2009).

Table 1. Recent changes in the higher taxonomy of the true freshwater crabs (after Cumberlidge and Ng, 2009)

A. According to Bott 1970b and Cumberlidge 1999

Pseudothelphusoidea Ortmann, 1893
- Pseudothelphusidae Ortmann, 1893
- Potamocarcinidae Ortmann, 1897

Potamoidea Ortmann, 1896
- Potamidae Ortmann, 1896
- Potamonautidae Bott, 1970
- Deckeniidae Ortmann, 1897
- Platythelphusidae Colosi, 1920
- Sinopotamidae Bott, 1970
- Isolapotamidae Bott, 1970

Gecarcinucoidea Rathbun, 1904
- Gecarcinucidae Rathbun. 1904
- Parathelphusidae Alcock, 1910
- Sundathelphusidae Bott, 1969

Portunoidea Rafinesque, 1815
- Trichodactylidae H. Milne Edwards, 1853

[4] Distribution often played a major role in creating the families, as is very obvious in Potamidae.

B. According to Martin & Davis 2001

Pseudothelphusoidea Ortmann, 1893
- Pseudothelphusidae Ortmann, 1893

Potamoidea Ortmann, 1896
- Potamidae Ortmann, 1896
- Potamonautidae Bott, 1970
- Deckeniidae Ortmann, 1897
- Platythelphusidae Colosi, 1920

Gecarcinucoidea Rathbun, 1904
- Gecarcinucidae Rathbun. 1904
- Parathelphusidae Alcock, 1910

Portunoidea Rafinesque, 1815
- Trichodactylidae H. Milne Edwards, 1853

C. According to Cumberlidge et al. 2008 and Ng et al. 2008

Pseudothelphusoidea Ortmann, 1853
- Pseudothelphusidae Rathbun, 1893

Potamoidea Ortmann, 1896
- Potamidae Ortmann, 1896
- Potamonautidae Bott, 1970

Gecarcinucoidea Rathbun, 1904
- Gecarcinucidae Rathbun, 1904
- Parathelphusidae Alcock, 1910

Trichodactyloidea H. Milne Edwards, 1853
- Trichodactylidae H. Milne Edwards, 1853

D. According to Cumberlidge and Ng, 2009

Potamoidea Ortmann, 1896
- Pseudothelphusidae Rathbun, 1893
- Potamidae Ortmann, 1896
- Potamonautidae Bott, 1970
- Gecarcinucidae Rathbun. 1904

Trichodactyloidea H. Milne Edwards, 1853
- Trichodactylidae H. Milne Edwards, 1853

The five freshwater crab families sensu Cumberlidge and Ng (2009) are presumably two monophyletic lineages. One lineage is the New World family, Trichodactylidae H. Milne Edwards, 1853. The other lineage consists of one New World family, Pseudothelphusidae Ortmann, 1893 and three Old World families, Potamidae Ortmann, 1896, Potamonautidae Bott, 1970 and Gecarcinucidae Rathbun, 1904. This latter lineage is now proposed as the superfamily,

Potamoidea Ortmann, 1896 (Cumberlidge & Ng, 2009). It is this Potamoidea, which is the subject of our study.

1.1.3 Phylogeny and distribution

The above-mentioned proposal of two monophyletic lineages and their internal relationships is the most recent view in a long series of hypotheses. At the beginning of this study the debate dealing with monophyly, polyphyly or paraphyly of the freshwater crabs was definitely less resolved than it is now. As we will show in chapter 3, more evidence is definitely necessary; the debate is far from finished.

Towards the end of this study and to a certain degree throughout this work, a consensus seems to be growing as to the freshwater crab relationships at the family level (molecular contributions are e.g., chapter three; Klaus et al., 2006, 2009; Daniels et al., 2006) and at the genus- and species-level for several taxa (e.g., Chapter four and five). However, the deepest relationships remain difficult to resolve, because the closest sistergroup or -groups of the true freshwater crabs remain elusive. To this date, there is still no consensus on the phylogenetic relationships within the true crabs or Brachyura (Ahyong et al., 2007 and references herein), because the use of morphological, fossil and molecular[5] markers infer contradicting phylogenies. Moreover, few studies (if any) included freshwater crab taxa.

[5] Even within molecular studies the different use of molecular markers causes contradictory phylogenies, but more on that in Material and Methods.

Fig. 1: distribution of true freshwater crabs

The global distribution of the 5 families is presented in Fig. 1. The (sub)tropical New World is home to two freshwater crab families: the Trichodactylidae and the Pseudothelphusidae. The Old World harbors the other three families. The Potamonautidae are restricted to the Afrotropical landmasses and islands. The Potamidae are divided in three zoogeographical regions (according to Cox, 2001): the Palaeartic region, including Europe and mainland Asia, the Oriental and the Afrotropical region. Members of the Gecarcinucidae are found in the Palaeartic and the Oriental region, but also occur in the Australasian region. Most freshwater crab species have narrow distributions and are thought to have poor dispersal capabilities. These features combined with their low fecundity and direct development, have lead to high levels of endemism (Chapter 3, 4 and 5 of this study, Yeo et al., 2008; Bossuyt et al., 2004; Shih et al., 2007; Schubart & Ng, 2008; Cumberlidge et al., 2009, Cumberlidge & Daniels, 2008). Most of their speciation has been attributed to allopatric speciation, but sympatric speciation has also been described (Marijnissen et al., 2006).

The unexpected enormous diversity, the high level of endemism and the growing threats to their typical habitats - (sub)tropical forest and aquatic ecosystems – have prompted researchers to assess their conservation status (Cumberlidge et al., 2009). In chapter 5 we investigate the importance of the freshwater crabs in conservation using species richness and phylogenetic diversity (PD), expressed

as evolutionary history, distributed over the elevational zones of Sri Lanka.

The increasing knowledge on taxonomy, phylogenetic relationships and contemporary distribution also motivated researchers to adjust their hypotheses on the origin and historical evolutionary relationships of freshwater crabs (Daniels et al., 2006; Klaus et al., 2006; 2009; Cumberlidge et al., 2008; Cumberlidge & Ng, 2009; Shih et al., 2009). Chapter 3 of this study is the first comprehensive phylogenetic study on Old World freshwater crabs that allows for an historical inference on a global scale.

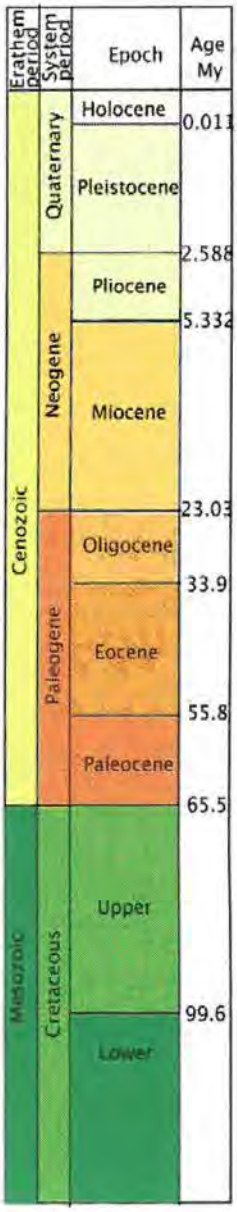
1.2. The adventures of the freshwater crabs: historical biogeography

1.2.1 Plate tectonics

To explain the current circumwide (sub)tropical distribution of the freshwater crabs, essentially two scenarios have been postulated. One implies migration, including transoceanic dispersal, which is not so straightforward for freshwater animals. The other is the vicariance drift scenario, which implies that the freshwater crabs are already an ancient lineage and geographical isolation has occurred through separation and drifting of the continents due to plate tectonics. The plate tectonic scenario outlined below, starts only at about 175 Mya, because the freshwater crabs most probably originated later in time (Feldmann et al., 2007; Sternberg and Cumberlidge, 2001; Ng et al, 1995; Porter et al., 2005). Fig. 3 illustrates the plate tectonic history outline described further in this chapter. Before 175 Mya, all landmasses were united in a supercontinent **Pangea**. The northern landmasses, **Laurasia**, began to drift from the Southern continental landmass, **Gondwana** in the Middle Jurassic (around 175 Mya; see Fig. 2). There is no extinct or extant evidence that freshwater crabs ever occurred in North America, which separated from the Eurasian continent during the Mid Cretaceous, or in the northern parts of Europe and Asia. In what follows, we focus on the Old World freshwater crabs.

Shortly after the break-up of Pangea, about 165-155 Mya ago (Briggs, 2003; Schettino & Scotese, 2005), Gondwana started to divide in **western and**

eastern Gondwana, consisting of South-America-Africa and Antarctica-Australia-India-Madagascar-Seychelles, respectively (Briggs, 2003).



Reconstructed from ICS Chart 2009

Fig 2: Geological times scale from the Cretaceous till now

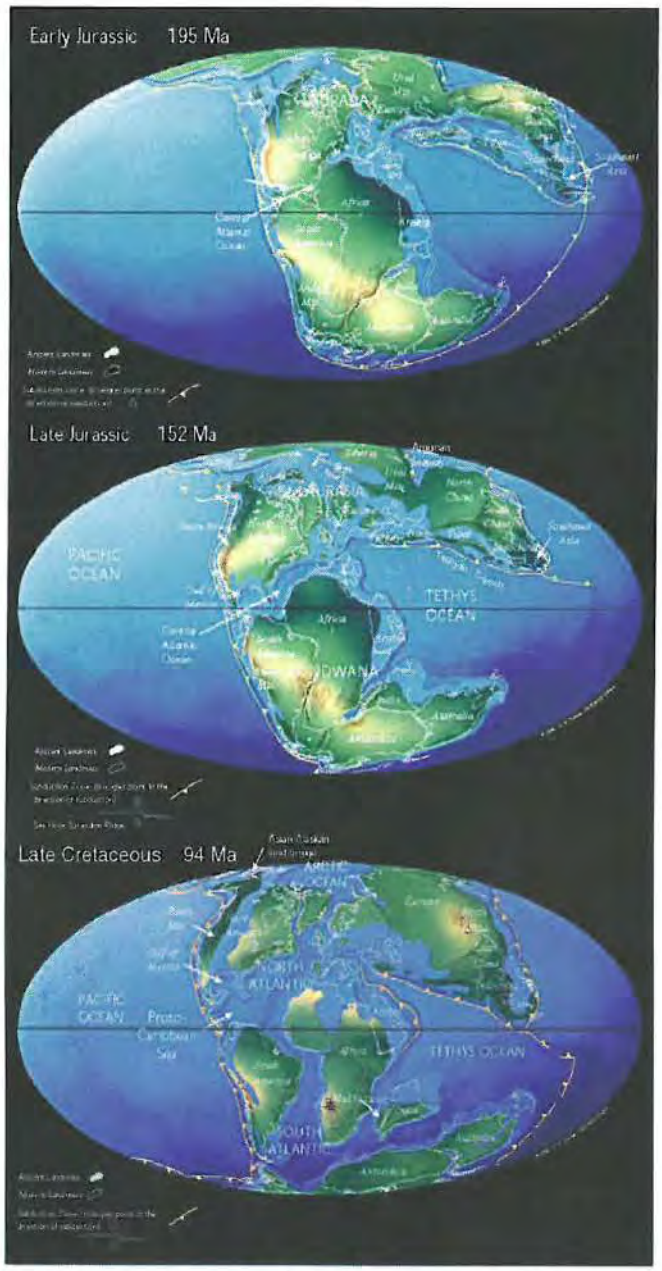


Fig. 3: Plate tectonics from 66 Mya till 14 Mya (source: Scotese, 2001)

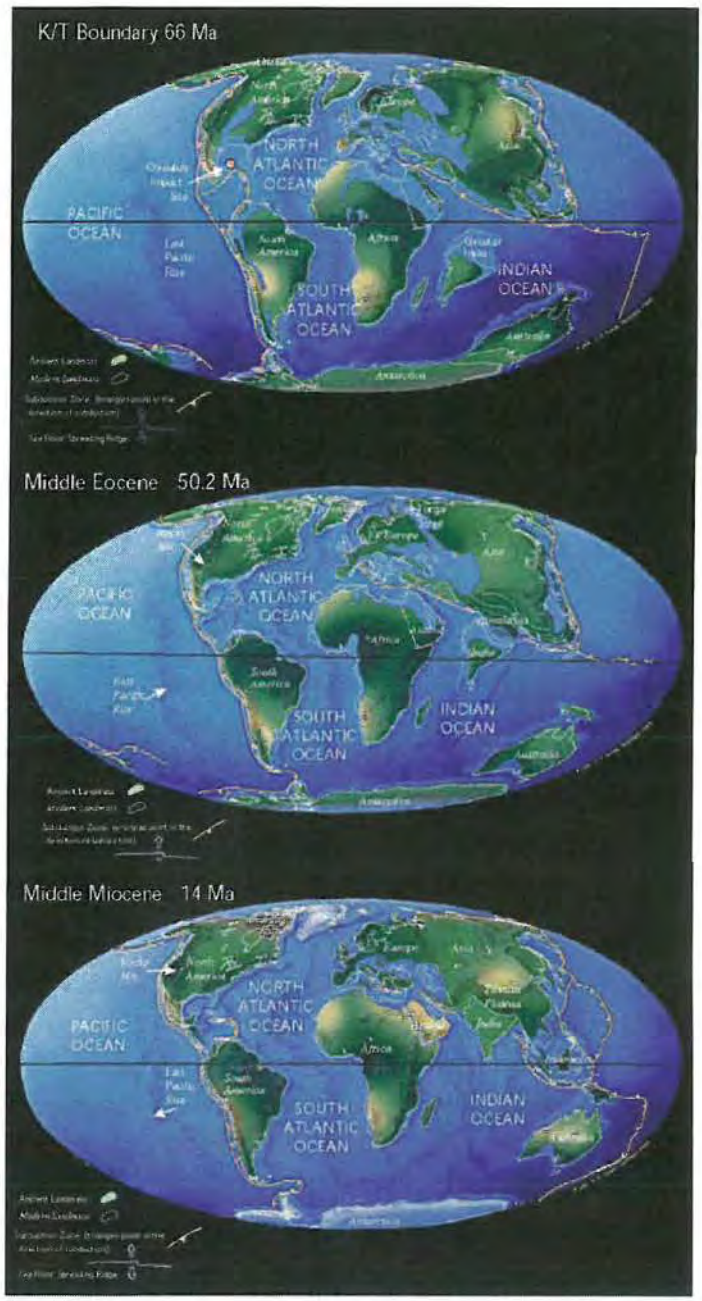


Fig. 3: continued – plate tectonics from 66 Mya till 14 Mya (Scotese, 2001)

Eastern Gondwana started to break up in the Mid-Cretaceous. Africa drifted slightly northward and South America started drifting more westward, and a little southward. The Indian/Madagascan block started drifting from the Antarctica-Australia block in the Early Cretaceous (around 135 mya; Powell et al., 1988; Brown et al., 2003). The former simultaneously drifted away from the African continental mass. Madagascar reached its current position in relation to Africa around 130-118 Mya (Harland et al, 1990, Seward et al., 2004). The separation between the Seychelles-India landmass and Madagascar happened between 100 and 88 Mya ago (100-95 according to Plummer, 1996; 97,6-80,3 according to Valsangkaret al., 1981; 91,2-88 according to Torsvik et al, 2000 and 87,6 according to Storey et al., 1995). The Seychelles-India landmass started drifting northeasterly, away from Madagascar, and separated from the Seychelles islands around 70 Mya. According to the traditional tectonic scenarios, India collided with Eurasia in the Paleocene (about 55 – 50Mya; e.g., Leech et al., 2005; Zhu et al., 2004), allowing overland dispersal between India and Eurasia from then on (see Fig. 4). Recent evidence provides a revision of this Indian plate collision. The collision of Greater India started indeed around 50 Mya, but the earliest period in which biological exchange could have been possible was presumably much later than 35 Mya (Ali & Aitchison, 2008).

Fig. 4: Drift and collision of Greater India with Eurasia (source: classroomsea.net)

Meanwhile, from 135 Mya onward (Briggs, 2003), the Antarctica-Australia block began drifting southward. This implies the end of terrestrial biotic exchange between Africa-India-Madagascar and Antarctica-Australia. This latter implication, however, has also been disputed, suggesting the existence of land bridges and/or stepping-stones between Antarctica and Indo-Madagascar (east) and Antarctica and South-America (west) around ca. 80 Mya (Buckley at al., 2000; Hay et al, 1999; Krause et al, 1997; Sampson et al., 1998, 2001). The Kerguelen Plateau (KP) and the Gunnerus Ridge (GR) are two of those potential exchange routes between Madagascar and South-America via Antarctica. Very recently, however, Ali & Aitchison (2008) contend against the existence of these land bridges. During the Early Eocene, Australia, which was still attached to Antarctica, began to move northward. During the Early Miocene (20 Mya) Antarctica was covered by ice and the world started taking its contemporary shape, although many landmasses at low altitudes, such as part of Asia, were flooded during this epoch (Scotese, 2004). The land bridges that were present during the ice ages and that connected some (continental) islands to the mainland, such as Sri Lanka to India, are discussed in more detail in chapter 4. However, many details of these geological plate tectonic reconstructions are still disputed, a debate mainly about the exact timings and routes.

Because of their freshwater habitat restriction, the marine environment is considered a major geographical and physiological barrier for dispersal for freshwater crabs. Therefore, studies have postulated that the present wide distribution of freshwater crabs might reflect past tectonic drift. However, recent indications show that the freshwater crabs are probably too young to link their global distribution entirely to the historical continental break up scenario. In this study (chapter 3) we will show, with the aid of molecular phylogenies, dating estimates and, if possible, fossil calibration points, that the latter theory, which implies oceanic dispersal, is a more plausible scenario for the current freshwater crab distribution.

1.2.2 Island biogeography and colonization

Islands are interesting biological environments. Which was already recognized by Darwin in his *'the Origin of Species'* (Darwin, 1859). Each island has its own age, size, geological and geographical structure, climatic conditions, and so on. Therefore unique evolutionary processes and biota might evolve over time. In this study we use the term island for systems that are isolated from continental landmasses by seawater. We refrain here from the island concept dealing with islands, consisting of an area within a continental landmass, which is isolated from other similar habitats by circumstances (e.g. geography), such as an oasis in a dessert.

Two chapters (4 and 5) entirely focus on the Sri Lankan freshwater crab diversity and interchange of this community with the continental mainland (i.e., the Indian Subcontinent). Chapter three discusses oceanic dispersal to and from islands. We briefly elaborate on the differences between, and consequently the properties and peculiarities of, islands. These differences often lead to adaptive radiation and concerns for conservation (Gillespie and Roderick, 2002). Therefore it is important to understand the history and isolation of the island concerned. Firstly 'Darwinian' or oceanic islands are landmasses that have never been in contact with existing continental landmasses. Apart from the well-known Galapagos Islands, other examples are Taiwan, the Hawaiian Islands and the Comoros. Initially there is no life on these islands, and all ecological niches of this type of island are available to new colonizers. Depending on their degree of isolation and time, the rate of adaptive radiation and colonization differs.

In contrast, continental islands were once part of a continental landmass. Many examples can be listed, but Sri Lanka, Madagascar and Borneo are just a few mentioned later on in this study. When a continental island separates from the main landmass, it already has all the ecological niches filled with continental faunal communities. Therefore it is very difficult for new colonizers to successfully settle on these islands. However, the original number of species will eventually decline, creating opportunities. More on this island concept and its

colonization and conservation can be found in a review article by Gillespie and Roderick (2002).

1.2.3 The continental island Sri Lanka

Sri Lanka is a relatively small-sized continental island (~66,500 km^2) situated 30 km southeast of the Indian peninsula (see Fig 5). It separated from the continental mainland, the Indian subcontinent, at least 10 Mya. The fauna and flora of the island is expected to show close relationships with the Indian peninsula and might have had all ecological niches already occupied before separation. Moreover, during the past ice ages, Sri Lanka has been connected several times to the mainland by land bridges (Rohling et al., 1998). These land bridges are regarded as ideal routes for interchange of faunas and floras. However, chapter 4 sheds a completely different light on this general idea. It demonstrates that despite the presence of land bridges during the ice ages, the accidental faunal interchange between India and Sri Lanka did not occur during the latter periods (chapter 4). The migrations that happened to and from Sri Lanka occurred earlier in time.

Fig. 5: Map of South-East Asia with a magnification of the Indian Subcontinent.

Sri Lanka's climate is under the influence of a monsoonal regime. Therefore it is often divided in four ecological zones, based primarily on isohyets: the wet, intermediate, dry and arid zones. For many organisms, the wet zone is most

species rich (plants: Wikramanayake *et al.* 2002; freshwater fish: Pethiyagoda 1991; amphibians: Manamendra-Arachchi & Pethiyagoda 2005, 2006; freshwater crabs: Bahir *et al.* 2005). Moreover, the island has a complex elevational structure. The extensive lowlands have the largest flat surface, but it is widely believed that the Sri Lanka highlands are harbouring most of the endemic species. The importance of Sri Lanka as part of a biodiversity hotspot is described in section 1.3.1.

1.2.4 Where are they buried? Fossil evidence and calibration points

Although contemporary freshwater crabs are found worldwide in the (sub)tropics, their historical biogeography is hard to unravel. Fossil records can be extremely informative as to the ancient distribution of the taxon concerned. The estimates of the nodal ages in phylogenetic trees, fossils and age estimates of biogeographical events (e.g., break ups of landmasses) are often used as calibration constraints. The use of these calibration points is not recommended, because it might cause circular reasoning (Conti et al., 2004) - a view we followed in this study. Nevertheless they are often used due to the lack of other reliable calibration points. The use of fossils is therefore recommended, but fossil evidence for freshwater crabs is scant. Whereas the oldest brachyuran crab lived in the middle Triassic (245-228 Mya – Schram & Dixon, 2003), the oldest currently known freshwater crab lived in the Oligocene (~ 35 Mya ago – Feldmann et al., 2007). Only few fossil records are known within the different families. In the Gecarcinucidae no fossil records are known at all(De Grave et al., 2009).

In Table 2 a list of currently known fossils of freshwater crabs is given (from Feldmann et al., 2007). Many of these fossil records are young or difficult to use as calibration constraints in dating estimates, because of uncertain taxonomic placement (assignment to an extinct genus) (see also section material and methods, 2.4). This has caused different nodal assignments of the same fossil constraint in time divergence estimates (Daniels et al., 2006; Shih et al., 2009).

Table 2. Checklist of fossil terrestrial and freshwater brachyurans (copied from Feldmann et al., 2007)

Superfamily Portunoidea Rafinesque, 1815
- Family Trichodactylidae H. Milne Edwards, 1853
  - *Sylviocarcinus* H. Milne Edwards, 1853
    - *Sylviocarcinus Piriformis* (Pretzmann, 1968);(Rodríguez 1997) – Miocene, Colombia

Superfamily Potamoidea Ortmann, 1896
- Potamidae Ortmann, 1896
  - *Potamon*savigny, 1816
    - *Potamo Nantiquum* Szombathy, 1916 – Late Pliocene, Hungary
    - *Potamon? Castellinense* (Szombathy, 1916) – Late Miocene, Italy
    - *Potamon Proavitum* Glaessner, 1928 – Early Pliocene, Austria
    - *Potamon Silvalense* Glaessner, 1933 – Miocene, India
  - *Architelphusa* Bott, 1955
    - *Architelphusa Punctata* (Heer, 1865) (Bott 1955) – Middle Miocene, Germany
  - *Geothelphusa* Stimpson, 1858
    - *Geothelphusa Tenuimanus* (Miyake & Minei, 1965) (Naruse Et Al. 2004) – Pleistocene, Japan
    - *Geothelphusa Dehaani* (White, 1847) (Karasawa 1997) – Pleistocene, Japan
  - *Proballaya* Bott, 1955
    - *Proballa Yaquenstedti* (Zittel, 1885) (Bott 1955; Schweigert Et Al.1997) – Early Miocene, Germany
  - *Propotamonautes* Bott, 1955
    - *Propotamonautes Speciosus* (V. Meyer, 1862) (Bott 1955) – Middle Miocene, Germany
- Potamonautidae Bott, 1970
  - *Potamonautes* Macleay, 1838
    - *Potamonautes Niloticus* (H. Milne Edwards, 1837) (Carriol & Secrétan 1994) – Late Miocene, Uganda
    - *Potamonautes Tugenensis* Morris, 1976 – Miocene, Kenya
    - Potamonautidae Gen. And Sp. Indet. (Martin & Trautwein 2003) – Mio/Pliocene, Kenya
  - *Tanzanonautes* Feldmann, O'connor, Stevens, Gottfried, Roberts, Ngasala, Rasmusson & Kapilima, 2007
    - *Tanzanonautes Tuerkayi* Feldmann Et Al., 2007 – Paleogene, Tanzania

Superfamily Pseudothelphusoidea Ortmann, 1893
- Family Pseudothelphusidae Ortmann, 1893
  - *Eudaniela* Pretzman, 1971
    - *Eudaniela Garmani* (Rathbun, 1898) (Rodriguez & Diaz 1977) – Subrecent, Venezuela

1.2.5 Historical biogeographical scenario's for freshwater crabs

As in many other faunal and floral groups (Bossuyt et al., 2006, Sanmartin, 2003; Yoder & Novak, 2006; Vences et al., 2003, Cowie & Holland, 2006), the discussion on the most plausible biogeographical scenario to explain the contemporary distributional pattern of the freshwater crabs is still ongoing. There are those who advocate vicariance and contest oceanic dispersal and *vice versa*. The same is true for the freshwater crabs. Nevertheless, besides the lack of fossil evidence, the freshwater crabs are a rather difficult group because their closest marine sister group is still not known. As a result the historical biogeographical pattern of freshwater crab distribution has been explained in various scenarios, from one ancestor that lived on Gondwana and diversified through plate tectonic vicariance events, to a post-Gondwana diversification with occasional transoceanic dispersal (Sternberg et al., 1999, Shih et al., 2006, 2009, Daniels et al., 2006, Cumberlidge et al., 2008, Feldmann et al., 2007, Klaus et al., 2006, 2009, Cumberlidge and Ng, 2009). Most studies focused on specific geographical regions or families and these results were then used to extrapolate and complete the hypothetical biogeographical scenario.

1.3. Too late for nature? - Biodiversity hotspots

1.3.1 What is a biodiversity hotspot?

Extinction is a natural process but human impact might cause a mass extinction comparable to previous natural mass extinctions. Alarmingly, between 5 and 10 species may disappear every day. Therefore, conservation should be an important issue on the political forum. Apparently only 12 countries harbour 70% of all the existing species! Not surprisingly, many of these countries constitute for large parts of islands. The answers to questions like 'What are the most immediately important areas for conserving biodiversity?' or 'How can we slow down most efficiently the extinction rate (mainly caused by human interaction)?' play a very important role in setting conservation priorities. In

1988, Norman Myers defined the concept of 'biodiversity hotspots'[6]. To qualify as a *hotspot*, a region must meet two strict criteria: it must contain at least 1,500 species of endemic vascular plants (> 0.5 percent of the world's total), and it has to have lost at least 70 percent of its original habitat. At this point 50% of all plant species and 42% of all terrestrial vertebrate species are endemic to 34 biodiversity hotspots. The data from the above paragraph are from http://www.biodiversityhotspots.org/ (retrieved on 7/9/2009).

Different diversity indices are used to quantify and calculate such figures. The use of different measures, such as species richness, endemic species richness, and the number of rare or threatened species for certain areas, has lead to the recognition of different biodiversity hotspots. The controversy regarding the use of indices to identify areas as hotspots (like hotspots of species richness, threat and endemism) has also consequences for the application of different conservation methods, including setting priorities for conservation. Currently there is incongruence in identifying areas of importance (*see* Orme et al., 2005) because policy makers are not always working with the same measures of biodiversity. In chapter five we show that using different diversity indices might indeed lead to different conclusions and consequently different measures for conservation planning.

Conservation biology studies are greatly hampered because of the lack of data, including insufficient data for several taxa. Vertebrates and vascular plants are currently used as reference species in conservation biology, partly because their taxonomy and distribution is best known. When the focus is on freshwater habitats, fish are mostly used as study species. Freshwater crabs could also be considered as an important (see paragraph 1) bio-indicator. Moreover, many freshwater crabs are highly threatened and 80% of them are considered to be at some level of risk (Cumberlidge et al., 2009, Bahir et al., 2005). As mentioned already, they are sensitive to water pollution, they are important, sometimes the only, food source for different faunal groups, they are recognized as important

[6]Parallel to biodiversity hotspots, other conservation systems have been developed, such as Endemic Bird Areas (EBA) or Global 200 Ecoregions.

nutrient recyclers and they are economically important. In this framework, the group deserves more attention as a study object within the biodiversity and conservation. This study contributes to the knowledge of the freshwater crabs in Sri Lanka (almost complete identification and relationships and well-known distributions). Furthermore, we use, combine and compare several measures, such as phylogenetic diversity, relationships, endemism, etc., so that the current conservation measures can also take into account at least one more invertebrate group, the true freshwater crabs.

1.3.2 Sri Lanka as (part of) a biodiversity hotspot

Sri Lanka is part of the Western Ghats-Sri Lanka hotspot, one of the 34 recognized biodiversity hotspots (Myers et al., 2000, Mittermeier et al., 2004), and it is also an endemism hotspot (Orme et al., 2005). Sri Lanka is a continental island of about 66,500 km^2. The Palk Strait separates Sri Lanka from the Indian subcontinent. Most probably Sri Lanka became an island around the Miocene, but during the past ice ages a land bridge (of at least 100km wide) connected the island to the subcontinent on several occasions (Rohling et al., 1998). As mentioned before, I will elaborate more on this in chapter four.

Sri Lanka is under severe anthropogenic stress. The ever-increasing population uses the land, protected areas included, for industrial farming, logging and poaching. Only fragmented remnants (1.5%) of the original forest remain. Still the biological diversity is enormous, and often unique.

Because of their apparently similar fauna and flora, the Western Ghats and Sri Lanka are considered as one biodiversity hotspot. However, for many groups it is demonstrated that genetic interchange occurred only occasionally between Sri Lanka and India (chapter 4 and 5). Those groups have experienced major radiations and endemism (chapter 3, 4, 5), which could be used as an argument to recognize Sri Lanka as an independent hotspot.

Moreover, the recent conservation assessments of freshwater crabs (Bahir et al., 2005; Cumberlidge et al., 2009) demonstrate their uniqueness and importance to conservation. In chapter 5, the evolutionary phylogenetic diversity (see more in Material & Methods) of the freshwater crabs is linked to the Red List IUCN

categories and the elevational zones of Sri Lanka. It appears that more than 50% of the freshwater crabs are threatened in all three zones, but more remarkably, the lowland zone, which receives far less attention from conservationists and policy makers, harbors at least 56 million years of unique evolutionary history. Since many of these crabs have a distribution wholly or substantially outside the protected areas, this calls for innovative conservation management actions, which should find a consensus between conserving the oldest, the most rare or most threatened species.

1.4. Objectives

Chapter 3, 4 and 5 aim to elucidate the Old World true freshwater crab phylogeny, and use this to tackle some evolutionary issues in their biogeography and biodiversity with some implications for conservation.

More specifically, in chapter 3, 'Historical biogeography of the Old World true freshwater crabs: significance of oceanic dispersal', we aim to provide a comprehensive phylogenetic framework and aim to propose, within a temporal framework, the most plausible biogeographical routes that explain the contemporary freshwater crab diversification and distribution.

In chapter 4, 'Local endemism in the western Ghats/Sri Lanka biodiversity hotspot', we aim to demostrate that Sri Lanka has maintained a fauna that is largely distinct from that of the Indian mainland, leading to local endemism and aim to provide evidence that dispersal between mainland India and Sri Lanka has been much more limited than previously assumed.

In chapter 5, 'Phylogenetic diversity of freshwater crabs and its implications for conservation', we focus on the freshwater crabs as possible important indicators for biodiversity conservation on Sri Lanka. We aim to demonstrate the possible important relationship between the evolutionary history of freshwater crabs and the elevational zones of Sri Lanka. We aim to show the importance of considering more than one index to measure biodiversity in conservation planning.

Chapter 02

Material & Methods

2. MATERIAL AND METHODS

In the following paragraphs, I do not intend to elaborate extensively on all material and methods used during this study. I merely wish to explain a couple of choices made, or differences in, methods used in chapters 3 to 5, which are not explained in the chapters themselves. Other topic-related methods are described in the chapters (3-5) themselves.

2.1. What you work with is what you get...

When is a dataset complete, or which data are most important? What is most important to infer the most likely tree or the best phylogenetic estimation: more taxa or more and/or longer sequences? Several researchers have focused on these questions, but contradictory results have been published (Graybeal, 1998; Rydin & Källersjö, 2002; Wiens, 2003, 2005).
In most cases, it is unrealistic to include all taxa and often difficult to obtain all sequences of those taxa in one phylogenetic study. The most realistic approach appears to include a good variety of (deliberately chosen) taxa and a good variety of gene loci, preferably also including morphological and fossil data. Much of the most recent software can handle different kinds of data (computationally and mathematically).

2.1.1 Choice of taxa

Many invertebrate groups have been neglected in many biological disciplines. When freshwater ecosystems are focused on, fish tend to be the main subject of these studies most of the time. Intensive explorations and renewed interest during the late 90's and early 2000 caused a spectacular increase in descriptions of freshwater crabs of Sri Lanka (Gecarcinucidae). It is rare that almost the complete inventory of different species estimated to occur in a specific area can

be assembled[1]. This collection of data enabled the study presented in two different chapters (4 and 5). The collection of sequences of the third chapter of this study (mainly the Old World) was partly retrieved from GenBank. The representatives were selected on the basis of their geographic distribution, taxonomical position and number of genes available. For the exact enumeration of the taxa, I refer to the respective chapters.

2.1.2 Choice of genes

At the beginning of this study, few molecular phylogenetic studies had been performed on Brachyura (Spears et al., 1992; Sturmbauer et al., 1996; Kitaura et al., 1998; Schubart et al., 1998, Harrison and Crespi, 1999; Schubart et al., 2000, 2001), and to my knowledge no molecular studies on *true* freshwater crabs were published before 2001. Therefore, the choice of my molecular markers is partly determined by the choice of genes of researchers that published earlier molecular studies on Crustaceans and Brachyura (Cunningham et al., 1992; Knowlton et al., 1993, and the above references).

There are advantages as well as disadvantages in using specific DNA markers. The mitochondrial genome (mtDNA) provides useful markers for phylogenetic analyses. It has the advantage that there are many copies in a cell (amplification advantage), that there is only little recombination and that it is inherited maternally (i.e., haploid), which causes less problems in phylogenetic analyses (all mitochondrial sequences of one organism should provide information for a single organismal tree) (Simon et al., 1994; Saccone et al., 1999). On the other hand, the maternal inheritance of all mitochondrial genes in a specimen also implies that these genes share a common evolutionary history. Therefore, phylogenetic inferences based only on mitochondrial genes are not independent (Moore, 1995). In general, mitochondrial genes are known to have high mutational rates, which might cause saturation (often caused by molecular convergence) and which causes lower support in case one intends to resolve

[1] I do not wish to take all the credit for this effort. It is mainly thanks to Mohomed Bahir, Peter Ng, Darren Yeo and Rohan Pethiyagoda that I managed to collect (and get permission to work on) these samples.

deeper divergences. The high AT frequency, which is typical for crustaceans, could increase the degree of homoplasy (Chu et al., 2009). I also noticed a high AT frequency, especially in the large 16S fragment, and performed a test to evaluate the effect of base-composition in phylogenetic methods (see Appendix 1). Nuclear rRNA has the same disadvantage regarding the alignment ambiguities as mitochondrial rRNA has. Moreover, it is more difficult to find longer fragments (they are often only 300 bp long). On the other hand, nuclear genes, which are protein coding, are easy to align[2]. Many nuclear genes have different evolutionary rates. In this study I used two mitochondrial fragments and one nuclear fragment.

Large ribosomal RNA gene (16S RNA)

The commonly, extracted and sequenced fragment of this mitochondrial gene is approximately 650 base pairs (bp) long, which was used in chapter three of this study. I extended this fragment towards 12S, consisting of a small part of 12S (~ 45 bp), the complete t-RNA sequence (~ 73 bp) and a large part of the 16S gene (~ 1,200 bp). To obtain this, I developed two new primers (see chapter 4).

Cytochrome c Oxidase 1 gene (COI)

This is a protein-coding mitochondrial gene. I extracted and amplified a fragment of ~ 650 bp. Since it is coding, it is easier to align with other similar sequences. Therefore, using the correct mitochondrial code (in this case the *Drosophila* mitochondrial DNA code) every three subsequent bp correspond to an amino acid. In case the alignment predicts frame-shifts mutations, the copy is non-functional; the sequence is ambiguous and should not be used. This occurred in three cases and is explained further in chapter 5.

Histone 3 (H3)

The extracted fragment of the nuclear protein-coding gene Histone 3 is ~ 320 bp, which codes for the histone 3 protein, a part of the chromatin construction. It is a slowly evolving gene, and therefore better suited for resolving deeper phylogenetic divergences (Chu et al., 2009).

[2] But so are mitochondrial protein coding genes.

From One to Multi-locus approach

In chapter 4, I used the long 16S fragment for the freshwater crabs and the shrimps, which resulted in high nodal support for most splits. In chapter 3 and 5 of this study, I applied a multi-locus approach. In chapter 5, I opted to add the COI fragment in the different analyses. In chapter 3, I used the usual 16S fragment and COI fragment, but also added H3, which indeed allowed for a better resolution for deeper splits than in any other previous study.

2.2. Multiple Sequence Alignment (MSA)

As if the choice of taxa, genes and phylogeny inference methods were not complicated enough already, nowadays even the choice of the right multiple alignment methods is complex. The amount of data (length and number of taxa and sequences) in data sets is increasing very fast. Currently, an enormous variety of multiple sequence alignment methods exist, which are becoming gradually computationally faster and can handle more complex problems. Moreover, the choice of alignment method might have a larger effect on the inference of phylogenetic trees than was previously expected (Wong et al., 2008). However, there is no 'best' method for all circumstances. The mathematical correctness of an alignment (and later phylogenetic inference) can be validated, but the biological correctness is still partly based on the skills of the expert. Often small 'manual' corrections are necessary, which makes it difficult to exactly repeat the same alignment. I refer to a recent review by Kemena and Notredame (2009) for an overview of multiple sequence alignment methods and the future challenges faced.

In the analyses of this study I made use of a very popular implementation of the traditional MSA methods, ClustalW (Thompson et al., 1994) and later ClustalX (Thompson et al., 1997). These traditional methods make use of progressive alignment. The sequences are first compared two by two in order to build up a distance matrix. A clustering algorithm is then applied onto this matrix to

generate a guide tree. The algorithm basically works from the tips towards the root, aligning each sequence pair belonging to a node.

In chapter 3, I implement a program of the next generation of MSA methods, which are consistency-based. The common idea behind this kind of methods is that pairwise alignments are evaluated through the comparison of a third sequence (i.e. considering an intermediate sequence). Since PRANK was specifically developed to optimize sequence alignments for evolutionary analyses, I made use of this program (with its graphical interface PRANKSTER; Loytynoja & Goldman, 2005, 2008). This probabilistic model is based on a novel algorithm that automatically takes evolutionary relations between sequences into account. It treats insertions correctly and avoids over-estimation of the number of deletion events. In addition, PRANK borrows ideas from maximum likelihood methods used in phylogenetics and correctly takes into account the evolutionary distances between sequences. For our DNA alignments, the default (Hasegawa-Kishino-Yano- HKY85) substitution model was used.

2.3 Phylogenetic analysis

2.3.1 Three different methods

The next step is finding the optimal method to infer the phylogeny. There are different types of methods on the market. Tree building methods that search for cladograms, instead of clustering data into a phenogram, can be tested for the fit between data and tree. They work with optimality criteria to choose among a set of all possible trees. The tree with the most optimal score is chosen as the best fit to represent the relationships between the taxa under study.

Among the discrete methods, which operate directly on sequences, which is why these methods lose less information than distance methods, three are commonly used: maximum parsimony (MP), maximum likelihood (ML) and the Bayesian inference method (BI).

Below I will very briefly outline the main principles and differences of the three approaches. There are several reviews, which elaborate on these methods (Page & Holmes, 1998, Whelan et al., 2001, Holder & Lewis, 2003, Felsenstein, 2004).

Maximum Parsimony chooses the tree(s) that requires the least *ad hoc*

assumptions of change. The tree that needed the least number of steps is preferred as the best tree. This method maximizes the amount of evolutionary similarity that can be explained as homologous similarity, i.e., it maximizes the similarity that we can attribute to common ancestry. The assumption made is that similarity is caused by homology and not by homoplasy. A particular weakness of parsimony analysis is the risk of long-branch attraction (Felsenstein, 1978, Huelsenbeck, 1997). Long branch attraction means that there is a strong unequal rate of substitution between the branches, causing long branches to be attracted to each other, while they do not have an immediate common ancestry.

The parsimony analyses themselves do not provide any means to estimate the sensitivity of the outcome (no confidence intervals). I used non-parametric bootstrapping (i.e., resampling the characters from the original dataset with replacement) to assess support. The support is the chance that the specific branch would be again recovered at the same position in the tree as it is for the preferred tree. In other words: What is the probability that these data would lead to the same clade? To avoid too much computational time, MP is employed under a heuristic hill-climbing algorithm (but so is Maximum Likelihood). I used the tree bisection/reconnection (TBR) method for 10,000 replicates.

The other two statistical methods used are likelihood-based methods, for which the user has to postulate a model of evolution (i.e. the program searches for the best tree that is consistent with both the given model and the observed data). The **Maximum Likelihood** phylogenetic inference provides the user with the single most likely tree (in our case according to the hill climbing algorithm, TBR). In other words the tree maximizes the probability of observing the data given the tree[3]. Apart from the incorporation of explicit models of evolution, this method also permits statistical tests of evolutionary hypotheses. The other likelihood-based statistical method is through Bayesian inference. Again the

[3] mathematically $L_D = Pr(D|H)$
for which L is the likelihood for a set of sequences; D are the data and H is a hypothetical phylogenetic tree. Thus the likelihood is the probability that given the inferred phylogenetic tree, the observed set of sequences is obtained.

given data (sequences) and an explicit model of evolution are needed to retrieve the best set of trees. The principle behind the **Bayesian approach** is that evidence is collected to be consistent or inconsistent with a given hypothesis. Bayesian analyses are robust to the *a priori* choice of distribution, the evolutionary model. After enough evidence (observations) is collected, the hypotheses with very high support should be accepted as true. In our case the evidence is collected via the Monte Carlo Markov Chain method (MCMC) and the quality of the approximate distribution will increase as the length of the MCMC increases. The support is expressed as a posterior probability, which gives us the highest probability that the given data would give us the observed clade. To know whether the posterior distribution obtained is 'good', MCMC analyses can be run more than once. Therefore, an obvious difference in inferring the phylogenetic relationships with ML or within a Bayesian framework is the fact that ML produces one most likely tree and the Bayesian inference produces a distribution (i.e., a set of trees). The 'Bayesian' tree is the consensus of the posterior distribution. Bayesian inference and Monte-Carlo techniques allow software to estimate many evolutionary parameters simultaneously a posteriori (Larget & Simon, 1999; Shoemaker et al., 1999; Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003).

There are advocates for each of these approaches. However, it is always ones intention to reach the most optimal phylogenetic values for the purpose and use the most appropriate methods. During this study I'm searching for the evolutionary history of the freshwater crabs, i.e. their real phylogeny. The maximum parsimony analysis infers the most optimal interpretation of the data, but assumes that evolutionary change is rare. It assumes that the tree that minimizes change is the best estimate of the actual phylogeny. It does not really take different evolutionary rates of substitution into account and has difficulties dealing with long branches. This gives a cladogram, but not necessarily a real phylogeny (especially for real data). Maximum Likelihood and the Bayesian inference implement a model of sequence evolution. Maximum Likelihood retrieves one tree and tries to optimize that tree. The Bayesian approach searches within a large tree landscape and finds the best set of optimal trees. This study makes explicitly use of the evolutionary character of the DNA

sequences. I use it not only for the phylogenetic inferences, but also for the time divergence estimates, the phylogenetic diversity estimates and for the ancestral range reconstructions.

I used the PAUP* 4.0 b10 (Swofford 1998) software package to calculate maximum parsimony and maximum likelihood with non-parametric bootstrap analyses to test the phylogenetic tree. Later on, to calculate the maximum likelihood, I used the newer and faster RAxML 0.0.7 (Stamatakis, 2006) software, which first performs rapid bootstrapping and consequently calculates the ML search. I also used the MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) software that allows for two simultaneous, completely independent runs to optimize the phylogenetic tree and calculate the posterior probabilities. MrBayes uses a Metropolis coupling (MC) to improve the MCMC sampling. We used four chains, three of which were heated and one of which was cold.

I only performed the analyses on the linear DNA sequences, and did not perform any analyses on the protein products or include any specific requirements that for instance describe specifities regarding the secondary structures (stem-loop). A simple prelimenary test indicated these precautions would not influence our results (appendix two).

2.3.2 Possible pitfall: choice of outgroup

A phylogeny might be a very good representation of the relationships between the present taxa. If rooted inaccurately (i.e., wrong choice of outgroup), however, the phylogeny inferences can no longer be correct and could also give low statistical branch support (Conti et al., 2004). However, the sister group(s) of the freshwater crabs is still not known (Cumberlidge & Ng, 2009). For chapter 4 & 5, I choose a close but safe outgroup within the same family (Gecarcinucidae), after preliminary tests with marine outgroups. In chapter 3, were I dealt with the Old World freshwater crabs and a few New World freshwater crabs, I opt for a number of marine representatives, and include two anomuran crabs as outgroup. In the literature different brachyuran outgroups have been used to infer phylogenies (Klaus et al., 2006; Daniels et al., 2006; Sternberg et al., 1999).

2.4 The temporal component of a phylogenetic tree: Time divergence estimates

The dating estimates of fossils are one way of providing insight into the evolutionary history of lineages. When molecular data are available, the time of origin of biological lineages can also be estimated with the help of molecular dating techniques. All these methods convert measures of genetic distance between sequences into estimates of the time at which the lineages diverged. I recommend the following overviews, from which I retrieved the summarized information: Bromham & Penny, 2003; Hedges and Kumar, 2003, 2004; Welch & Bromham, 2005; Graur and Martin, 2004.

Initially the molecular clock, which is the assumption that genetic change accumulates steadily over time (Zuckerlandl & Pauling, 1965), was the basic assumption underlying the first dating techniques. Thus, synonymous and non-synonymous sites were assumed to have a constant rate of substitutions. However, apparently not many gene lineages have a constant rate of substitution, often demonstrated with the maximum likelihood ratio test (Felsenstein, 1981), which is a statistical test of the goodness of fit between two models[4]. This rate of molecular evolution is influenced by many factors, such as population size, body size, temperature and adaptive radiation (Benton, 1999).

If the substitution rate is not constant, an alternative method, such as the *relaxed molecular clock*, can be implemented. This technique allows for the incorporation of multiple gene fragments and accounts for rate variation (i.e., the rate can vary across the tree, thus rate heterogeneity) when estimating divergence times. I employed the Multidivtime software (Thorne & Kishino, 2002), which needs a rooted input topology[5] and uses a Bayesian approach to model the variation in rate of substitution along the tree. This method used a kind of 'rate smoothing ' approach (i.e. many small changes are more likely than large changes) (Sanderson, 1997). This rate is supposed to vary in an auto-

[4] The likelihood ratio statistic will test the likelihood of an alternative (nested) hypothesis to the null hypothesis. $\Delta = \log L_1 - \log L_0$.

[5] One of the main assumptions is the input of a correct phylogeny

correlated manner. The prior assumptions are expressed in a probability distribution (patterns that deviate more from these assumptions get lower probability values). These prior distributions and their variances should be specified for a selected divergence time (rttm and rttmsd) and rates of change (rtrate and rtratesd). Together with other (often default) parameters the times estimates for the different nodes (the posteriors) can be estimated. With the amount of sequence data used, the influence of the prior diminishes (Holder & Lewis, 2003; Huelsenbeck et al., 2002; Douzery et al., 2004). Yet, this relaxed molecular time estimate method still expects an input topology and parameters and on top of this, it is model-dependent. Extra calibration constraints can help to define a reasonable distribution of rates.

The relaxed molecular clock method implemented by Multidivtime (Thorne and Kishino, 2002) requires at least one calibration point to be set as an upper limit, the rttm, in order to be able to transfer relative divergence times to absolute divergence times. Calibration dates can also be used to interpolate, or more commonly extrapolate, but uncertainty increases with the distance between this calibration point and the estimated node (Springer, 1995). The choice and number of calibration dates is crucial to the accuracy of molecular dating (see Brohman et al., 1999; Graur and Martin, 2004). Calibration dates should come from independent information, such as geological events or fossil evidence. In our study I did not implement geological barriers, such as the island formation, to avoid circular reasoning in our biogeographical inferences (Conti et al., 2004; Magallòn, 2004). I use fossils as the oldest known representatives for a certain lineage, which corresponds to the minimum age (as recommended by Sanderson, 1998).

Several studies have focused on problems related to choice and use of calibration points and even developed specific cross-validation tests (Rutschmann et al., 2007, Near et al., 2005, Won & Renner, 2006, Conti et al., 2004). However, many of them are not applicable to our study because they assume rather a 'large' selection of available calibration points.

As mentioned above, I do not implement geographical barriers in my divergence age estimates, to avoid circular reasoning. I already pointed out that freshwater crabs do not have a plethora of fossils available (see introduction table 2) and

this incompleteness might lead to an underestimation of the lineage ages. Moreover, estimating the age of fossil evidence is also a science in evolution, meaning that its accuracy is often doubtful, also within freshwater crabs (Feldmann et al., 2007), which again makes it difficult to reliably position the fossil e.g., whether at the crown or stem group node (Benton & Ayala, 2003).

The relaxed molecular dating method (Multidivtime) used in this study, uses the prior distribution and calibration constraints to estimate the posterior distribution for the evolutionary rates and times per sequence. Closely related species share similar rates and will be under more influence from nearby constraints. Ideally, several well-spread fossil constraints should be included in the analysis. Adding several fossil calibration points within the same clade does not influence the posterior estimates of the distinct clades much more than adding only one.

I selected the fossil evidence of *Potamonautes niloticus* (Potamonautidae) from late Miocene and set the minimum evolutionary time at 5.3 Mya, corresponding to the ISC Chart (Gradstein et al., 2004; Ogg et al., 2008). Additionally I also used the fossil evidence of a '*Potamon*' specimen (Potamidae) (Glaessner, 1969) dated back to early Miocene. For this fossil record I set the minimum age at 23.03 Mya (Gradstein et al., 2004; Ogg et al., 2008). There are no fossil records for the Gecarcinucidae.

In chapter four I use the rate of substitution calculated for the genus *Sesarma* (Schubart et al., 1998) as a prior rate of substitution. This is the best available alternative when a reliable prior distribution age is absent. The prior rate and time are inversely related to one another (rtrate = X/rttm - Thorne & Kishino, 2002), with X being the median of the substitution rates from the tips to the root. I thus use the published rate (Schubart et al., 1998) and the median of substitution rates to deduce the prior distribution of our ingroup. I carefully interpret the absolute posterior time estimates and preferably compare them in a rather relative framework.

In chapter five I perform similar analyses, but also test the results against a larger topology, which includes the ingroup of the study. For this extended group there is no prior ingroup distribution available either. However, in this case I include two fossil calibration points. Again, I carefully interpret the

absolute posterior time estimates and preferably compare them in a rather relative framework.

In chapter three I employ the regular methodology (Thorne & Kishino, 2002), i.e., using the prior ingroup distribution to deduce the prior rate of substitution. I also include additional calibration constraints. Age estimates are clearly older than the estimates of chapter four and five. A combination of several causes could explain this phenomenon: (1) the geographical and taxonomical restriction of the other studies; (2) the published rate of evolution was used as a prior rate of evolution, which is a slower rate than the currently estimated prior rate; (3) denser taxon sampling; and (4) more data partitions often cause the inference of an older age for the root of the clade (see Poux et al., 2008).

2.5 Phylogenetic Diversity

In ecology and conservation biology several quantitative indices are used to estimate biodiversity. The most common and simple way is through the use of species richness. This is a quantitative measure that calculates the number of species per defined area. Other indices are often derived from this measure. However, within this kind of measures there is no account for evolutionary processes. Phylogenetic Diversity (PD) is an alternative biodiversity index that estimates 'feature diversity' on a phylogenetic tree, i.e., it measures variation of changes (features) along the branches, which is a finer scale than species richness. The PD for a subset of taxa is the sum of the branch lengths of the minimal sub-tree that spans all considered taxa of this set (Vane-Wright et al., 1991; Faith, 1992) from the root of the tree (Faith, 2006). The root of the tree corresponds to outgroup changes. Therefore, PD measures the length of the evolutionary pathways of a given set of taxa. The PD measures the amount of evolutionary clade history (Sechrest et al., 2002).

When PD is calculated on a chronogram (a phylogeny with time divergence estimates), the scores are derived in time units (e.g., million years). It provides an explanation and comparison of the evolutionary history of a group of organisms. Within chapter 5, the subset of taxa corresponds to a certain area,

such as, country, lowland, upland or highland. Therefore, the clade evolutionary history is equal to the amount of branch length uniquely represented in this area. It reflects the amount of PD inevitably lost if this area is lost (Sechrest et al., 2002).

We employed the software PDA (Minh et al., 2006) to calculate the different phylogenetic diversity values in chapter 5. We use this measure of diversity and compare it to species richness calculations for the same areas. From these analyses we conclude that both measures are equally important in conservation. They can lead to different priorities to be set in conservation actions.

Chapter 03

Historical biogeography of the Old World true freshwater crabs: the significance of oceanic dispersals

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Historical biogeography of the Old World true freshwater crabs: significance of oceanic dispersal

Abstract

Currently mainly two biogeographical scenarios are used to explain the contemporary distribution of the freshwater crabs. One hypothesizing the current distribution is the result of an ancient lineage that diversified through Gondwanan vicariance, the other postulating a post-Gondwanan diversification. We compiled a multi-locus data set of 228 specimens of freshwater crabs, mainly from the Old World. We used 3 gene fragments: two mitochondrial fragments (COI and 16S) and one nuclear fragment (H3). After a preliminary analysis, we remained with a data set of 107 species, of which 102 were Old World freshwater crab species. We provide a comprehensive phylogenetic framework and explain within a temporal framework the freshwater crab diversification and distribution. The results support the monophyly of the Old World freshwater crabs and the higher-level family classification. Using this temporal phylogeny and ancestral area reconstruction, we postulate that the most plausible biogeographical pattern that resulted in the contemporary freshwater crab distribution, is a diversification that started about mid Eocene, including several oceanic dispersal events.

3. 1 Introduction

Approximately one fifth (~1280 species) of the globally known brachyuran species are true freshwater crabs (Yeo et al., 2008; Ng et al., 2008; Cumberlidge et al., 2009). Old World freshwater crabs are believed to form a monophyletic group consisting of three families: Potamidae (with 505 species in 90 genera), Gecarcunicidae (with 345 species in 57 genera) and Potamonautidae (with 132 species in 18 genera) (Yeo et al., 2008; Cumberlidge et al., 2009; Klaus et al., 2009). Together with the New World Pseudothelphusidae, the Old World freshwater crabs are currently considered a monophyletic group (Cumberlidge & Ng, 2009).

At the species level, freshwater crab species distributions are often very restricted, with many species endemic to small areas (Yeo et al., 2008). As a group, however, and despite their poor dispersal capacities, the true freshwater crabs have an extremely wide distribution, occurring in all (sub)tropical continental areas worldwide, and even on some volcanic islands (Yeo et al., 2008, Cumberlidge et al., 2009). The marine environment is generally considered a major geographical barrier for successful oceanic dispersal for freshwater organisms. Although a number of studies have shown that to some extent and for limited periods of time, a few species of freshwater crabs are tolerant to seawater (Bott, 1970; Morris and Van Aardt, 1998; Esser, 2007). This limited tolerance might not be sufficient to fully explain the occurrence of close relatives occurring on geographical landmasses widely separated by marine environments.

Different scenarios have been proposed to explain the observed biogeographical patterns. One hypothesis postulates that the present true freshwater crab distribution was the result of divergence from a single Gondwanan ancestor through the sequential fragmentation and drift of the continental plates (Rodriguez, 1986, Ng and Rodriguez, 1995, Ng et al., 1995). More recently, several authors, mostly working with specific clades or within relatively limited geographical ranges, have postulated a post-Gondwanan dispersal, especially for Old World freshwater crabs based on molecular, morphological and geological results (Sternberg et al., 1999, Shih et al., 2006, 2009, Daniels et al., 2006, Cumberlidge et al., 2008, Feldmann et al., 2007,

Klaus et al., 2006, 2009; Cumberlidge and Ng, 2009). However, this discussion cannot be solved because a comprehensive phylogeny of the true freshwater crabs is still lacking.

This study aims to compile a comprehensive molecular multi-locus data set to resolve the phylogenetic relations of the Old World freshwater crabs. Moreover, this study also aims to infer the most plausible biogeographical patterns within a temporal framework and with mainly geological evidence for the Potamoidea (*sensu* Cumberlidge and Ng, 2009; Shih et al., 2009).

3.2 Material and Methods

3.2.1 Data and sequence collection

A data matrix of 228 terminal taxa and three different gene fragments was constructed. The fragments are two mitochondrial DNA fragments: a ca. 560 base pair (bp) region of the 16S rRNA gene, and a ca. 650 bp fragment of the Cytochrome *c* Oxidase subunit 1 gene (COI). The third fragment is a partial gene sequence of ca. 350 bp of the Histone 3 gene (H3). Most of the sequences were retrieved from GenBank, while others were sequenced for this study. An overview of all species, haplotypes, voucher numbers, location and gene data (accession numbers) is given as supplementary material (S1). For several species one or two of the three gene fragments are lacking. Therefore, after a preliminary phylogenetic analysis of this concatenated dataset, 107 taxa were selected to be included in a smaller dataset for further analysis. Selection of these taxa was based on their position within the phylogenetic tree of the preliminary analysis and/or in published phylogenies (Shih et al., 2004, 2007, 2009, Beenaerts et al., in press, Daniels et al., 2006, Klaus et al., 2009, Cumberlidge et al., 2008). The selected taxa were chosen as representatives of clearly monophyletic groups consisting of species that were considered very closely related (i.e. congeneric) in classical taxonomy, for which the most complete sequence data were available. We included three brachyuran marine species and two anomuran species as outgroups (see 'Choice of outgroup').

3.2.2 Alignment and phylogenetic analysis

The sequences for the protein coding fragments COI and H3 were aligned using the ClustalX_V1_81 software (Thompson et al., 1997). These sequences were compared with their amino acid sequences. Consequently, ambiguous fragments were excluded from subsequent analyses. To optimize the alignment of the 16S fragment, for which ambiguous alignment problems can be expected, the probabilistic alignment software PRANK (Löytynoja & Goldman, 2005, 2008) was preferred. This method keeps a record of the proposed insertions and deletions, which should be more efficient than the traditionally used progressive, multiple alignment methods (Loytynoja and Goldman, 2008). It outperforms all alternative multiple alignment software when tested on simulation and real data (Benavides et al., 2007; Kemena & Notredame, 2009).

Bayesian analyses were performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using a locus-based data partitioned GTR + Γ + I-model, as this was identified as the best fitting evolutionary model by Modeltest v3.7c (Posada and Crandall, 1998). Two independent runs of four MCMC chains (one cold, three heated) each were run simultaneously for 10,000,000 generations. They were sampled every 1000 generations and the first 4,000 trees were discarded as the "burn-in". Bayesian posterior probabilities (BPP) were estimated as the 50% majority-rule consensus of the last 6,000 sampled trees. A rapid bootstrapping search followed by a thorough Maximum Likelihood (ML) search was performed in RAxML 7.0.4 (Stamatakis, 2006). The rapid bootstrap analyses were conducted with 1000 replications. All free model parameters are estimated by RAxML 7.0.4 under the GTR + Γ + I-model of rate heterogeneity and ML estimate of the alpha-parameter tree. The above phylogenetic analyses were performed for the three individual loci separately (results not shown), for the complete concatenated data set (228 taxa) and for the concatenated subsample (107 taxa).

Alternative branching scenarios were evaluated by a nonparametric approximately unbiased (AU) test (Shimodaira, 2002) and by the more conservative Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999). The alternative hypotheses were represented by candidate trees estimated under ML using RAxML. Both the AU-test and SH-test, aim to provide

better control of type 1-errors by comparison of multiple hypotheses. Site-wise log-likelihoods estimated by RAxML for all candidate trees were used as input for the software package CONSEL 0.1g (Shimodaira and Hasegawa, 2001). Constraints were chosen on the basis of conflicting biogeographical hypotheses (see Table 2).

3.2.3 Choice of outgroup

Low statistical support and inconsistencies in phylogenies estimated by phylogenetic methods can be a consequence of the choice of outgroup (Conti et al., 2004). The sister group of the Old World freshwater crabs is still subject to debate (see Cumberlidge and Ng, 2009) and several marine species belonging to different families have been used as outgroups in phylogenitic studies (e.g., Klaus et al., 2006; Daniels et al., 2006; Sternberg et al., 1999). Moreover, there is still lack of consensus on the phylogenies of the Decapoda and Brachyura (Ahyong and O'Meally, 2004, Porter et al., 2005, Brösing et al., 2007, Ahyong et al., 2007, Ng et al., 2008, Tsang et al., 2008, Brösing, 2008, Crandall et al., 2009), although most studies show Anomura as the sister group to Brachyura (e.g., Scholtz & Richter, 2003; Dixon et al., 2003; Ahyong & O'Meally, 2004; Porter et al., 2005; Tsang et al., 2008). Only a few brachyuran or decapod phylogenetic studies have included freshwater crab taxa and then only one or two species were included (Spears et al., 1992; Porter et al., 2005; Brösing et al., 2008, Crandall et al., 2009). Hence, we used two representatives of Aeglidae (Anomura) as outgroup and representatives of some marine crab families, i.e., *Carcinus maenas, Pachygrapsus marmoratus* and *Cancer pagurus* (with the extra asset that their families have fossil age estimates).

3.2.4 Divergence time estimates

The hypothesis of the molecular clock was rejected using the likelihood ratio test (LRT; df=106; p=0.05; Felsenstein 1981). Consequently, divergence times were estimated with the Bayesian multi-locus relaxed molecular clock method implemented in Multidivtime (Thorne and Kishino, 2002), which uses an MCMC procedure to derive the posterior distributions of rates and times. Prior gamma distributions were specified through the mean and standard deviation of

the root age (rttm and rttmsd), the root rate and the rate autocorrelation (Thorne and Kishino, 2002).

We performed two time divergence analyses (see below and Table 1) on the concatenated subsample tree topology. For both analyses markov chains were run for 1,100,000 generations with sampling intervals of 100 generations and burn-in corresponding to the first 100,000 generations. Both analyses were repeated once to confirm successful convergence towards the proper distributions for divergence ages.

The uncertainty of brachyuran and decapod phylogenies (see 'Choice of outgroup') and uncertainties in calibration procedures (Near et al., 2005; Rutschmann et al., 2007; Hedges and Kumar, 2004; Benton and Donoghue, 2007) lead us to run two time divergence estimates. The time divergence analyses differ in the prior distribution used. In the first analysis (A1), we used the minimum time estimate of the Portunidae as the mean of the root age (107 Mya: Porter et al., 2005, Crandall et al, 2009) in a 95% credibility interval. However, the phylogenetic relationship between Cancridae and Portunidae is not well resolved (Schubart and Reuschel, 2009). Hence, we performed a second analysis (A2), in which we excluded the Portunidae from the data set and used the minimum age for the Cancridae (49 Mya, cf. Brösing 2008) as the ingroup prior.

We avoided the use of biogeographical calibration dates to minimize the risk of circular reasoning when inferring biogeographical scenarios (Conti et al., 2004; Magallòn, 2004). However, there is a notoriously sparse freshwater crab fossil record, with the oldest freshwater crab fossil dating back to the Oligocene of Tanzania (about 35 Mya) (Feldmann et al., 2007). We did not used this record, since it is a doubtful fossil record (new genus) to place in the phylogeny of our extant specimen. We used the following calibration points (1) a fossil of *Potamon* of 24 Mya, (2) a fossil of *Potamonautes niloticus* of 6 Mya sediments. Additionally we used a third calibration point, being 40.4 Mya as the minimum age of Cancridae (mid Eocene; Schweitzer et al., 2002) in the first time divergence analysis (A1) and, in the alternative analysis (A2), we used 40.4 Mya as a minimum age for Grapsidae (Brösing, 2008). This analysis discards the Portunidae from the analysis.

3.2.5 Biogeographical reconstructions

The locations (countries) of our specimens can be found as supplementary material, Table S1 (from Bossuyt et al., 2004, Beenaerts et al., in press, Ng and Tay, 2001; Bahir & Ng, 2005; Bahir and Yeo, 2005, 2007; Daniels et al., 2006; Klaus et al., 2009; Shih et al., 2004, 2006, 2009; Schubart & Ng, 2008). All ingroup taxa were coded for their present distribution across the following ranges: Africa, Madagascar, Seychelles, Indian Subcontinent, Palaearctical area, Thailand-Maleysia, Sunda Shelf, Wallacea, Australia and the Neotropics. The probability method, implementing a Dispersal – Extinction - Cladogenesis (DEC) model (Lagrange - Ree et al., 2005; Ree & Smith, 2008) is employed to infer the most likely ancestral range reconstruction. We used the unconstrained model, with the assumption that the ancestor only occurred in one or two areas (Ree et al., 2005; Ree & Smith, 2008) and allowed the model to estimate the most likely dispersal and extinction probabilities for the phylogeny in a temporal framework. In case more than one dispersal route is suggested, only the pattern with the highest relative probability is shown.

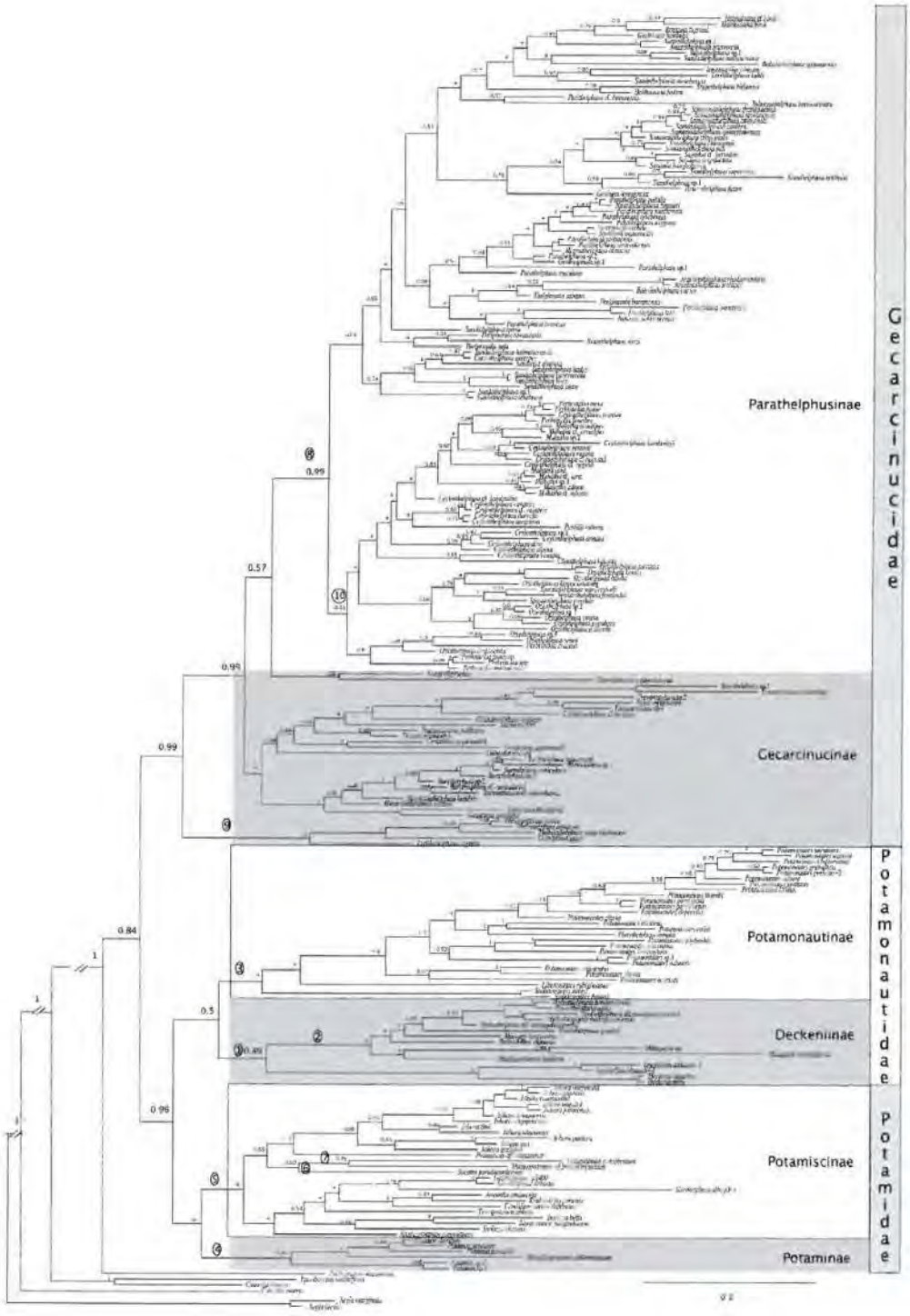
3.3 Results

3.3.1 Sequence characteristics

The results of the analyses on the partitioned datasets were consistent with, though less resolved than, the results of the analyses on the concatenated dataset. The partitions contained 165 (16S), 129 (COI), and 138 (histone 3) fragments, respectively. The results of the partitioned alignments and phylogenetic analyses are not shown. After removal of the ambiguous sites, the concatenated data set consisted of 228 haplotypes with 1,267 nucleotide sites. Inferring the correct phylogenetic analysis is still possible with missing data (Wiens, 2003, 2005; Philippe et al., 2004). Our preliminary analysis indeed demonstrates that the inferred phylogeny largely corresponds to the one of the subsample, but some discrepancies due to the lack of sequences are observed, such as the positions of *Perithelphusa borneensis1* and *Perithelphusa borneensis2*. Other more cryptic discrepancies might also be present in the large

concatenated data set. For the concatenated subsample, the data set consisted of 107 species. After exclusion of 614 nucleotide sites due to ambiguities in the alignments, the total data set consisted of 1,236 characters.

Figure 1 (next page): The 50% consensus tree for the concatenated (COI, H3 and 16S) dataset of 228 putative species, using Bayesian inference under the best-fitting model, GTR + I + G. The tree was rooted using two Aeglidae/Anomura and three marine Brachyura species. The posterior probability values are indicated on the branches. Splits supported less than 0.6 (BI) are indicated with an asterisk. Classification (family and subfamily) on the right follows Cumberlidge & Ng (2009). The rectangular lenses correspond to the respective clade. Nodes 1-10 correspond to clades mentioned in the text.


Gecarcinucidae
Parathelphusinae
Gecarcinucinae
Potamonautidae
Potamonautinae
Deckeniinae
Potamidae
Potamiscinae
Potaminae
0.99
0.57
0.99
0.84
1
1
0.5
0.96
0.2

3.3.2 *Phylogeny and phylogeography*

The results of the analyses are presented as the Bayesian 50% majority rule consensus topology for the entire data set (Fig. 1) and for the subsample data set (Fig. 2), including clade confidence values as posterior probabilities (MrBayes >50%) and maximum likelihood bootstrap support values (bp >50%). The Bayesian and ML analysis provided similar topologies, only differing in those nodes (Fig. 1) for which ML bootstrap results are not indicated (for instance in Potamidae, *Potamiscus* aff. *yunnanense*). In the ML analysis, there is low nodal support (<50%) for several relationships at low taxonomical level, which is probably a consequence of using two partitions of protein coding genes (Toon et al., 2009). Yet, supporting evidence for most species relationships is available in published phylogenies (see M&M). It was not possible to check misidentifications correlated to some sequences.

The inferred topology (Fig. 2) represents a well-resolved molecular higher-level phylogenetic relationship of the Old World freshwater crabs. It shows three monophyletic clades, representing the three currently accepted families, Potamonautidae, Potamidae and Gecarcinucidae (Klaus et al., 2009; Cumberlidge & Ng, 2009). These findings corroborate recent results of phylogenetic analyses that focus on the above clades separately (Potamonautidae: Daniels et al., 2006; Cumberlidge et al., 2008; Potamidae: Shih et al., 2009; and Gecarcinucidae: Klaus et al., 2009). Furthermore, Potamidae and Potamonautidae are sister groups in our study. Within the three recognized families, several lineages experienced explosive (endemic) radiations, such as those from Borneo, Sri Lanka, Madagascar and Sulawesi (see also Bossuyt et al., 2004; Beenaerts et al., in press; Schubart & Ng, 2008; Cumberlidge & Sternberg, 2002; Klaus et al., 2009). The alternative phylogeny, as proposed by Klaus et al. (2009), which places the Potamidae basal to the other Old World freshwater crabs, was not rejected by the AU or SH tests (Table 2).

Table 2: Statistical confidence (P-values) values for the approximately unbiased (AU) test, the Shimodaira-Hasegawa (SH) test and the posterior probablities (PP) (α = 0.05), for six alternative hypotheses.

Alternative hypothesis	**AU**	**PP**	**SH**
H0: Topology this study (Fig. 1)	**0.906**	**1.000**	**0.987**
H1: Potamidae (Gecarcinucidae, Potamonautidae) – Klaus et al. (2009)	**0.142**	<0.001	**0.413**
H2: *Socotra* within Potaminae	**0.107**	<0.001	**0.408**
H3: *Sundathelphusa* basal to SE Asian Gecarcinucidae	<0.001	<0.001	0.005
H4: *Socotra* with Potamonautidae (sister to Deckeniinae) and *Sundathelphusa* basal	<0.001	<0.001	0.006
H5: Potamidae, Potamonautidae, (Deckeniinae (India, rest Gecarcinucidae) – Klaus et al. (2006)	<0.001	<0.001	0.002
H6: Traditional tectonic theory	<0.001	<0.001	<0.001

Potamonautidae

Within this monophyletic, entirely Afrotropical clade, the split (clades 1 and 3) between the African Potamonautinae (*sensu* Ng et al., 2008) and Deckeniinae-clade, with representatives on Madagascar, Seychelles and the African continent, is highly supported, as are most relationships within the African lineage. All endemic Malagasy genera of Deckeniinae form a monophyletic group (clade 2; see Cumberlidge & Sternberg, 2002; Daniels et al., 2006; Klaus et al., 2006). The other Deckeniinae, which occur on the Seychelles and East-Africa, form a monophyletic sister group to the former (clade 3), which supports the decision to include the Deckeniinae within the Potamonautidae (Cumberlidge et al., 2008). *Plathythelphusa armata*, which represents the monophyletic plathythelphusid clade (Marijnissen et al., 2006), is nested within the genus *Potamonautes*. This shows that *Potamonautes* is not a monophyletic genus, unless *Platythelphusa* is synonymized with *Potamonautes* (see also Daniels et al., 2006; Marijnissen et al., 2006, Cumberlidge et al., 2008).

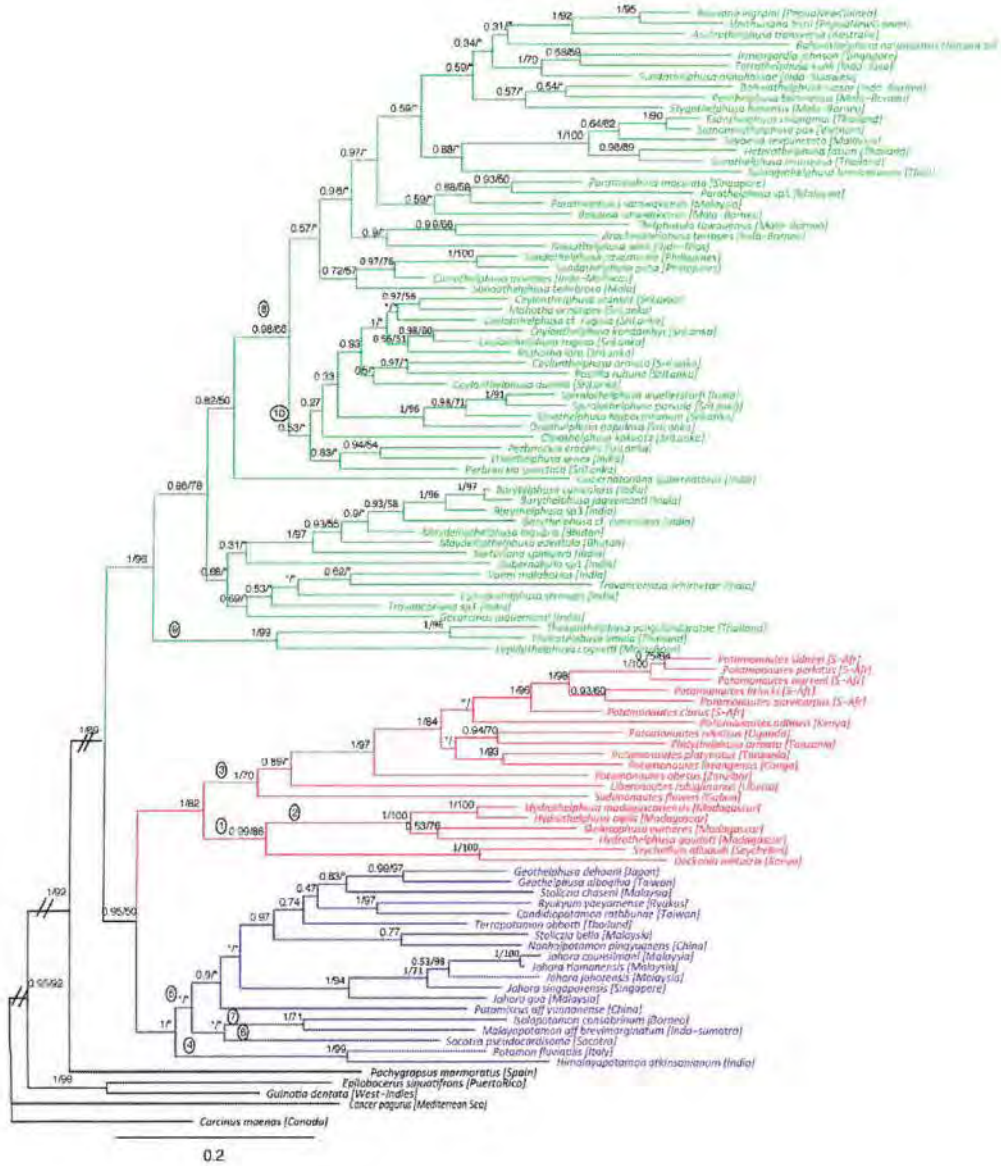


Fig.2: The 50% consensus tree for the concatenated (COI, histone 3 and 16S) dataset of 107 putative species using Bayesian inference under the best-fitting model, GTR + I + G. The tree was rooted using two Aeglidae/Anomura and three marine Brachyura species. The posterior probability values (BI) and bootstrap values (ML) are indicated on the branches (BI/ML). When splits were supported less than 60% (ML) or 0.6 (BI) they are indicated with an asterisk. The colours correspond to the respective family: green – Gecarcinucidae, red – Potamonautidae and blue - Potamidae. The geographical position of the specimen is in parentheses. Nodes 1-10 correspond to clades mentioned in the text.

Potamidae

In general, our results reflect the phylogenetic relationships retrieved in a recent study by Shih et al. (2009). The monophyletic Potaminae *sensu* Yeo & Ng (2003) (clade 4), represented by *Potamon* and *Himalayapotamon*, has a West Palaearctical distribution (except Myanmar). The phylogenetic position of the taxa from Socotra, represented by *Socotra pseudocardisoma* (clade 6), remains enigmatic (Yeo et al., 2007, Shih et al., 2009). Although this species is consistently positioned within Potamiscinae *sensu* Yeo and Ng (2003; clade 5), it has a different phylogenetic position in both topologies (Figs. 1, 2), both with only weak support. In the likelihood ratio test, relocation of *Socotra pseudocardisoma* within the Potaminae-clade was not rejected for the AU and SH-test (Table 2). Because the fauna and flora of Socotra is often linked to African biota (e.g., Nagy et al., 2003; Kürschner, 2000; Kürschner et al. 2001; Thiv & Meve, 2007), we also tested the relocation of *Socotra pseudocardisoma* in the Afrotropical family Potamonautidae, but both AU and SH tests rejected this phylogeny (Table 2). The insular Potamiscinae (clade 7; here presented by the Bornean and Sumatran *Isolapotamon* and *Malayopotamon)* are basal to the clade represented by *Johora* from the Sundaic Malay peninsula and associated islands (Fig. 1). However, in the topology of the subsample (Fig. 2), they are, together with *Socotra,* the basal clade for the Potamiscinae.

Gecarcinucidae

Within Gecarcunicidae, Parathelphusinae (clade 8) appears monophyletic, while Gecarcinucinae *sensu* Klaus et al. (2006) appears to be paraphyletic (see also Klaus et al., 2009). Clade 9, with a distribution in India, Malaysia, Thailand and Bhutan, is highly supported as the basal clade for the Gecarcinucidae. Our results also support the recent study of Indian freshwater crabs by Bahir and Yeo (2007), revealing high gecarcinucid diversity in India. The well-resolved monophyletic Sri Lankan radiation (clade 9) most probably evolved from an Indian ancestor, which also lies basal to the Southeast Asia diversification. Gecarcunicidae is the only lineage that shows the independent crossing of the Wallace line in several occasions, e.g., the clade containing *Currothelphusa* and *Sendleria*, the clade represented by *Sundathelphusa*

minahassae, and the clade represented by *Rouxana-Holtuisana-Austrothelphusa* (see discussion).

Monophyly of a clade consisting of the Old World freshwater crabs and the New World family Pseudothelphusidae, as postulated by many authors (Sternberg et al., 1999; Cumberlidge & Ng, 2009, and several references herein), is not confirmed in this study. The intertidal marine crab, *Pachygrapsus marmoratus* (Grapsidae, Ng et al., 2008) consistently takes a position between both clades (Fig. 1).

3.3.3 Divergence estimates and biogeographical analysis

The ultrametric trees (Fig. 3 and 4) are based on the Bayesian topology of the concatenated subsample for the Old World freshwater crabs. The first time divergence analysis (A1; Fig. 3) represents the time divergence phylogeny with a prior distribution of 107 Mya and, the second analysis (A2; Fig. 4) represents the inferred chronogram using the prior distribution of 49 Mya. Both figures show different time divergence estimates, with their 95% credibility interval (CI), for 16 biogeographical events (node A – P). These posterior time estimates for the two approaches including their 95% credibility interval are also shown in Table 1.

The most likely ancestral range reconstructions for both approaches are also reflected in Fig. 3 (A1) and 4 (A2). Only the highest relative probabilities for the likelihood values are shown. The overall likelihoods (-ln) are 182.6 (A1) and 181.4 (A2), respectively. De overall dispersal rates are 0.053 (A1) 0.097 (A2) and the overall extinction rates are 0.098 (A1) and 0.219 (A2), respectively. Both figures show different circumstances of range inheritance. They show that range expansion (e.g., splits C and K), vicariance (e.g., split K) and colonization (e.g., splits B, F and J) events often occurred. The results typically show lower relative probability values for deeper splits, and therefore more ambiguity towards the root of the tree (Ree et al., 2005).

Table 1: Posterior divergence estimates in million years (node A-0 are the biographical events reflected in Fig. 5 and 6) with upper and lower bounds for major splits under a credibility interval (CI) of 0.95% of the Old World freshwater crabs for two time divergence approaches under a relaxed molecular clock using three calibration points (see section 2.4). Approach 1 (A1) reflects the estimates for the data set including the representative of the Portunidae (*Carcinus maenas*) (105 ingroup species). The prior time distribution is set to 107 Mya. In approach 2 (A2) this representative is eliminated; the ingroup has 104 species. The prior time distribution (rttm) is set to 49 Mya.

	Biogeographical event	A1 Rttm = 107 Mya	CI A1 0.95%	A2 Rttm = 49 Mya	CI A2 0.95%
A	Origin Old World Freshwater crabs	79.0	64.1–94.1	43.6	40.5–48.7
B	Sundaland - Africa	68.6	56.7–81.3	38.2	33.7–42.8
C	Sundaland – India	59.7	48.4–72.2	33.3	28.7–38.3
D	Africa/Palaearctic - Palaearctic	61.4	49.5–74.1	34.6	29.9–39.3
E	Africa - Madagascar	55.2	42.1 -69.1	30.1	23.9–36.3
F	Africa - Seychelles	25.7	9.7–46.4	13.8	6.0–23.8
G	Sunda shelf – Thailand/Malaysia	41.2	31.2-52.3	22.9	18.0-28.1
H	Sunda shelf - Australia	30.0	20.7–40.5	16.7	12.2–21.7
I	Sunda shelf - Wallacea	21.0	10.1–32.5	12.1	7.0–17.7
J	Sunda shelf – Philippines (Wallacea)	40.3	27.6-53.4	21.9	16.0-28.2
K	India - Palaearctic	35.1	23.1-48.2	20.0	13.9-26.5
L	Sunda shelf – Thailand/Malaysia	47.7	28.9-66.7	27.6	18.7-36.3
M	Palaearctic - Africa	56.0	44.7-68.1	31.6	27.0-36.4
N	Africa – Sunda shelf	52.2	40.9–64.6	28.9	23.5-34.3
O	Palaearctic – India	30.7	23.5-42.4	24.2	23.1-27.3
P	Philippines - Wallacea	28.6	14.9–43.2	15.9	9.1–23.3

3.4 Discussion

3.4.1 Phylogenetic inferences

In the most recent classification of the true freshwater crabs (Cumberlidge & Ng, 2009), three Old World families are recognized: Potamidae, Gecarcinucidae, and Potamonautidae. These families, together with the Pseudothelphusidae, a monophyletic family of Neotropical freshwater crabs, are classified into the superfamily Potamoidea (Cumberlidge & Ng, 2009). The remaining Neotropical true freshwater crabs belong to an unrelated clade, the Trichodactylidae (Cumberlidge & Ng, 2009; Schubart & Reuschel, 2009).

Our results cast some doubt on the monophyly of the taxon Potamoidea *sensu* Cumberlidge & Ng (2009), as the marine species *Pachygrapsus marmoratus* (Grapsidae) takes a position in between Pseudothelphusidae and the three families of Old World freshwater crabs. This suggests that either *P. marmoratus* represents a clade that has secondarily returned to the marine environment, or two independent invasions of freshwater habitats have occurred, one in the Neotropics (Pseudothelphusidae) and one in the Palaeotropics (common ancestor of the Potamidae, Gecarcinucidae, and Potamonautidae).

The monophyly of each of the three Old World families of freshwater crabs is very well supported (Fig. 2), as is the sister group relationship between the Potamidae and Potamonautidae. Although the lower level relationships are not the main interest of this contribution, we want to emphasize that many of the genera do not appear to be monophyletic and at least one subfamily, the Gecarcunicinae, may be paraphyletic. The latter is supporting the findings of Klaus et al. (2009). Our data therefore suggests that the lower-level taxonomy of the Old World freshwater crabs is in need of revision if the taxonomy is to better reflect phylogenetic relationships within families and subfamilies.

3.4.2 Biogeographical routes in a temporal framework

For many groups of animals and plants, it is sill unclear whether their distribution is best explained by vicariance or by long distance dispersal, which leads to vigorous debates in literature (e.g., de Queiroz, 2005; Teeling et al.,

2005; Bossuyt et al., 2006; Noonan & Chippindale, 2006; Waters and Craw, 2006; Yoder and Novak, 2006; Azuma et al., 2008). Also for fresh water crabs, it is still unclear how they attained their present day distribution, and analyses specifically aiming at elucidating this problem are scarce (Daniels et al., 2006; Klaus et al., 2006, 2009; Feldmann et al., 2007). By using the ancestral area reconstructions performed on the two time divergence approaches [further indicated as A1 (prior 107 Mya) and A2 (prior 49 Mya)], which reflect the uncertainty as to the closest (marine) relative to the Potamoidea (*sensu* Cumberlidge and Ng, 2009; Shih et al., 2009), we will postulate the most plausible historical biogeographical pattern for the Old World true freshwater crabs (Potamoidea).

Our results show that the Old World freshwater crabs evolved from a common ancestor that occurred on the Asian Sunda Shelf (split A, Fig 3 and 4). These findings contradict an African origin for the Old World freshwater crabs (contra Klaus et al., 2006; Feldmann et al., 2007; Table 3). The drifting of landmasses from their original Gondwanan position towards their current position started at least 175 Mya (Scotese, 2001; Hedges, 2003). The Antarctica-Australia-Madagascar-Seychelles-India block broke off from Africa-South-America around 165-155 Mya ago (Briggs, 2003; Schettino & Scotese, 2005). Africa and South-America started drifting apart in the Mid-Cretaceous. The Indian/Madagascan block started drifting from the Antarctica-Australia block already in the Early Cretaceous (around 135 mya; Powell et al., 1988; Brown et al., 2003) and the India-Seychelles block drifted away from Madagascar between 100 and 88 Mya (see overview in Yoder & Nowak, 2006). In case vicariance was the main cause of speciation for the major lineages of the freshwater crabs, the evolutionary relationships should at least reveal these successive events, e.g. the phylogeny would show a sister-group relationship between the Indian and the Malagasy fauna. Neither the phylogenetic relationships nor the posterior dating estimates (Table 1) reflect this pattern of events. Even alternative tectonic models, which suggest other temporary land bridges or narrower distances between continents during drifting (Patriat and Segoufin, 1988; Krause et al., 1997; Chatterjee and Scotese, 1999; Hay et al., 1999; Briggs, 2003; Rage, 2003, Noonan & Chippindale, 2006), are not congruent with our time estimates.

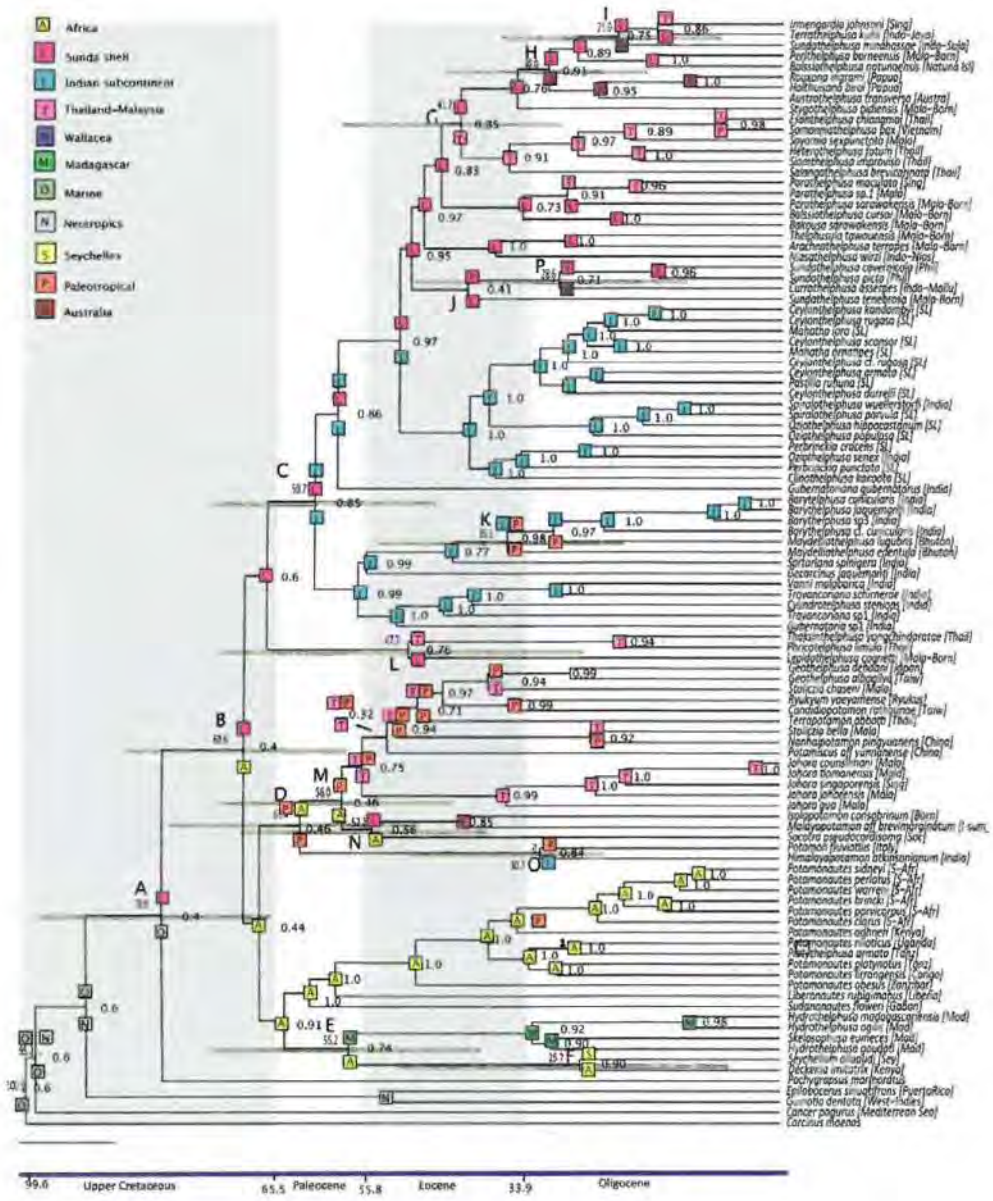


Fig. 3: see legend next page

Fig.3: The chronogram of the Bayesian topology for the subsample (107 taxa). The tree is rooted with a mean prior distribution (rttm) of 107 Mya with the 95% credibility interval (CI). The following minimum calibration constraints were used (1) a fossil of *Potamon* of 24 Mya, (2) a fossil of *Potamonautes niloticus* of 6 Mya sediments and (3) the minimum age of Cancridae (mid Eocene-Schweitzer et al., 2002). The geographical position (country) of the specimen is indicated in parentheses after the species name (abbreviations: SL-Sri Lanka; Mala-Malaysia; Papua-Papua New Guinea; Thail-Thailand; S-AFr-South-Africa; Soc-Socotra; Mad-Madagascar; Tanz-Tanzania; Sey-Seychelles; Indo-Indonesia; Sing-Singapore; I-sum-Indonesia, Sumatra; Taiw-Taiwan; Indo-Born-Indonesia, Borneo; Indo-Sul-Indonesia, Sulawesi; Phil-Philippines). The maximum likelihood ancestral range reconstruction (under a DEC-model; Lagrange, Ree et al., 2005; Ree & Smith, 2008) is plotted on the chronogram. On the right side of the node, the highest relative probabilities of the ancestral range reconstruction are shown. Abbreviations of the ranges are indicated in the figure. Splits A - O represent biogeographical events described in the text and Table 1. On the left side of these splits the estimated age and the 95% CI is given.

Fig. 4: The chronogram of the Bayesian topology for the subsample, with the elimination of *Carcinus maenas* (106 taxa). The tree is rooted with a mean prior distribution (rttm) of 49 Mya, with the 95% credibility interval (CI). The following minimum calibration constraints were used (1) a fossil of *Potamon* of 24 Mya, (2) a fossil of *Potamonautes niloticus* of 6 Mya sediments and (3) the minimum age of Grapsidae (Brösing, 2008). The geographical position (country) of the specimen is indicated in parentheses (abbreviations: SL-Sri Lanka; Mala-Malaysia; Papua-Papua New Guinea; Thail-Thailand; S-AFr-South-Africa; Soc-Socotra; Mad-Madagascar; Tanz-Tanzania; Sey-Seychelles; Indo-Indonesia; Sing-Singapore; I-sum-Indonesia, Sumatra; Taiw-Taiwan; Indo-Born-Indonesia, Borneo; Indo-Sul-Indonesia, Sulawesi; Phil-Philippines). The maximum likelihood ancestral range reconstruction (under a DEC-model; Lagrange, Ree et al., 2005; Ree & Smith, 2008) is plotted on the chronogram. On the right side of the node, the highest relative probabilities of the ancestral range reconstruction are shown. Abbreviations of the ranges are indicated in the figure. Splits A - O represent biogeographical events described in the text and Table 1. On the left side of these splits the estimated age and the 95% CI (grey bars) is given.

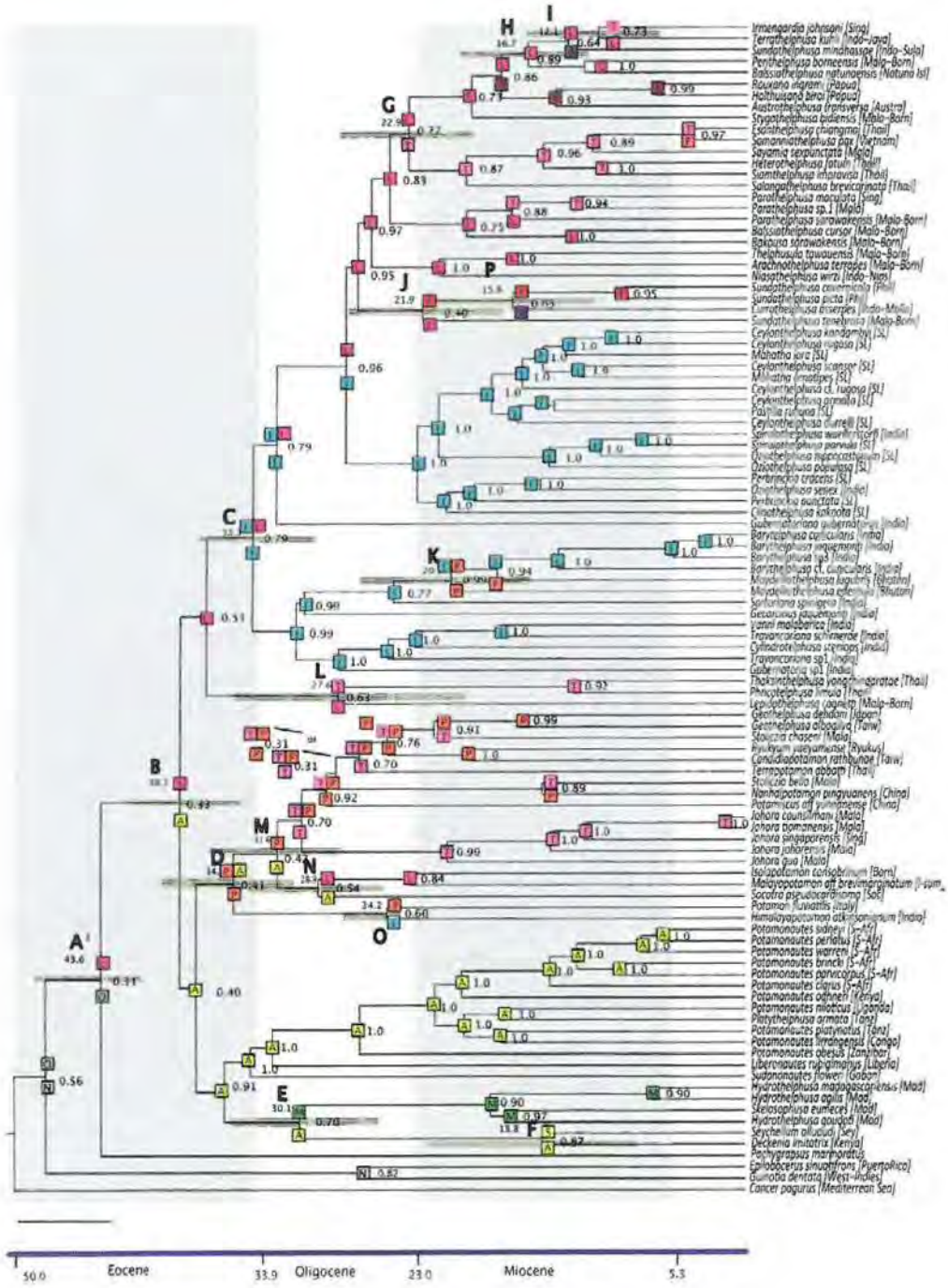


Fig. 4: see previous page

Therefore, a post-Gondwanan dispersal is the most likely scenario explaining the present day distribution of the Old World freshwater crabs, which started diversifying around late Eocene (split B; A2, Table 1 and Fig. 4). In what follows, we will elaborate on specific (possibly constraining) historical biogeographical patterns, which imply several instances of long distance dispersal. Moreover, we will discuss that in most instances the dispersal events better fit a more recent time estimate (A1), then an older one (A2) (Table 1 and Fig. 3).

The Old World freshwater crabs started diversifying from a common ancestor, occurring on the Sunda shelf, in the late Eocene. This ancestor gave rise to two clades, one of which dispersed to Africa (split B; Table 1 and Fig. 3 and 4). This dispersal can only be explained by an overseas dispersal event, even when the oldest time estimate approach (A2) is considered. Both landmasses were never in close proximity within both time frames.

This 'African' lineage diversified shortly afterwards into two lineages. The first lineage, the Potamonautidae diversified further on the African continent. Within this clade, two overseas dispersal events can be seen both within the subclade Deckeniinae. The first occurs in the early Oligocene (split E; Fig. 4 and Table 1; 30.1 Mya; 23.9–36.3), when Madagascar is colonized, followed by an endemic radiation on this island (Fig. 1; Cumberlidge et al., 2008). This colonization event was possible through the land bridge between Madagascar and East-Africa between 45–26 Mya (McCall 1997). The estimated time frame of the older time divergence analysis (A1) covers this 'land bridge' period only marginally (A1: 55.2 Mya; 42.1 -69.1; Fig. 3) and is therefore less likely. A second overseas dispersal event is the colonization of the Seychelles, out of Africa (split F; Table 1 and Fig. 3 and 4).

The second lineage that originates from the original 'African ' lineage, is the emnophyletic Potamidae. This Potamidae almost immediately expanded its range towards the Palaearctic (split D). The African continent was isolated from other tropical landmasses through the late Cretaceous and only came into close proximity to Eurasia (i.e., the Iberian peninsula) by the late Eocene (39-36 Mya), which corresponds well with the most recent time estimates (A2; Table 1).

Again the older estimates (A1) would need oceanic dispersal to explain this range expansion. The overland diversification of one lineage within Potamidae from the Palaearctic range towards the Thai-Malaysian peninsula (split M) can easily be explained by overland dispersal and hence vicariance.. The sister-group of this lineage, on the other hand, dispersed in Africa and eventually even returns to the Sunda landmasses. This back migration from Africa to the Sunda Shelf (split O) can be inferred because of the consistent sister-group relationship between the species of Potamidae of Socotra Island (Yemen) in the Indian Ocean and the Southeast Asian potamiscines (clade 7 - split N). Within both time approaches (A1 and A2; Table 1 and Fig. 3 and 4) this migration can only be explained by supposing oceanic dispersal. Socotra has an interesting biogeographical position, lying on the boundaries of the Afrotropical, Oriental and Palearctic zones. Socotra has Gondwanan origins and drifted from Africa on the Arabian plate about 40 Mya. In the Eocene era (41-34 Mya; Braithwaite, 1987) Socotra started drifting towards its current position. Even if the Socotra-based genera would be the actual sister clade to clade 4 (Fig. 2) (a phylogenetic relationship that could not be rejected by the AU and SH test for *Socotra pseudocardisoma*; Table 2), only an oceanic dispersal event can explain the patterns shown by A1 and A2. Within the same family, the representatives of Potamidae ocuuring in the Palaearctic (split O; Table 1 and Fig. 3 and 4) colonized India, which concurs with the same biogeographical events noticed in the Gecarcinucidae (split C; see explanation below).

The ancestors of the Gecarcinucidae (Fig. 1 and 2) occurred on the Sunda Shelf and diversified further on the Asian-Austrialian continent (Fig. 3 and 4). The ancestral area reconstruction indicates the expansion towards the Indian Subcontinent (split C), followed by an explosive radiation on this subcontinent. This conclusions rather supports an 'Into-India'-hypothesis for the freshwater crabs. Therefore, for our group, the popular 'Out-of-India' hypothesis (Bossuyt & Millinkovitch, 2001; Conti et al., 2002), which refers to the rafting of Gondwanan lineages on India and dispersal into Southeast Asia after collision of India (about 50 Mya) with the Eurasian continent (Metcalfe, 1999) or about 35 Mya (Ali & Aitchison, 2008), does not hold. The latter revised hypothesis even predicts a short collision with the Sundaland area. The most recent time estimates for this

earliest freshwater crab colonization on the Indian Subcontinent support the overland colonization from Sundaland on the Indian Subcontinent.

Within the same family, the Gecarcinucidae, but within the lineage that dispersed and radiated on South- (East) Asia, several oceanic dispersals, including the crossing of the Wallace line occurred (split H, J, P and I of Fig. 3 and 4).

Within all three families, several migrations to and from continental islands have occurred, often followed by extensive radiation and high levels of endemism. Denser sampling would be needed to provide enough data to predict migration over sea or via landbridges, but at least time estimates predate already the often-used explanations of the quaternary (Pleistocene) low-level sea periods, e.g., Sri Lanka, Taiwan, Madagascar, Socotra (see also Bossuyt et al., 2004; Shih et al., 2006, 2009; Daniels et al., 2006).

The divergences that lead to the contemporary distribution of the Old World freshwater crabs are too recent to be the result of continental drift vicariance. We infer a post-Gondwanan cladogenesis, which originated in mid Eocene. The posterior time estimates from the analysis with the prior of 49 Mya (A2), supports the contemporary distribution in a less complex historical biogeographical framework (i.e. less oceanic dispersals) than does the time frame estimated with the prior of 107 Mya. However, we conclude that multiple oceanic dispersals - probably via rafting (see also Shih et al., 2004; Yeo et al., 2007) - occurred independently in all three major clades. The limited tolerance to salt water supports these inferences. In a future prospective, several time constraints (e.g., age of volcanic islands, such as Taiwan) (Fig. 3 and 4) could be included in the probability model (Ree & Smith, 2008). Additionally, the discovery of new relevant fossils and the resolution of the closest marine ancestor of this Old World freshwater crab clade would evidently improve the evaluation of the above-reconstructed biogeographical routes. As a final note we would like to mention that not only the above extra data, but also the continuous evolution within the geological and paleogeographical reconstructions will probably allow for more precise inferences in the future.

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Supplementary information:

S1: List of putative genus, species, voucher number, family, geographic location of sample and the absence or presence of the gene fragment for each locus (fragments of 16S, COI and histone 3) used in the analyses. For taxa with the same sequences, but different submissions on Genbank, only one was used since RAxML (likelihood estimates for the phylogenetic tree) does not accept duplicates.

Sources for our samples are Bossuyt et al., 2004, Beenaerts et al., 2009, Ng & Tay, 2001; Bahir & Ng, 2005; Bahir and Yeo, 2005, Daniels et al., 2006, Klaus et al., 2009, Shih et al., 2004, 2006, 2009, Schubart & Ng, 2008.

	Genus	species	Country	Family	HISTON	16S	COI
*	Arachnothelphusa	terrapes	Borneo	Cecarcinucidae	-	GenBank	GenBank
*	Austrothelphusa	transversa	Australia	Gecarcinucidae	FM178896	FM180122	-
*	Bakousa	sarawakensis	Mala-Borneo	Gecarcinucidae	FM178899	FM180124	-
*	Balssiathelphusa	cursor	Mala-Borneo	Gecarcinucidae	FM178900	FM180126	-
*	Balssiathelphusa	natunaensis	Natuna	Cecarcinucidae	-	GenBank	GenBank
*	Barytelphusa	cunicularis	India	Cecarcinucidae	-	AY708065	GQ289634
*	Barythelphusa	cf. cunicularis	India	Gecarcinucidae	AY919132	AY919090	AY919113
*	Barythelphusa	jacquemonti	India	Gecarcinucidae	AY919129 of AY919128	AY919088	AY919110
*	Barythelphusa	sp.3	India	Gecarcinucidae	AY919130	AY919092	AY919116
*	Cancer	pagurus	Marine	Cancridae	DQ079668/FM208806	DQ079708	AF060771
*	Candidiopotamon	rathbunae	Taiwan	Potamidae	AB290668	AB208591	AB290649
*	Carcinus	maenas	Marine	Portunidae	AY919138	AY919095	DQ523685
*	Ceylonthelphusa	armata	SL	Cecarcinucidae	-	AY708075	GQ289641
*	Ceylonthelphusa	cf. rugosa	SL	Gecarcinucidae	AY803716	AY803587	AY803560
*	Ceylonthelphusa	durrelli	SL	Cecarcinucidae	-	AY708091	GQ289660

*	Ceylonthelphusa	kandambyi	SL	Cecarcinucidae	-	AY708084	GQ289653
*	Ceylonthelphusa	rugosa	SL	Cecarcinucidae	-	GQ289594	GQ289643
*	Ceylonthelphusa	scansor	SL	Cecarcinucidae	-	AY708052	GQ289617
*	Clinothelphusa	kakoota	SL	Cecarcinucidae	-	GQ289608	GQ289664
*	Currothelphusa	asserpes	Indo-Moluccas	Gecarcinucidae	FM178902	GenBank	-
*	Cylindrotelphusa	steniops	India	Cecarcinucidae	FM178903	AY708073	GQ289639
*	Deckenia	imitatrix	Kenya	Potamonautidae	-	AY803544	AY803576
*	Epilobocera	sinuatifrons	Puerto Rico	Pseudothelphusidae	FM208830	AJ130810	EU004939
*	Esanthelphusa	chiangmai	Thailand	Cecarcinucidae	-	GenBank	GenBank
*	Gecarcinus	jaquemonti	India	Gecarcinucidae	AY919126	AY919086	AY919108
*	Geothelphusa	albogilva	Taiwan	Potamidae	AY803715	AY803559	AY803586
*	Geothelphusa	dehaani	Japan	Potamidae	AB290667	AB290630	AB290648
*	Gubernatoria	sp.1	India	Cecarcinucidae	-	AY708087	GQ289655
*	Gubernatoriana	gubernatorus	India	Gecarcinucidae	AY919131	AY919089	AY919112
*	Guinotia	dentata	West Indies	Pseudothelphusidae	AY803723	-	AY803593
*	Heterothelphusa	fatum	Mala	Gecarcinucidae	FM178905	FM180131	-
*	Himalayapotamon	atkinsonianum	India	Potamidae	AB290670	AB290632	AB290651
*	Holthuisana	cf. biroi	New	Gecarcinucidae	-	FM180132	-

			Guinea				
*	Hydrothelphusa	aff. madagascariensis2	Madagascar	Potamonautidae	AY803703		
*	Hydrothelphusa	agilis	Madagascar	Potamonautidae	AY803700	AY803546	AY803578
*	Hydrothelphusa	goudoti	Madagascar	Potamonautidae	AY803702	AY803548	AY803579
*	Irmengardia	johnsoni	Singapore	Gecarcinucidae	FM178908	-	+
*	Isolapotamon	consobrinum	Mala-Borneo	Potamidae	AY803713	AY803557	-
*	Johora	counsilmani	Mala	Potamidae	AB290652	-	-
*	Johora	gua	Mala	Potamidae	AB290655	AB290617	-
*	Johora	johorensis	Mala	Potamidae	AB290657	-	-
*	Johora	singaporensis	Singapore	Potamidae	FM178886	FM180114	AB290641
*	Johora	tiomanensis	Mala	Potamidae	AB290663	AB290626	AB290644
*	Lepidothelphusa	cognetti	Mala-Borneo	Gecarcinucidae	FM178909	FM180134	-
*	Liberonautes	rubigimanus	Liberia	Potamonautidae	AY803697	AY803543	-
*	Mahatha	adonis	SL	Cecarcinucidae	-	GQ289586	GQ289614
*	Mahatha	cf. iora	SL	Cecarcinucidae	-	AY708055	GQ289620
*	Mahatha	sp. 2	SL	Cecarcinucidae	-	AY708078	GQ289644
*	Malayopotamon	aff.	Indo-	Potamidae	FM178887	SUBMITTEN	-

		brevimarginatum	Sumatra				
*	Maydelliathelphusa	edentula	Bhutan	Gecarcinucidae	FM178911	FM180136	-
*	Maydelliathelphusa	lugubris	Bhutan	Gecarcinucidae	FM178912	FM180137	-
*	Nanhaipotamon	pingyuanens	China	Potamidae	-	-	AB265249
*	Niasathelphusa	wirzi	Indo- Nias	Gecarcinucidae	FM178913	FM180138	-
*	Oziothelphusa	hippocastanum	SL	Cecarcinucidae	-	GQ289612	GQ289668
*	Oziothelphusa	populosa	SL	Cecarcinucidae	-	AY708060	GQ289631
*	Oziothelphusa	senex	India	Gecarcinucidae	AY919133	-	AY919114
*	Pachygrapsus	marmoratus	Spain	Grapsidae	AY919137	DQ079728	-
*	Parathelphusa	maculata	Singapore	Gecarcinucidae	FM178917	FM180142	-
*	Parathelphusa	sarawakensis	Mala-Borneo	Gecarcinucidae	FM178920	FM180145	-
*	Parathelphusa	sp.1	Malaysia	Gecarcinucidae		AY803561	AY803588
*	Pastilla	ruhuna	SL	Cecarcinucidae	-	AY708082	GQ289651
*	Perbrinckia	cracens	SL	Cecarcinucidae	-	GQ289606	GQ982589
*	Perbrinckia	punctata	SL	Cecarcinucidae	-	GQ289598	GQ289648
*	Perithelphusa	borneensis	Mala-Borneo	Gecarcinucidae	FM178921	FM180146	-
*	Phricotelphusa	limula	Thailand	Gecarcinucidae	AY803721 of FM178926	GenBank	GenBank

*	Platythelphusa	armata	Tanzania	Potamonautidae	FM178893	FM180120	-
*	Potamiscus	aff. yunnanense	China	Potamidae	AB290666	AB290629	AB290647
*	Potamon	fluviatilis	Italy	Potamidae	AY803710	AY803554	AY803584
*	Potamonautes	brincki	S-Afr.	Potamonautidae	AY803674	AY042244	AF510875
*	Potamonautes	clarus	S-Afr.	Potamonautidae	AY803676	AY042241	AF510872
*	Potamonautes	lirrangensis	DRC	Potamonautidae	AY803682	AY803534	AY803568
*	Potamonautes	niloticus	Uganda	Potamonautidae	AY803685	AY803536	N/a
*	Potamonautes	obesus	Zanzibar	Potamonautidae	AY803686	AY803537	AY803570
*	Potamonautes	odhneri	Kenya	Potamonautidae	AY803687	AY803538	AY803571
*	Potamonautes	parvicorpus	S-Afr.	Potamonautidae	AY803689	AY042252	AF510869
*	Potamonautes	perlatus	South-Africa	Potamonautidae	AY803690	AY042249	AF510874
*	Potamonautes	platynotus	Tanzania	Potamonautidae	AY803691	AY803539	AY803572
*	Potamonautes	sidneyi	S-Afr.	Potamonautidae	AY803693	AY042245	AF510871
*	Potamonautes	warreni	S-Afr.	Potamonautidae	AY803695	AY042251	AF510880
*	Rouxana	ingrami	New Guinea	Gecarcinucidae	AY803720	AY803563	AY803589
*	Ryukyum	yaeyamense	Okinawa	Gecarcinucidae	AB290669	AB290631	AB290650
*	Salangathelphusa	brevicarinata	Thailand	Gecarcinucidae	FM178928	GenBank	-

*	Sartoriana	spinigera	India	Gecarcinucidae	FM178930/AY803722	GQ289604	GQ289661
*	Sayamia	sexpunctata	Malaysia	Gecarcinucidae	FM178932	AY803564	AY803590
*	Seychellum	alluaudi	Seychelles	Potamoidea incert...sedis	FM178894	AM234653	-
*	Siamthelphusa	improvisa	Thailand	Gecarcinucidae	FM178934	GenBank	-
*	Skelosophusa	eumeces	Madagascar	Potamonautidae	-	AY803553	AY803583
*	Socotra	pseudocardisoma	Socotra	Potamidae	-	AY803555	AY803585
*	Somanniathelphusa	pax	Viet Nam	Cecarcinucidae	-	GenBank	-
*	Spiralothelphusa	parvula	India	Cecarcinucidae	-	GQ289613	GQ289669
*	Spiralothelphusa	wuellerstorfi	SL	Cecarcinucidae	-	AY708090	GQ289658
*	Stoliczia	bella	Mala	Potamidae	FM178889	FM180117	-
*	Stoliczia	chaseni	Mala	Potamidae	AB290664	AB290627	AB290645
*	Stygothelphusa	bidiensis	Mala	Gecarcinucidae	FM178943	FM180170	-
*	Sudanonautes	floweri	Bioko	Potamonautidae	AY803696	AY803541	AY803574
*	Sundathelphusa	cavernicola	Philippines	Gecarcinucidae	FM178937	FM180162	-
*	Sundathelphusa	minahassae	Indo-Sulawesi	Gecarcinucidae	FM178939	AM234651	-
*	Sundathelphusa	picta	Philippines	Gecarcinucidae	FM178940	FM180166	-
*	Sundathelphusa	tenebrosa	Mala-Borneo	Gecarcinucidae	FM178942	FM180169	-

*	Terrapotamon	abbotti	Thailand	Potamidae	AB290665	AB290628	AB290646
*	Terrathelphusa	kuhli	Indo- Java	Gecarcinucidae	FM178945	FM180172	-
*	Thaksinthelphusa	yongchindaratae	Thailand	Gecarcinucidae	FM178946	FM180173	-
*	Thelphusula	tawauensis	Mala-Borneo	Gecarcinucidae	FM178950	FM180177	-
*	Travancoriana	schirnerae	India	Cecarcinucidae	-	AY708066	GQ289635
*	Travancoriana	sp. 1	India	Cecarcinucidae	-	AY708072	GQ289638
*	Vanni	malabarica	India	Gecarcinucidae	FM178951	FM180180	-
	Aegla	laevis	S-AM	Aeglidae/Anomura	-	AY050037	AY050083
	Aegla	marginata	S-AM	Aeglidae/Anomura	-	AY595896	AY595642
	Amamiku	amamense	Ryukus	Potamidae	-	AB208630	-
	Arachnothelphusa	rhadamanthysi	Mala-Borneo	Gecarcinucidae	FM178895	FM180121	-
	Austrothelphusa	sp.1	Australia	Gecarcinucidae	FM178897	FM180123	-
	Barytelphusa	sp.1	India	Cecarcinucidae	-	AY708071	GQ289637
	Barythelphusa	cf. cunicularis	India	Gecarcinucidae	AY919127	-	-
	Barythelphusa	sp.	India	Gecarcinucidae	AY919134 of AY919130	-	-
	Barythelphusa	sp.2	India	Gecarcinucidae	AY919135	-	-
	Ceylonthelphusa	alpina	SL	Cecarcinucidae	-	GQ289587	GQ289623

Ceylonthelphusa	cavatrix	SL	Cecarcinucidae	-	AY708057	GQ289624
Ceylonthelphusa	cf. cavatrix	SL	Cecarcinucidae	-	GQ289590	GQ982586
Ceylonthelphusa	cf. kandambyi	SL	Gecarcinucidae	FM178901	-	-
Ceylonthelphusa	cf. rugosa2	SL	Cecarcinucidae	-	AY708056	GQ289622
Ceylonthelphusa	diva	SL	Cecarcinucidae	-	GQ289588	GQ289626
Ceylonthelphusa	sanguinea	SL	Cecarcinucidae	-	GQ982590	GQ289621
Ceylonthelphusa	sentosa	SL	Cecarcinucidae	-	AY708081	GQ289649
Ceylonthelphusa	sp.1	SL	Cecarcinucidae	-	AY708051	GQ289616
Ceylonthelphusa	venusta	SL	Cecarcinucidae	-	GQ289610	GQ289666
Cylindrotelphusa	aff. steniops	India	Gecarcinucidae	FM178903	-	-
Deckenia	mitis	Tanzania	Potamonautidae	FM178890	FM180118	-
Gecarcinus	jaquemonti	India	Gecarcinucidae	FM178904	-	-
Geelvinkia	holthuisi	New Guinea	Gecarcinucidae	FM178898	FM180129	-
Geithusa	lentiginosa	Mala	Cecarcinucidae	-	FM180130	-
Geothelphusa	sp.1	X	Potamidae	DQ079677	-	-
Holthuisana	biroi	New Guinea	Gecarcinucidae	FM178906	-	-
Holthuisana	festiva	New Guinea	Gecarcinucidae	FM178907	FM180133	-

Hydrothelphusa	aff. madagascariensis1	Madagascar	Potamonautidae	-	AY803549	AF399972
Hydrothelphusa	madagascariensis	Madagascar	Potamonautidae: Dec keniina	FM178891	AY803549	AY803580
Hydrothelphusa	bombetokensis	Madagascar	Potamonautidae	AY803701	-	-
Johora	gapensis	Mala	Potamidae	AB290653	-	-
Johora	grallator	Mala	Potamidae	AB290654	-	-
Johora	intermedia	Mala	Potamidae	AB290656	-	-
Johora	murphyi	Mala	Potamidae	AB290658	-	-
Johora	punicea	Mala	Potamidae	AB290659	-	-
Johora	tahanensis	Mala	Potamidae	AB290661	-	-
Johora	thoi	Mala	Potamidae	AB290662	-	-
Liotelphusa	gagei	Bhutan	Gecarcinucidae	FM178910	FM180135	-
Madagapotamon	humberti	Madagascar	Potamonautidae	AY803704	AY803550	-
Mahatha	cf. ornatipes	SL	Cecarcinucidae	-	GQ289607	GQ289663
Mahatha	iora	SL	Cecarcinucidae	-	AY708074	GQ289640
Mahatha	ornatipes	SL	Cecarcinucidae	-	GQ289599	GQ289650
Mahatha	sp.1	SL	Cecarcinucidae	-	AY708059	GQ289627
Mahatha	sp.2	SL	Cecarcinucidae	-	GQ289591	GQ289628

Malagasya	antongilensis	Madagascar	Potamonautidae	-	AY803551	-
Malagasya	SAD-2004	Madagascar	Potamidae	-	-	AY803581
Marojejy	longimerus	Madagascar	Potamonautidae	-	AY803552	AY803582
Migmathelphusa	olivacea	Indo-Sulawesi	Gecarcinucidae	-	AM292917	-
Nautilothelphusa	zimmeri	Indo-Sulawesi	Gecarcinucidae	-	AM292907.	-
Oziothelphusa	aurantia	SL	Cecarcinucidae	-	GQ289603	GQ289659
Oziothelphusa	biloba	India	Cecarcinucidae	-	GQ289600	GQ289654
Oziothelphusa	ceylonensis	SL	Gecarcinucidae	FM178914		
Oziothelphusa	gallicola	SL	Cecarcinucidae	-	GQ289597	GQ289647
Oziothelphusa	kerala	SL	Cecarcinucidae	-	AY708062	GQ982587
Oziothelphusa	sp.1	SL	Cecarcinucidae	-	AY708083	GQ289652
Oziothelphusa	sp.2	SL	Cecarcinucidae	-	AY708053	GQ289618
Oziothelphusa	sp.3	SL	Gecarcinucidae	FM178915	-	-
Oziothelphusa	stricta	SL	Cecarcinucidae	-	AY708086	GQ289615
Parathelphusa	celebensis	Indo-Sulawesi	Gecarcinucidae	-	AM292922	-
Parathelphusa	convexa	Indo-Sulawesi	Gecarcinucidae	FM178916	FM180141	-

Parathelphusa	oxygona	Mala-Borneo	Gecarcinucidae	FM178918	FM180143	-
Parathelphusa	pallida	Indo-Sulawesi	Gecarcinucidae	-	AM292914	-
Parathelphusa	pantherina	Indo-Sulawesi	Gecarcinucidae	FM178919	FM180144	-
Parathelphusa	possoensis	Indo-Sulawesi	Gecarcinucidae	-	AM292916	-
Parathelphusa	sp.2	Malaysia	Gecarcinucidae	AY803718	-	-
Perbrinckia	fenestra	SL	Cecarcinucidae	-	AY708076	GQ289642
Perbrinckia	morrayensis	SL	Cecarcinucidae	-	AY708058	GQ289625
Perbrinckia	nana	SL	Cecarcinucidae	-	AY708054	GQ289619
Perbrinckia	rosae	SL	Cecarcinucidae	-	GQ289601	GQ289657
Perbrinckia	uva	SL	Cecarcinucidae	-	GQ289593	GQ289630
Perithelphusa	borneensis	Mala-Borneo	Cecarcinucidae	-	GenBank	-
Perithelphusa	lehi	Mala-Borneo	Gecarcinucidae	FM178922	FM180147	-
Phricotelphusa	amnicola	Malaysia	Gecarcinucidae	FM178923	FM180148	-
Potamon	ibericum	Turkey	Potamidae	-	AM234645	-
Potamon	sp.2	?	Potamidae	-	DQ028733	-

Potamon	persicum	Iran	Potamonautidae	FM178888	FM180116	-
Potamon	sp.1	?	Potamidae	-	AM234647	-
Potamonautes	bayonianus	X	Potamonautidae	-	-	AF510868
Potamonautes	calcaratus	S-Afr.	Potamonautidae	AY803675	AY042242	AF510867
Potamonautes	dentatus	S-Afr.	Potamonautidae	AY803677	AY042246	AF510878
Potamonautes	depressus	S-Afr.	Potamonautidae	AY803678	AY042247	AF510877
Potamonautes	ecorssei	Mali	Potamonautidae	AY803679	-	-
Potamonautes	emini	DRC	Potamonautidae	AY803680	AY803533	N/a
Potamonautes	granularis	S-Afr.	Potamonautidae	AY803681	AY042254	AF510876
Potamonautes	lividus	S-Afr.	Potamonautidae	AY803683	AY042248	AF510879
Potamonautes	parvispina	S-Afr.	Potamonautidae	AY803688	AY042253	AF510873
Potamonautes	raybouldi	Tanzania	Potamonautidae	AY803692	AY803540	AY803573
Potamonautes	unispinus	S-Afr.	Potamonautidae	AY803694	AY042250	AF510870
Potamonautes	sp.1	S-Afr.	Potamonautidae	AY803684	-	-
Sartoriana	blandfordi	Iran	Gecarcinucidae	FM178929	-	-
Sayamia	bangkokensis	Thailand	Gecarcinucidae	FM178931	FM180155	-
Sayamia	cf. germaini	Viet Nam	Gecarcinucidae	-	-	AB265250
Sendleria	gloriosa	New Guinea	Gecarcinucidae	FM178933	FM180157	-

Seychellum	alluaudi	Seychelles	Potamoidea incert...sedis	AY803699	AY803545	AY803577
Siamthelphusa	holthuisi	Thailand	Gecarcinucidae	-	AM234650	-
Siamthelphusa	sp.1	Thailand	Gecarcinucidae	FM178935	-	-
Sinopotamon	yangtsekiense	China	Potamidae	-	-	EU676303
Snaha	escheri	India	Gecarcinucidae	-	FM180160	-
Somanniathelphusa	amoyensis	China	Gecarcinucidae	-	-	AB265242
Somanniathelphusa	qiongshanensis	China	Gecarcinucidae	-	-	AB265248
Somanniathelphusa	taiwanensis	Taiwan	Gecarcinucidae	-	-	AB265241
Somanniathelphusa	zanklon	China	Gecarcinucidae	-	-	AB265244
Somanniathelphusa	zhangpuensis	China	Gecarcinucidae	-	-	AB265243
Somanniathelphusa	zhapoensis	China	Gecarcinucidae	-	-	AB265246
Spiralothelphusa	fernandoi	SL	Cecarcinucidae	-	GQ289611	GQ289667
Sudanonautes	aubryi	Nigeria	Potamonautidae	-	AY803542	AY803575
Sundathelphusa	boex	Philippines	Gecarcinucidae		GenBank	-
Sundathelphusa	cf. tenebrosa	Mala-Borneo	Gecarcinucidae	-	FM180169	-
Sundathelphusa	hades	Philippines	Gecarcinucidae	FM178938	FM180164	-
Sundathelphusa	halmaherensis	Indo-Halmahera	Gecarcinucidae	-	FM180165	-

Sundathelphusa	molluscivora	Indo-Sulawesi	Gecarcinucidae	-	AM292918	-
Sundathelphusa	rubra	Indo-Sulawesi	Gecarcinucidae	FM178941	FM180167	-
Sundathelphusa	sp.1	Indo-Sulawesi	Potamonautidae	-	AM292919	-
Syntripsa	flavichela	Indo-Sulawesi	Gecarcinucidae	-	AM292921	-
Syntripsa	matannesis	Indo-Sulawesi	Gecarcinucidae	-	AM234643	-
Thelphusula	baramensis	Mala-Borneo	Gecarcinucidae	FM178947	FM180174	-
Thelphusula	hulu	Mala-Borneo	Gecarcinucidae	FM178948	FM180175	-
Thelphusula	sabana	Mala-Borneo	Gecarcinucidae	FM178949	-	-
Travancoriana	pollicaris	India	Gecarcinucidae	-	FM180179	-
Travancoriana	sp. 2	India	Cecarcinucidae	-	AY708088	GQ289656
Travancoriana	sp. 4	India	Cecarcinucidae	-	AY708063	GQ289632
Vanni	nilgiriensis	India	Gecarcinucidae	-	FM180181	-

Chapter 04

Local endemism in the Western Ghats/Sri Lanka biodiversity hotspot

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LOCAL ENDEMISM IN THE WESTERN GHATS/SRI LANKA BIODIVERSITY HOTSPOT

Abstract

The apparent biotic affinities between the mainland and the island in the Western Ghats/Sri Lanka biodiversity hotspot have been interpreted as the result of frequent migrations during periods of low sea level. Here we show, using molecular phylogenies of two invertebrate and four vertebrate groups, that biotic interchange between these areas has been much more limited than hitherto assumed. Despite several extended periods of land connection during the past 500,000 years, Sri Lanka has maintained a fauna that is largely distinct from that of the Indian mainland. Future conservation programs for the subcontinent should take into account such patterns of local endemism at the finest scale at which they may occur.

4. 1. Introduction

Island biota typically are closely related to the source of colonists when both areas have been in regular contact (*1-3*). The level of endemism on continental islands is therefore expected to reflect the number and duration of ocean-level lowstands that allowed exchange with the mainland (*4*). Sri Lanka is a relatively large island (*c.* 66,000 km2) in the Indian Ocean on the same shallow (maximum 70 m depth) continental shelf as India (*5*). During Pleistocene ice ages, Sri Lanka was intermittently connected to mainland India (*6*), until sea-level rise created the present disruption ~ 10,000 years ago (*7*) (Fig. 1). Classical comparisons of faunal elements from both sides of the Palk Strait indicate a high degree of morphological similarity in several groups, suggesting abundant, recent biotic interchange with southern India (*8-12*). Similar observations prompted Wallace (*13*) more than a century ago to recognize a Ceylonese (or Lankan) biogeographic region, associating Sri Lanka with the southernmost part of the Western Ghats, a hill range along the west coast of India (Fig 1A). Today, both areas are united in the Western Ghats/Sri Lanka biodiversity hotspot because they form "a community of species that fits together as a biogeographic unit" (*14*).

4.2. Material and methods

4.2.1. Sampling

Specimens of the six groups were sampled from 125 and 70 locations in Sri Lanka and southern India, respectively (see supplementary Table 1). For each group, a wide variety of both morphologically similar and divergent specimens from both sides of the oceanic barrier were randomly selected.

4.2.2. DNA methods and alignment

Mitochondrial DNA fragments were PCR-amplified and sequenced on both strands. Primers used in this study are provided in supplementary table S2. The following fragments were amplified and sequenced: for *treefrogs:* (i) a ~580 bp segment of the Cytb gene, (ii) a ~500 bp segment including portion of the ND1 gene, the complete $tRNA^{Ile}$ and $tRNA^{Gln}$ genes, and portion of the $tRNA^{Met}$ gene, (iii) a ~370 bp segment of the 12S rRNA gene; for caecilians: a ~375 bp segment of the 12S rRNA gene, a ~535 bp segment of the 16S rRNA gene and a ~690 bp segment of the Cytb gene; snakes: a ~375 bp segment of the 12S rRNA gene and a ~505 bp segment of the 16S rRNA gene; fishes: a ~590 bp segment of the 16S rRNA gene and a ~540 bp segment of the Cytb gene; shrimps and crabs: a ~1310 bp segment including portion of the 16S rRNA gene, $tRNA^{Val}$ and portion of the 12S rRNA gene.

4.2.3 Phylogenetic analyses

Sequences were aligned using ClustalX 1.64 (*S2*) and ambiguous sections were excluded for subsequent analyses. Plots of transitions and transversions against uncorrected and GTR-corrected pairwise distances indicated that none of the fragments showed saturation. Appropriate likelihood models were determined with Modeltest 3.06 (*S3*). An overview of the results of parsimony and Modeltest 3.06 analyses are provided in table S3.

Maximum parsimony (MP) analyses were performed using PAUP* 4.0b10 (*S4*). Heuristic maximum parsimony (MP) searches were performed with 10,000 replicates each with a random addition-starting tree (all characters unordered and equally weighted). Clade support under MP was calculated using nonparametric bootstrapping (*S5*) in 10,000 replicates (Fig S1). For our maximum likelihood analyses, we conducted 250 replicated metaGA searches using MetaPIGA 1.0.2b (*S6*), each with strict consensus pruning among four populations, using a HKY+G+I model (the most parameter-rich model

implemented in MetaPIGA) with the Ti/Tv ratio optimized every 200 generations. The 1,000 resulting trees were used to compute metaGa branch support values and thus estimate posterior probabilities of branches (Fig. 2).

The Bayesian analyses were performed using MrBayes v.3.0b4 (*S7*) under the models proposed by Modeltest 3.06. Four chains were run simultaneously for 2,000,000 generations and trees were sampled every 200 cycles. We discarded the first 2,000 trees as the "burn in". Hence Bayesian posterior probabilities were estimated as the 50% majority-rule consensus tree of the 8,000 last sampled trees (Fig. S2). Repeated runs confirmed the successful convergence to the posterior parameter distribution.

4.2.4. Time estimation

To evaluate whether biotic exchange between southern India and Sri Lanka occurred during late Pleistocene sea-level lowstands, we calculated conservative interval estimates for dates of divergence at every node that represents a split between Indian and Sri Lankan lineages (Table S4). As reliable calibration points were not available, we determined the median (and minimum and maximum) of all pairwise divergences between taxa on either side of each corresponding node. Because rates are unknown for most of our specific taxa, we based our divergence time estimates on a range of published mtDNA clock rates in each taxonomic group separately. Brachyuran rates reported for rDNA are fairly comparable, ranging from 0,65-0,88% (*S8*) to 0,9% (*S9*). The same range was used for shrimps (*S10*). We applied a broad range of divergence rates, between 0.65% per Myr (*S11*) and 1,25-1,32% per Myr reported for *Barbus* freshwater fishes in *cytb* (*S12*), to estimate divergences in *Puntius*. In amphibians, a range of divergence rates has been published: 0,38 % per Myr for rDNA and 0,77 % per Myr for *cytb* in newts (*S13*), 0,69% per Myr in the ND1-ND2 region of Bufonidae (*S14*) and 1% in 16S rDNA of Ranidae (*S15*). For snakes, we used a rate interval of 0.47 to 1.32 per Myr (*S16*).

Two of the nodes in our trees are backed by published estimates: first, the estimated divergence of 21 - 43 mya between *Philautus charius* and (*P. microtympanum, P. wynaadensis*) in (*S17*) corresponds to node 1 in our Fig. S2. Second, ref. (*S18*) estimated a minimum of 10-15 million years divergence between Sri Lankan and Indian Uropeltids, which corresponds to node 4 in Fig. S2.

	Node	Sequence divergence (%) Median	Min.	Max.	Range for mtDNA clock rate [Ref.]	Estimated time (Myr) Calculated	Published	Minimum age (Myr)	Score
Treefrogs	1	0.201	0.177	0.230	0.38 - 1.00 [12, 13, 14]	20.1 - 52.9	21-43	17.74	**
	2	0.095	0.081	0.122	0.38 - 1.00 [12, 13, 14]	9.6 - 25.3	-	9.11	**
Caecilians	3	0.099	0.093	0.105	0.38 - 1.00 [12, 13, 14]	9.9 - 26.0	-	9.25	**
Snakes	4	0.078	0.071	0.086	0.47 - 1.32 [15]	5.9 - 16.7	10-15	5.42	**
Fishes	5	0.107	0.101	0.108	0.65 - 1.32 [10, 11]	8.1 - 8.6	-	7.68	**
	6	0.048	0.047	0.050	0.65 - 1.32 [10, 11]	3.6 - 3.8	-	3.56	*
	7	0.098	0.086	0.126	0.65 - 1.32 [10, 11]	7.4 - 7.8	-	6.54	**
	8	0.035	0.031	0.058	0.65 - 1.32 [10, 11]	2.6 - 2.8	-	2.35	*
	9	0.005	0.004	0.005	0.65 - 1.32 [10, 11]	0.4 - 0.4	-	0.28	#
	10	0.030	0.030	0.032	0.65 - 1.32 [10, 11]	2.3 - 2.4	-	2.28	*
	11	0.024	0.023	0.024	0.65 - 1.32 [10, 11]	1.8 - 1.9	-	1.71	*
Shrimps	12	0.008	0.007	0.009	0.65 - 0.90 [7, 8, 9]	0.9 - 1.2	-	0.81	#
	13	0.094	0.080	0.128	0.65 - 0.90 [7, 8, 9]	10.4 - 14.4	-	8.88	**
	14	0.113	0.113	0.113	0.65 - 0.90 [7, 8, 9]	12.5 - 17.3	-	12.51	**
	15	0.052	0.052	0.052	0.65 - 0.90 [7, 8, 9]	5.8 - 8.0	-	5.78	**
Crabs	16	0.107	0.092	0.120	0.65 - 0.90 [7, 8, 9]	11.9 - 16.5	-	10.18	**
	17	0.055	0.029	0.066	0.65 - 0.90 [7, 8, 9]	6.1 - 8.4	-	3.20	*

Table S4. - Percent sequence divergences and time estimates. The range of nodal ages reflects the median divided by the max. and min. % divergence, *resp.*, for biotic exchange events indicated by purple numbers in Fig. S2. We also calculated a conservative minimum age (*i.e.*, the minimum sequence divergence divided by the maximum rate). Based on the latter calculation, 10 out of the 17 estimated times predate the sea-level lowstands of the past 500,000 years by an order of magnitude (*i.e.*, > 10 times, indicated by **), and five more by threefold (indicated by *). Only two exchange events are estimated younger than 1 million year (indicated by #).

We calculated a conservative minimum age of divergence (Table S4) as the minimum sequence divergence for a split in our data divided by the maximum published rate. Our estimates of nodal times corresponding to biotic exchange events pre-date the border of 500,000 years by an order of magnitude (*i.e.*, 10-fold) in 10 nodes and by three-fold in 5 nodes. These approximations should be viewed with caution, given that they are likely

subject to several problems discussed in the literature (*S19*). Additionally, some calculated splits may not represent the actual biotic exchange event between the mainland and the island Sri Lanka, because we can not rule out closer relationships of Asian taxa with Indian or Sri Lankan taxa in some groups (*cf.* remark 16 in References and Notes). Nevertheless, our cautious estimates indicate that the late Pleistocene sea-level lowstands had little influence on the dispersal of several of Sri Lanka's prominent faunal groups.

4.3. Discussion and conclusions

Here we explore the evolutionary relationships between the subcontinent's island and mainland fauna in two invertebrate and four vertebrate groups. The selected taxa are freshwater crabs (Parathelphusidae and Gecarcinucidae), freshwater shrimps (*Caridina*, Atyidae), treefrogs (*Philautus*, Rhacophorinae, Ranidae), caecilian amphibians (Ichthyophiidae and Uraeotyphlidae), shieldtail snakes (Uropeltidae) and freshwater fish (*Puntius*, Cyprinidae). These animals occupy a diverse range of habitats (terrestrial, subterranean, semi-aquatic and strictly aquatic, Table 1) and are thus a sample of a broad range of ecologies and life histories. To get unbiased partitions of genetic diversity, individuals were sampled randomly from 125 and 70 different locations (Table S1) in Sri Lanka and the Western Ghats of southern India, respectively. We sequenced fragments of mitochondrial DNA for each specimen and then selected one individual per unique haplotype / per geographic region for further phylogenetic analyses (*15*).

Our analyses indicate that the Sri Lankan fauna is derived from an evolutionary diverse faunal stock on the Indian mainland (*16*). However, the inferred phylogenetic trees also demonstrate that the overall limited biotic interchange has left both areas with an unexpectedly large number of endemics. For example, the Sri Lankan *Philautus* treefrogs (Fig. 2A) are the

result of an extensive radiation on the island (*17*), and a small clade of deeply nested Indian treefrogs provides evidence for back dispersal of a single lineage to southern India. Similarly, our analyses revealed a radiation into several endemic genera of parathelphusids on Sri Lanka, followed by limited dispersal to India in the lowland-associated clade (*Oziothelphusa* and *Spiralothelphusa*) (Fig. 2F). In accord with morphological studies (*18, 19*), no gecarcinucids *sensu stricto* were found on Sri Lanka, leaving no extant evidence for successful colonization of the island. The uniqueness of the Sri Lankan fauna is most noticeably illustrated by caecilians and shieldtail snakes: In both cases, all sampled island species represent endemic monophyletic groups (Fig. 2, B and C). Finally, although the pattern of limited biotic exchange is less apparent in strictly aquatic groups (Table 1), part of Sri Lanka's fish- and shrimp species nevertheless form distinct clades (Fig. 2, D and E).

Taxon	**Total number of specimens**	**Unique haplotypes**	**Habitat**
treefrogs	44	34	terrestrial
caecilians	35	28	subterranean
uropeltid snakes	33	22	subterranean
freshwater fishes	51	41	strictly aquatic
freshwater crabs	77	40	semi-aquatic
freshwater shrimps	44	33	strictly aquatic

Table 1. - List of taxa included in this study.

These observations jointly indicate that exchange between southern India and insular Sri Lankan faunas has been severely restricted, despite the recurrent existence of a broad (>100 km) land bridge (*5*) during several episodes of sea level lowstands (Fig. 1B).

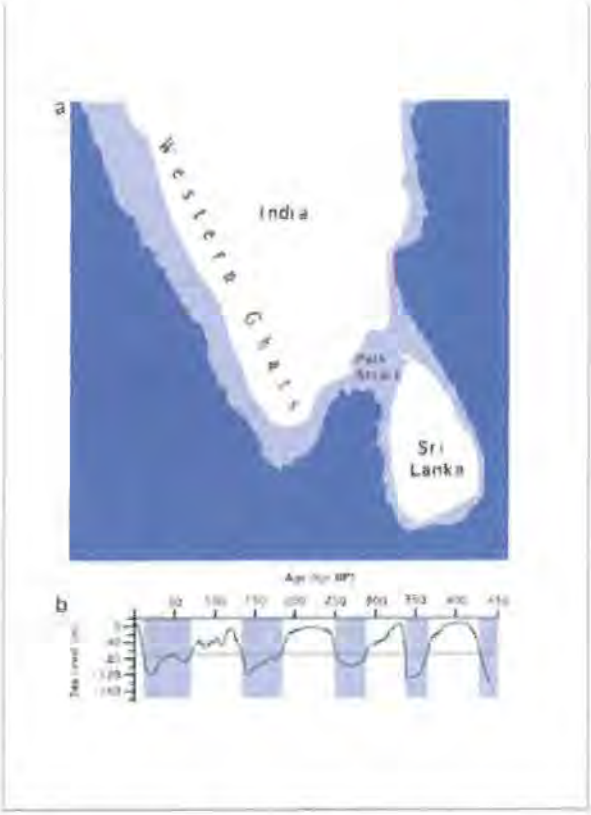

Fig. 1. - (A) India and Sri Lanka (current outline in white) are part of the same continental shelf (light gray), that does not exceed 70 m (light gray/dark gray border) in depth at its maximum. - (B) During the past 500,000 years, sea-level variations (*6*) dropping below -70 m (indicated by the horizontal line) caused, on several occasions, Sri Lanka to be connected to India (periods indicated in light gray) by a >100 km broad land bridge.

We used the sequence data to estimate the age of biotic exchange events (Fig. S2, purple numbers) in each of the six groups. Our calculations (Table S4) preclude a late Pleistocene origin for all but two splits, indicating that the corresponding events occurred prior to the multiple sea-level lowstands of the last 500,000 years. These results are reinforced by the fact that field surveys

and phylogenetic analyses did not reveal conspecific populations in India and Sri Lanka in the four terrestrial, subterranean and semi-aquatic groups (Table 1). This was unexpected because, throughout their taxonomic history, there have been many instances in which populations on both sides of the oceanic barrier have been regarded as conspecific (*8-10, 12*).

Our analyses show that the numerous rainforest species form endemic clades, clearly identifying the Western Ghats and Sri Lanka's wet zone as distinct units. There are two possible reasons why biologists may have overlooked the differentiation between Indian and Sri Lankan faunas. First, incorrect systematic affiliations of specimens is understandable a posteriori, because our analyses identify homoplasy in coloration and general morphology in all groups. Second, the Sri Lankan fauna comprises a widely distributed dry low-country element, and a more diverse but restricted rainforest component (*20*). Because the former contains several species common to the dry-zones of northern Sri Lanka and southern India that are likely Pleistocene dispersers, it has been assumed that this pattern could be generalized across the whole region.

Exact causes for the restricted dispersal between India and Sri Lanka remain speculative, but our findings highlight the importance of less conspicuous factors as important barriers to terrestrial dispersal. The faunal insularity between the wet zone of Sri Lanka and the moist forests of the Western Ghats likely results from the inability of rainforest organisms to disperse across the intervening dry lowlands. Although the climatic history of South Asia remains poorly understood, our results and the current climatic correlation between the plains of northern Sri Lanka and southern India (*21*) are possibly indicative of similar conditions during the late Pleistocene,

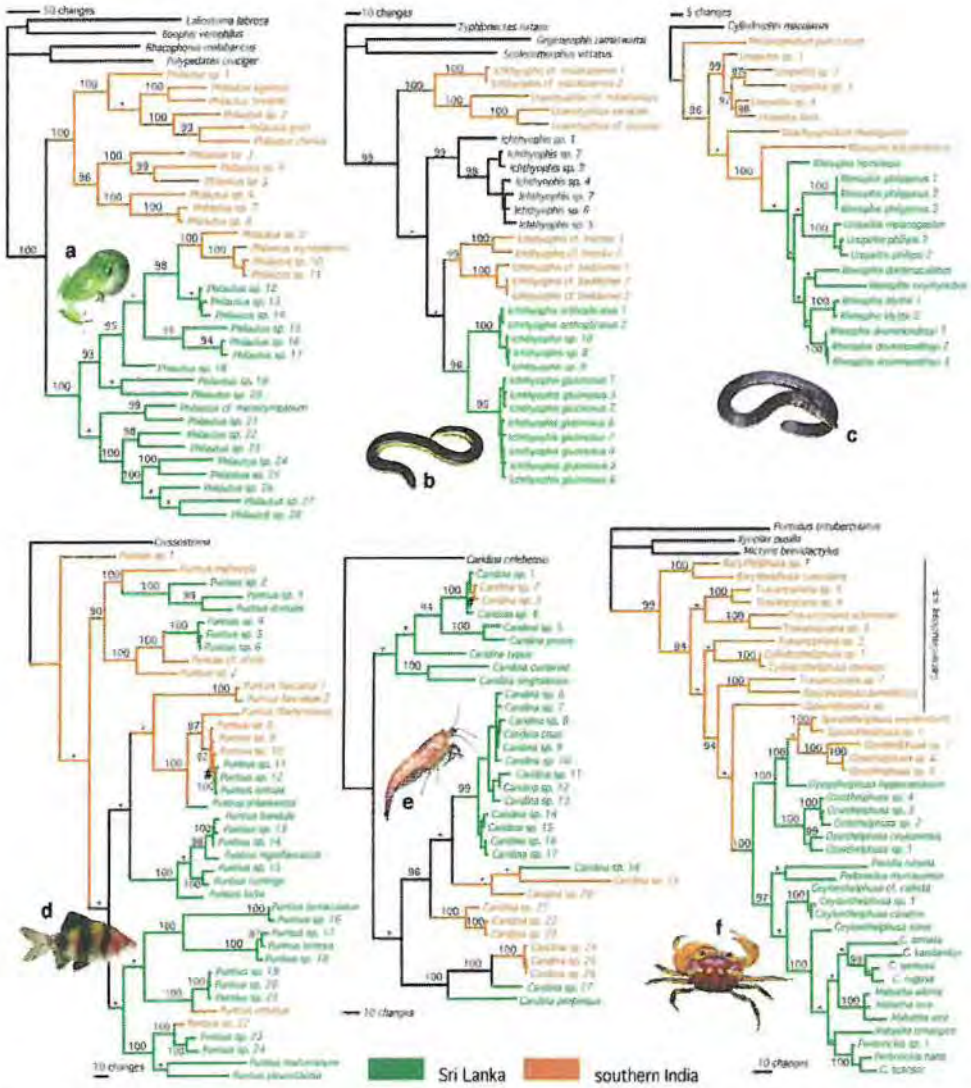

Fig. 2. Phylogenetic relationships among Indian (orange) and Sri Lankan (green) species as revealed by the (single, or one of the, see table S3) most parsimonious trees for (A) treefrogs, (B) caecilians, (C) uropeltid snakes, (D) freshwater fishes, (E) freshwater shrimps and (F) freshwater crabs. Black names represent outgroup species, except for *Ichthyophis*, which are South-East Asian taxa. Numbers on branches and asterisks indicate metaGa branch support values ≥90% and <90%, respectively. Parsimony bootstrap values and Bayesian posterior probabilities are given in fig S1 and S2, respectively. Numerical designations of OTUs indicate different haplotypes for mitochondrial DNA, not necessarily different species. Splits indicated with # represent recent exchanges between the mainland and the island.

contrary to the idea that rainforest spread onto the land bridge during periods of low sea level (*22*). Hence, montane areas and their associated climate and vegetation, rather than the present-day coastal outline, may constitute isolated islands in which the rainforest-adapted fauna has been trapped for long periods (*23*, *24*). We therefore expect that similar patterns of restricted dispersal exist elsewhere on the subcontinent, such as between opposite sides of the Palghat gap, a broad valley that traverses the southern Western Ghats. The high degree of endemicity in some species of the subcontinent is compatible with this prospect: treefrogs, uropeltids and freshwater crabs, for example, include point endemics with distributions of often just a few square kilometers (*25-27*). Thus, treating the Western Ghats and Sri Lanka as a single hotspot carries with it the danger of overlooking strong biogeographic structure within this region (*28*, *29*). Conservation management of the Indian subcontinent will benefit from further characterization of the heterogeneity of biodiversity down to a very local scale.

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4.4 References and Notes

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16. The geographic origin and/or direction of dispersal of a clade can only be established if sufficient sampling is available from the whole distribution area. As such, a single mainland origin of Sri Lankan lineages is currently indicated in three of the six examined groups, because of their nested position with respect to Indian and/or Asian lineages: caecilians and uropeltid snakes (both indicated by our analyses), and *Philautus* treefrogs (not evident from our tree, but shown in reference *17*). A peninsular origin for Sri Lankan clades is not contradicted in the three other groups as well, but will only be unambiguously confirmed when more inclusive phylogenies are available for these groups (*e.g.*, including Asian Parathelphusidae for freshwater crabs).

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Additional remark:

Although the conclusions made in this chapter still stand. The freshwater crab taxonomy and phylogeny has changed since the publication of this chapter. Recently the Parathelphusidae have been suggested to be synonymized under Gecarcinucidae (Klaus et al., 2009), hence the statement "...no gecarcinucids sensu stricto were found on Sri Lanka, leaving no extant evidence for successful colonization of the island" no longer applies in the strict sence. Similarly, in S1 Table 1: the "Parathelphusidae" could be changed to "Gecarcinucidae (formerly Parathelphusidae)" or "Gecarcinucidae (=Parathelphusidae)", which we did not carry through in this work to keep the authenticity of the publication.

Supplementary material chapter 4

Table S1: List which gives haplotypes, species, description of their geographical location and country in which the specimens were collected.

Hapl.	*Genus*	*Species*	Locality	Country
Caecilians				
H1	*Uraeotyphlus*	*cf. malabaricus*	near Vandiperiyar, Kerala	India
H2	*Uraeotyphlus*	*cf. oxyurus*	near Payyanur, Kerala	India
H3	*Uraeotyphlus*	*narayani*	Kannam, Kerala	India
H4	*Ichthyophis*	*cf. malabarensis2*	Palod, Kerala	India
H5	*Ichthyophis*	*cf. malabarensis*	near Thodupuzha, Kerala	India
H5	*Ichthyophis*	*cf. malabarensis1*	Thodupuzha, Kerala	India
H6	*Ichthyophis*	*orthoplicatus 2*	near Passara, Uva Province	Sri Lanka
H7	*Ichthyophis*	*orthoplicatus 1*	Bibilegama, Uva Province	Sri Lanka
H8	*Ichthyophis*	*cf. tricolor 1*	near Vandiperiyar, Kerala	India
H9	*Ichthyophis*	*cf. tricolor 2*	near Punalur, Kerala	India
H10	*Ichthyophis*	*cf. beddomei 2*	near Periya, Kerala	India
H10	*Ichthyophis*	*cf. beddomei 2*	near Sulthan Bathery, Kerala	India
H11	*Ichthyophis*	*cf. beddomei 1*	Subramanya, Karnataka	India
H12	*Ichthyophis*	*sp.2*	Ban Tung Tao, Surat Thani Province	Thailand
H13	*Ichthyophis*	*sp.3*	Hat Yai, Songkhla Province	Thailand
H14	*Ichthyophis*	*sp.6*	Ban Na Sabaeng, Ubon Ratchathani Province	Thailand
H15	*Ichthyophis*	*sp.5*	Mae Saivalley, Chiang Mai Province	Thailand
H16	*Ichthyophis*	*sp.7*	Longling, Yunnan Province	China
H17	*Ichthyophis*	*sp.4*	Tam Dao, Vinh Phuv Province	Vietnam
H18	*Ichthyophis*	*sp.1*	Mang Xang	Vietnam
H19	*Ichthyophis*	*glutinosus 1*	Western Province, Kalutara District, nr. Palawatta	Sri Lanka
H19	*Ichthyophis*	*sp. 10*	near Haldummula, Sabaragamuwa Province	Sri Lanka
H20	*Ichthyophis*	*glutinosus 2*	near Nakiyadeniya, Southern Province	Sri Lanka
H20	*Ichthyophis*	*glutinosus 2*	near Galle, Southern Province	Sri Lanka
H21	*Ichthyophis*	*glutinosus 3*	near Opata, Southern Province	Sri Lanka
H21	*Ichthyophis*	*glutinosus 3*	near Opata, Southern Province	Sri Lanka
H21	*Ichthyophis*	*glutinosus 3*	near. Morawaka, Southern Province	Sri Lanka
H22	*Ichthyophis*	*glutinosus 4*	Suudagala, Sabaragamuwa Province	Sri Lanka
H22	*Ichthyophis*	*glutinosus 4*	Pussellawa, Central Province	Sri Lanka
H23	*Ichthyophis*	*glutinosus 5*	near Rattota, Central Province	Sri Lanka
H23	*Ichthyophis*	*glutinosus 5*	Gammaduwa, Central Province	Sri Lanka
H24	*Ichthyophis*	*glutinosus 6*	near Peradeniya, Central Province	Sri Lanka
H25	*Ichthyophis*	*glutinosus 7*	near Rattota, Central Province	Sri Lanka
H26	*Ichthyophis*	*glutinosus 8*	Bibilegama, Uva Province	Sri Lanka
H27	*Ichthyophis*	*sp. 8*	near Haldummula, Sabaragamuwa Province	Sri Lanka
H28	*Ichthyophis*	*sp. 9*	near Haldummula, Sabaragamuwa Province	Sri Lanka
Out	*Typhlonectes*	*natans*	unknown	unknown
Out	*Gegeneophis*	*ramaswamii*	unknown	India
Out	*Scolecomorphus*	*vittatus*	unknown	unknown

Table S1. List of specimens sampled (continued).

Hapl.	*Genus*	*Species*	Locality	Country
Treefrogs				
H1	*Philautus*	*sp. 1*	Chikmalagur-Bhadra Reservoir Road	India
H1	*Philautus*	*sp. 1*	Madikeri, Karnataka	India
H2	*Philautus*	*signatus*	Ooty, Tamil Nadu	India
H2	*Philautus*	*signatus*	Sims Park, Coonoor, Tamil Nadu	India
H2	*Philautus*	*signatus*	Ooty, Tamil Nadu	India
H3	*Philautus*	*tinniens*	Ooty, Tamil Nadu	India
H4	*Philautus*	*sp. 2*	Coonoor, Tamil Nadu	India
H5	*Philautus*	*griet*	Munnar, Kerala	India
H5	*Philautus*	*griet*	Munnar, Kerala	India
H6	*Philautus*	*charius*	Chikmalagur-Bhadra Reservoir Road	India
H6	*Philautus*	*charius*	Madikeri, Karnataka	India
H7	*Philautus*	*sp. 3*	Munnar, Kerala	India
H8	*Philautus*	*sp. 4*	Trivandrum - Ponmudi Road, Kerala	India
H9	*Philautus*	*sp. 5*	Madikeri, Karnataka	India
H10	*Philautus*	*sp. 6*	Madikeri, Karnataka	India
H11	*Philautus*	*sp. 7*	Sultans Battery, Kerala	India
H12	*Philautus*	*sp. 8*	Ponmudi, Kerala	India
H13	*Philautus*	*sp. 9*	Neyyar Dam, Kerala	India
H13	*Philautus*	*sp. 9*	Ponmudi, Kerala	India
H13	*Philautus*	*sp. 9*	Neyyar Dam, Kerala	India
H13	*Philautus*	*sp. 9*	Ponmudi, Kerala	India
H14	*Philautus*	*wynaadensis*	Sultans Battery, Kerala	India
H15	*Philautus*	*sp.12*	around Kandy, Central Province	Sri Lanka
H16	*Philautus*	*sp. 13*	Unawatuna, Southern Province	Sri Lanka
H17	*Philautus*	*sp.14*	Kitulgala, Sabaragamuwa Province	Sri Lanka
H18	*Philautus*	*sp.15*	Unknown	Sri Lanka
H19	*Philautus*	*sp. 16*	Kitulgala, Sabaragamuwa Province	Sri Lanka
H20	*Philautus*	*sp. 17*	Unawatuna, Southern Province	Sri Lanka
H21	*Philautus*	*sp.18*	Kitulgala, Sabaragamuwa Province	Sri Lanka
H22	*Philautus*	*sp.19*	Kottawa, Southern Province	Sri Lanka
H22	*Philautus*	*sp.19*	Kottawa, Southern Province	Sri Lanka
H23	*Philautus*	*sp.20*	Unknown	Sri Lanka
H24	*Philautus*	*microtympanum*	Nuwara Eliya, Central Province	Sri Lanka
H25	*Philautus*	*sp.21*	Unknown	Sri Lanka
H26	*Philautus*	*sp.22*	Unknown	Sri Lanka
H27	*Philautus*	*sp.23*	Unknown	Sri Lanka
H28	*Philautus*	*sp. 24*	Nuwara Eliya, Central Province	Sri Lanka
h28	*Philautus*	*sp. 24*	Nuwara Eliya, Central Province	Sri Lanka
H29	*Philautus*	*sp.25*	Unknown	Sri Lanka
H30	*Philautus*	*sp 26*	Nuwara Eliya, Central Province	Sri Lanka
H31	*Philautus*	*sp. 27*	Knuckles, Central Province	Sri Lanka
H32	*Philautus*	*sp.28*	Unknown	Sri Lanka
H33	*Philautus*	*sp. 10*	Kumarakum, Kerala	India
H34	*Philautus*	*sp. 11*	Thekkady, Kerala	India
Out	*Laliostoma*	*labrosa*	unknown	Madagascar
Out	*Boophis*	*xerophilus*	unknown	Madagascar
Out	*Rhacophorus*	*malabaricus*	Ponmudi, Kerala	India
Out	*Polypedates*	*cruciger*	unknown	Sri Lanka

Table S1. List of specimens sampled (continued).

Hapl.	*Genus*	*Species*	Locality	Country
Caecilians				
H1	*Uraeotyphlus*	*cf. malabaricus*	near Vandiperiyar, Kerala State	India
H2	*Uraeotyphlus*	*cf. oxyurus*	near Payyanur, Kerala State	India
H3	*Uraeotyphlus*	*narayani*	Kannam, Kerala State	India
H4	*Ichthyophis*	*cf. malabarensis2*	Palod, Kerala State	India
H5	*Ichthyophis*	*cf. malabarensis*	near Thodupuzha, Kerala State	India
H5	*Ichthyophis*	*cf. malabarensis1*	Thodupuzha, Kerala State	India
H6	*Ichthyophis*	*orthoplicatus 2*	near Passara, Uva Province	Sri Lanka
H7	*Ichthyophis*	*orthoplicatus 1*	Bibilegama, Uva Province	Sri Lanka
H8	*Ichthyophis*	*cf. tricolor 1*	near Vandiperiyar, Kerala State	India
H9	*Ichthyophis*	*cf. tricolor 2*	near Punalur, Kerala State	India
H10	*Ichthyophis*	*cf. beddomei 2*	near Periya, Kerala State	India
H10	*Ichthyophis*	*cf. beddomei 2*	near Sulthan Bathery, Kerala State	India
H11	*Ichthyophis*	*cf. beddomei 1*	Subramanya, Karnataka State	India
H12	*Ichthyophis*	*sp.2*	Ban Tung Tao, Surat Thani Province	Thailand
H13	*Ichthyophis*	*sp.3*	Hat Yai, Songkhla Province	Thailand
H14	*Ichthyophis*	*sp.6*	Ban Na Sabaeng, Ubon Ratchathani Province	Thailand
H15	*Ichthyophis*	*sp.5*	Mae Saivalley, Chiang Mai Province	Thailand
H16	*Ichthyophis*	*sp.7*	Longling, Yunnan Province	China
H17	*Ichthyophis*	*sp.4*	Tam Dao, Vinh Phuv Province	Vietnam
H18	*Ichthyophis*	*sp.1*	Mang Xang	Vietnam
H19	*Ichthyophis*	*glutinosus 1*	Western Province, Kalutara District, nr. Palawatta	Sri Lanka
H19	*Ichthyophis*	*sp. 10*	near Haldummula, Sabaragamuwa Province	Sri Lanka
H20	*Ichthyophis*	*glutinosus 2*	near Nakiyadeniya, Southern Province	Sri Lanka
H20	*Ichthyophis*	*glutinosus 2*	near Galle, Southern Province	Sri Lanka
H21	*Ichthyophis*	*glutinosus 3*	near Opata, Southern Province	Sri Lanka
H21	*Ichthyophis*	*glutinosus 3*	near Opata, Southern Province	Sri Lanka
H21	*Ichthyophis*	*glutinosus 3*	near. Morawaka, Southern Province	Sri Lanka
H22	*Ichthyophis*	*glutinosus 4*	Suudagala, Sabaragamuwa Province	Sri Lanka
H22	*Ichthyophis*	*glutinosus 4*	Pussellawa, Central Province	Sri Lanka
H23	*Ichthyophis*	*glutinosus 5*	near Rattota, Central Province	Sri Lanka
H23	*Ichthyophis*	*glutinosus 5*	Gammaduwa, Central Province	Sri Lanka
H24	*Ichthyophis*	*glutinosus 6*	near Peradeniya, Central Province	Sri Lanka
H25	*Ichthyophis*	*glutinosus 7*	near Rattota, Central Province	Sri Lanka
H26	*Ichthyophis*	*glutinosus 8*	Bibilegama, Uva Province	Sri Lanka
H27	*Ichthyophis*	*sp. 8*	near Haldummula, Sabaragamuwa Province	Sri Lanka
H28	*Ichthyophis*	*sp. 9*	near Haldummula, Sabaragamuwa Province	Sri Lanka

Table S1. List of specimens sampled (continued).

Hapl.	*Genus*	*Species*	Locality	Country
Snakes				
H1	*Brachyophidium*	*rhodogaster*	Shembagganur, Tamil Nadu State	India
H2	*Melanophidium*	*punctatum*	Valparai, Tamil Nadu State	India
H3	*Rhinophis*	*drummondhayi 2*	near Passara, Uva Province	Sri Lanka
H3	*Rhinophis*	*drummondhayi 2*	Talawakella, Central Province	Sri Lanka
H3	*Rhinophis*	*drummondhayi 2*	above Namunkula	Sri Lanka
H4	*Rhinophis*	*drummondhayi 1*	Madulsima, Uva Province	Sri Lanka
H4	*Rhinophis*	*drummondhayi 1*	Madulsima, Uva Province	Sri Lanka
H5	*Rhinophis*	*drummondhayi 3*	Pindarawatta	Sri Lanka
H6	*Uropeltis*	*sp. 2*	Ooruvasal, Kerala State	India
H7	*Uropeltis*	*sp. 3*	Munnar, Kerala State	India
H8	*Uropeltis*	*sp. 1*	unknown	India
H9	*Uropeltis*	*sp. 4*	Munnar, Kerala State	India
H10	*Uropeltis*	*liura*	unknown	India
H10	*Uropeltis*	*liura*	unknown	India
H11	*Rhinophis*	*philippinus 1*	near Rattota, Central Province	Sri Lanka
H11	*Rhinophis*	*philippinus 1*	Kalugalterna	Sri Lanka
H11	*Rhinophis*	*philippinus 1*	Kalugalterna	Sri Lanka
H11	*Rhinophis*	*philippinus 1*	near Rattota, Central Province	Sri Lanka
H11	*Rhinophis*	*philippinus 1*	Palatenne	Sri Lanka
H12	*Rhinophis*	*dorsimaculatus*	Marichchikkadi	Sri Lanka
H13	*Rhinophis*	*travancoricus*	Palod, Kerala State	India
H14	*Uropeltis*	*melanogaster*	Nicapota, North Western Province,	Sri Lanka
H15	*Uropeltis*	*phillipsi 1*	near Gammaduwa, Central Province	Sri Lanka
H16	*Uropeltis*	*phillipsi 2*	Gammaduwa, Central Province	Sri Lanka
H17	*Rhinophis*	*oxyrhynchus*	unknown	Sri Lanka
H17	*Rhinophis*	*oxyrhynchus*	Polonaruwa	Sri Lanka
H18	*Rhinophis*	*homolepis*	near Rakwana, Sabaragamuwa Province	Sri Lanka
H19	*Rhinophis*	*philippinus 2*	Palatenne	Sri Lanka
H20	*Rhinophis*	*philippinus 3*	Palatenne	Sri Lanka
H21	*Rhinophis*	*blythii 1*	Talawakella, Central Province	Sri Lanka
H22	*Rhinophis*	*blythii 2*	Ingestre Estate	Sri Lanka
H22	*Rhinophis*	*blythii 2*	Ingestre Estate	Sri Lanka
H22	*Rhinophis*	*blythii 2*	Ingestre Estate	Sri Lanka
Out	*Cylindrophis*	*maculatus*	near Palawatta, Western Province	Sri Lanka

Hapl.	Genus	Species	Locality	Country
Crabs				
H1	*Ceylonthelphusa*	*sentosa*	Kannelliya, Southern Province	Sri Lanka
H1	*Ceylonthelphusa*	*sentosa*	Pitadeniya, Uva Province	Sri Lanka
H2	*Oziothelphusa*	*sp. 1*	Puwakpitiya Knuckles, Central Province	Sri Lanka
H2	*Oziothelphusa*	*sp. 1*	Tanamalwila, Uva province	Sri Lanka
H2	*Oziothelphusa*	*sp. 1*	Pathanegala	Sri Lanka
H3	*Ceylonthelphusa*	*sarar*	Bogowantalawa-Balangoda road	Sri Lanka
H3	*Ceylonthelphusa*	*sarar*	Bogowantalawa-Balangoda road	Sri Lanka
H3	*Ceylonthelphusa*	*sarar*	Bogowantalawa-Balangoda road	Sri Lanka
H4	*Ceylonthelphusa*	*scansor*	Hantane, Sabaragamuva Province	Sri Lanka
H4	*Ceylonthelphusa*	*scansor*	Hantane, Sabaragamuva Province	Sri Lanka
H5	*Oziothelphusa*	*sp. 2*	Tanamalwila, Uva Province	Sri Lanka
H5	*Oziothelphusa*	*sp. 2*	Tanamalwila, Uva Province	Sri Lanka
H5	*Oziothelphusa*	*sp. 2*	Tanamalwila, Uva Province	Sri Lanka
H6	*Perbrinckia*	*nana*	Kannelliya, Southern Province	Sri Lanka
H7	*Mahatha*	*iora*	Kumbalwela, Uva Province	Sri Lanka
H7	*Mahatha*	*iora*	Kumbalwela, Uva Province	Sri Lanka
H8	*Ceylonthelphusa*	*kandambyi*	Udugama, Sabaragamuva Province	Sri Lanka
H9	*Ceylonthelphusa*	*rugosa*	Gannoruwa, Central Province	Sri Lanka
H9	*Ceylonthelphusa*	*rugosa*	Puwakpitiya Knuckles, Central Province	Sri Lanka
H9	*Ceylonthelphusa*	*rugosa*	Daraweila	Sri Lanka
H9	*Ceylonthelphusa*	*rugosa*	Hantane, Sabaragamuva Province	Sri Lanka
H10	*Ceylonthelphusa*	*cf. callista*	Knuckles, Central province	Sri Lanka
H11	*Ceylonthelphusa*	*cavatrix*	Pathanegala	Sri Lanka
H11	*Ceylonthelphusa*	*cavatrix*	Pathanegala	Sri Lanka
H11	*Ceylonthelphusa*	*cavatrix*	Pathanegala	Sri Lanka
H11	*Ceylonthelphusa*	*cavatrix*	Pathanegala	Sri Lanka
H11	*Ceylonthelphusa*	*cavatrix*	Pathanegala	Sri Lanka
H12	*Perbrinckia*	*morrayensis*	Tillerie Estate	Sri Lanka
H12	*Perbrinckia*	*morrayensis*	Tillerie Estate	Sri Lanka
H12	*Perbrinckia*	*nn*	Adams Peak, Sabaragamuwa Province	Sri Lanka
H13	*Pastilla*	*ruhuna*	Hirimbura, Southern Province	Sri Lanka
H13	*Pastilla*	*ruhuna*	Hirimbura, Southern Province	Sri Lanka
H13	*Pastilla*	*ruhuna*	unknown	Sri Lanka
H14	*Oziothelphusa*	*sp. 3*	Denhyaya, Southern Province	Sri Lanka
H15	*Mahatha*	*sp.*	Bibilegama, Uva Province	Sri Lanka
H16	*Oziothelphusa*	*sp. 4*	Richmond hill	Sri Lanka
H17	*Oziothelphusa*	*hippocastanum*	unknown	Sri Lanka
H17	*Oziothelphusa*	*hippocastanum*	unknown	Sri Lanka
H18	*Spiralothelphusa*	*wuellerstorfi*	Madras, Tamil Nadu	India
H19	*Gubernatoriana*	*sp.*	Ponmudi, Kerala State	India
H19	*Gubernatoriana*	*sp.*	Ponmudi, Kerala State	India
H19	*Gubernatoriana*	*sp.*	Ponmudi, Kerala State	India
H20	*Oziothelphusa*	*ceylonensis*	Colombo, Western Province	Sri Lanka
H21	*Travancoriana*	*sp. 3*	Ualur-Nangery Road, Tamil Nadu	India
H22	*Oziothelphusa*	*sp. 5*	Kottarakkar-Trivandrum Road, Kerala State	India
H22	*Oziothelphusa*	*sp. 5*	Kolaththuppuzha-Tenmalai Road, Kerala State.	India
H23	*Travancoriana*	*sp. 4*	Munnar-Pollachchi Road, Kerala State	India
H24	*Spiralothelphusa*	*sp. 1*	Trisur-Chalakudy Road, Kerala State	India
H24	*Oziothelphusa*	*sp. 1*	unknown	India
H24	*Spiralothelphusa*	*sp. 1*	Trisur, Kerala State	India
H25	*Barytelphusa*	*cunicularis*	Manjery-Trissur Road, Kerala State	India
H25	*Barytelphusa*	*cunicularis*	Manjery-Trissur Road, Kerala State	India
H26	*Travancoriana*	*schimerae*	Mettupalayam-Ooti Road, Tamil Nadu	India
H26	*Travancoriana*	*schimerae*	Mettupalayam-Ooti Road, Tamilnadu.	India
H27	*Oziothelphusa*	*sp. 6*	Kolaththuppuzha-Tenmalai Road, Kerala State.	India
H28	*Travancoriana*	*sp. 5*	Kumerli-Munnar Road, Kerala State	India
H29	*Oziothelphusa*	*sp. 7*	Angamali-Thoduppusa Road, Kerala State	India
H29	*Oziothelphusa*	*sp. 7*	Trissur-Chalakudy Road, Kerala State	India
H29	*Oziothelphusa*	*sp. 7*	near Angamali, Kerala State	India
H29	*Oziothelphusa*	*sp. 7*	Angamali- Thoduppusa Road, Kerala State	India
H30	*Barytelphusa*	*lamellifrons*	Between Ranni-Kumerli, Kerala State	India
H30	*Barytelphusa*	*lamellifrons*	unknown	India
H31	*Barytelphusa*	*sp. 1*	Chathankodu, Kerala State	India
H31	*Barytelphusa*	*sp. 1*	Chathankodu, Kerala State	India
H32	*Travancoriana*	*sp. 1*	Ponmudi, Kerala State	India
H32	*Travancoriana*	*sp. 1*	Ponmudi, Kerala State	India
H33	*Cylindrotelphusa*	*steniops*	Chathenkodu, Kerala State	India
H34	*Mahatha*	*iora*	Dunhinda Falls, Uva Province	Sri Lanka
H34	*Mahatha*	*iora*	Duhinda Falls, Uva Province	Sri Lanka
H35	*Cylindrotelphusa*	*sp. 1*	Gammaduwa Knuckles, central Province	India
H36	*Ceylonthelphusa*	*armata*	Kaduganawa, Central Province	Sri Lanka
H37	*Perbrinckia*	*sp. 1*	Batadomba cave, Kuruwita, Sabaragamuva Province	Sri Lanka
H37	*Perbrinckia*	*sp. 1*	Batadomba cave, Kuruwita, Sabaragamuva Province	Sri Lanka
H38	*Ceylonthelphusa*	*cavatrix*	Pathanegala	Sri Lanka
H38	*Ceylonthelphusa*	*sp. 1*	Batambakuruwita	Sri Lanka
H39	*Travancoriana*	*sp. 2*	Ponmudi, Kerala State	India
H40	*Mahatha*	*ornatipes*	Navinna, Southern Province	Sri Lanka

Table S1. List of specimens sampled (continued).

Hapl.	*Genus*	*Species*	Locality	Country
Shrimps				
H1	*Caridina*	*typus*	Rumassala, Southern Province	Sri Lanka
H2	*Caridina*	*cruzi*	Imaduwa, Southern Province	Sri Lanka
H2	*Caridina*	*cruzi*	Kamburupitiya, Southern Province	Sri Lanka
H2	*Caridina*	*cruzi*	Kosmulla, Southern Province	Sri Lanka
H3	*Caridina*	*cumariae*	Rozella, Central Province	Sri Lanka
H4	*Caridina*	*pristis*	Pussellawa, Central Province	Sri Lanka
H4	*Caridina*	*pristis*	Peradeniya, Central Province	Sri Lanka
H5	*Caridina*	*propinqua*	Ratgama lake, Southern Province	Sri Lanka
H6	*Caridina*	*singhalensis*	Horton Plains, Central Province	Sri Lanka
H6	*Caridina*	*singhalensis*	Galpalama, Central Province	Sri Lanka
H7	*Caridina*	*sp. 1*	Kandy Lake, Central Province	Sri Lanka
H8	*Caridina*	*sp. 10*	Elledola, Southern Province	Sri Lanka
H9	*Caridina*	*sp. 11*	Bataldtta, Sabaragamuwa Province	Sri Lanka
H10	*Caridina*	*sp. 12*	Lelkada, Southern Province	Sri Lanka
H10	*Caridina*	*sp. 12*	Thalduwa, Sabaragamuwa Province	Sri Lanka
H10	*Caridina*	*sp. 12*	Battaluoya	Sri Lanka
H10	*Caridina*	*sp. 12*	Babbarugahana ella, Central Province	Sri Lanka
H10	*Caridina*	*sp. 12*	Wellemedan, Southern Province	Sri Lanka
H11	*Caridina*	*sp. 13*	Midigahamulla, Sabaragamuwa Province	Sri Lanka
H12	*Caridina*	*sp. 14*	Kandy Lake, Central Province	Sri Lanka
H13	*Caridina*	*sp. 15*	Wasgomuwa, Northern Central Province	Sri Lanka
H14	*Caridina*	*sp. 16*	Madukotanarawa	Sri Lanka
H15	*Caridina*	*sp. 17*	Moneragala, Uva Province	Sri Lanka
H16	*Caridina*	*sp. 18*	Modera, Western Province	Sri Lanka
H16	*Caridina*	*sp. 18*	Wakwella, Southern Province	Sri Lanka
H17	*Caridina*	*sp. 19*	Vikom, Kerala State	India
H18	*Caridina*	*sp. 2*	Porunthanur, Kerala State	India
H19	*Caridina*	*sp. 20*	near Sanchipuram, Tamil Nadu State	India
H20	*Caridina*	*sp. 22*	Between Kanjirappalli-Palai, Kerala State	India
H21	*Caridina*	*sp. 23*	near Thamarahulam, Kerala State	India
H22	*Caridina*	*sp. 24*	Kattakada, Kerala State	India
H23	*Caridina*	*sp. 25*	Vellikkunnam, Kerala State	India
H24	*Caridina*	*sp. 26*	Kumarakom, Kerala State	India
H24	*Caridina*	*sp. 26*	Achchankovil River, Kerala State	India
H25	*Caridina*	*sp. 21*	unknown	India
H26	*Caridina*	*sp. 27*	Kottawa, Southern Province	Sri Lanka
H27	*Caridina*	*sp. 3*	Vellikkunnam, Kerala State	India
H27	*Caridina*	*sp. 3*	Near Kanjirappalli, Kerala State	India
H28	*Caridina*	*sp. 4*	Mawanana, Southern Province	Sri Lanka
H29	*Caridina*	*sp. 5*	Parhenegedera dola, Central Province	Sri Lanka
H30	*Caridina*	*sp. 6*	Ekneligoda, Sabaragamuwa Province	Sri Lanka
H31	*Caridina*	*sp. 7*	Mawanana, Southern Province	Sri Lanka
H32	*Caridina*	*sp. 8*	Rumassala, Southern Province	Sri Lanka
H33	*Caridina*	*sp. 9*	Mawanana, Southern Province	Sri Lanka

Table S2. - Primers used in chapter 4.

Alias	Primer sequence (5' - 3')	Reference
Cytb-A	CCATGAGGACAAATATCATTYTGRGG	Bossuyt & Milinkovitch (2000) *PNAS* 97: 6585-6590
Cytb-B	CTTCTACTGGTTGTCCTCCGATTCA	Bossuyt & Milinkovitch (2000) *PNAS* 97: 6585-6590
Cytb-C	CTACTGGTTGTCCTCCGATTCATGT	Bossuyt & Milinkovitch (2000) *PNAS* 97: 6585-6590
Cytb-D	TATGTTCTACCATGAGGACAAATATC	Simon et al. (1994) *Ann. Entomol. Soc. Am.* 87: 651-701
Cytb-E	ACCTCTCATCCTTATGAAACTTTGG	this study
12V16-A	ACAAGCGCCAGGGWAYTACGAGC	Bossuyt & Milinkovitch (2000) *PNAS* 97: 6585-6590
12V16-B	TTCATTGTTATTTAATCTTTCCC	Bossuyt & Milinkovitch (2000) *PNAS* 97: 6585-6590
12V16-C	AAACTGGGATTAGATACCCCACTAT	Richards & Moore (1996) *Mol. Phylogenet. Evol.* 5: 522-532.
12V16-D	GAGGGTGACGGGCGGTGTGT	this study
12V16-E	GCTAGACCATKATGCAAAAGGTA	Richards & Moore (1996) *Mol. Phylogenet. Evol.* 5: 522-532.
16S-A	CGCCTGTTTATCAAAAACAT	Simon et al. (1994) *Ann. Entomol. Soc. Am.* 87: 651-701
16S-B	CCGGTYTGAACTCAGATCAYGT	Simon et al. (1994) *Ann. Entomol. Soc. Am.* 87: 651-701
NDH-A	GCCCCATTTGACCTCACAGAAGG	this study
NDH-D	GGTATGGGCCCAAAAGCTT	this study
MIT01-A	GTACATATCGCCCGTCGCTT	Kitaura et al. (1998) *Mol. Biol. Evol.* 15: 626-637
MIT01-C	CATGTACATATCGCCCGTCG	this study
MIT01-B	TTGCACGGTCATAATACCGC	this study

The use of mitochondrial DNA. - Biogeographic studies that are based solely on mitochondrial DNA should be interpreted with caution, because patterns in mitochondrial haplotypes can become at least partially decoupled from that in chromosomal DNA (*S1*). However, decoupling of mtDNA and nuDNA loci is a problem that generally occurs at a recent time scale (populations within species). In this study, differential sorting might cause some disagreements between mitochondrial and nuclear trees *within* each Sri Lankan/Indian clade, but it is hard to imagine the process to cause discrepancies between nuclear and mitochondrial loci regarding the existence of these clades.

Table S3. An overview of the results of parsimony and Modeltest 3.06 analyses

	Shrimps	Crabs	Snakes	Frogs	Caecilians	Fishes
Number of specimens sequenced	44	77	33	44	35	51
Unique haplotypes	33	40	22	34	28	41
Total data matrix (bp)	1363	1430	910	1489	1659	1153
Unambiguously aligned (bp)	836	764	815	1369	1366	1065
Parsimony informative (bp)	227	247	115	623	414	316
Constant (bp)	524	423	634	654	830	679
Number of MP trees	8	108	1	6	6	6
Length of MP trees (10,000 reps)	833	1126	372	4099	1479	1400
Hierarchical chi-square test	HKY+G+I	GTR+G+I	TrN+G+I	TrN+G+I	GTR+G+I	TrN+G+I
Akaike transformation criterion	TVM+G+I	GTR+G+I	GTR+G+I	HKY+G+I	GTR+G+I	GTR+G+I

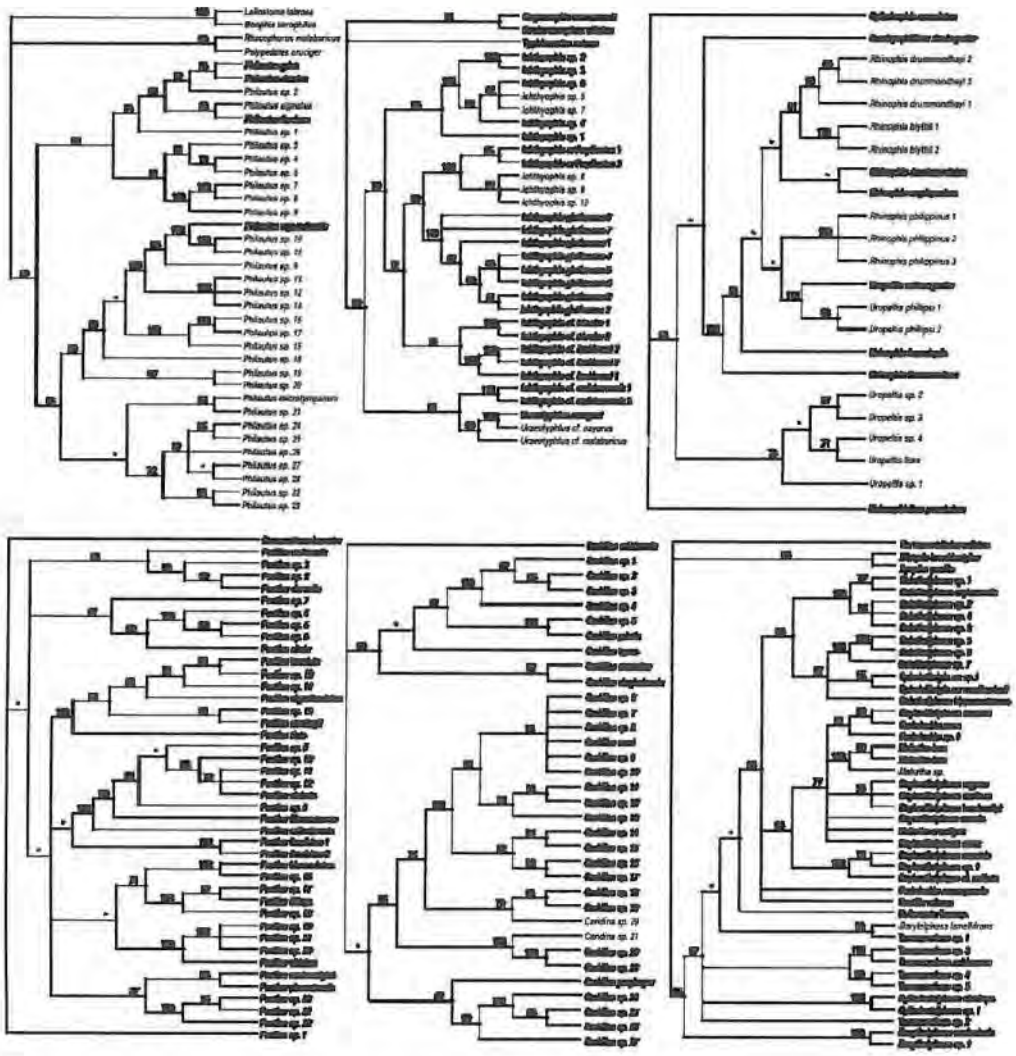

Fig. S1. - Strict consensus of equally parsimonious trees for each of the six groups. The numbers on the branches indicate MP bootstrap values for 10,000 replicates.

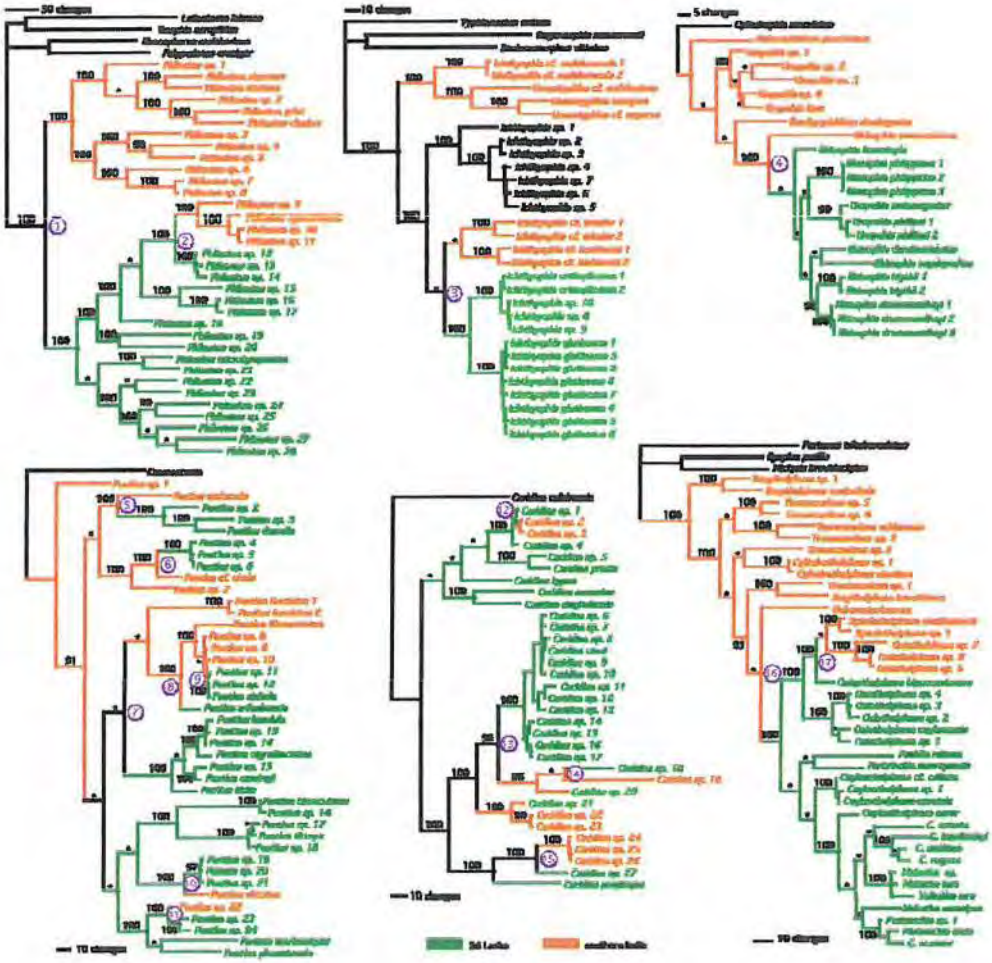

Fig. S2. - Phylogenetic relationships among Indian (orange) and Sri Lankan (green) species. Numbers on branches and asterisks indicate bayesian posterior probability values ≥90% and <90%, respectively. Numbers in purple are biotic exchange events between India and Sri Lanka, *cf.* Table S4.

Chapter 05

Phylogenetic diversity of the Sri Lankan freshwater crabs and its implications to conservation

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PHYLOGENETIC DIVERSITY OF SRI LANKAN FRESHWATER CRABS AND ITS IMPLICATIONS FOR CONSERVATION

Abstract

As part of a Global Biodiversity Hotspot, the conservation of Sri Lanka's endemic biodiversity warrants special attention. With 51 species (50 of them endemic) occurring in the island, the biodiversity of freshwater crabs is unusually high for such a small area (65,600 km^2). Freshwater crabs have successfully colonized most moist habitats and all climatic and elevational zones in Sri Lanka. We assessed the biodiversity of these crabs in relation to the different elevational zones (lowland, upland and highland) based on both species richness and phylogenetic diversity. Three different lineages appear to have radiated simultaneously, each within a specific elevational zone, with little interchange thereafter. The lowland and upland zones show a higher species richness than the highland zone while—unexpectedly—phylogenetic diversity is highest in the lowland zone, illustrating the importance of considering both these measures in conservation planning. The diversity indices for the species in the various IUCN Red List categories in each of the three zones suggest that risk of extinction may be related to elevational zone. Our results also show that overall more than 50% of Sri Lanka's freshwater crab species (including several as yet undescribed ones), or approximately 72 million years of evolutionary history, are threatened with extinction.

5.1. Introduction

Sri Lanka is situated in one of the world's 34 biodiversity 'hotspots' (Mittermeier *et al.* 2004) and is a recognized reservoir of unique evolutionary history (Sechrest *et al.* 2002; Bossuyt *et al.* 2004). Despite the island's small size (65,600 km^2), its true freshwater-crab fauna is remarkable for containing five endemic genera and 50 endemic species, i.e., four percent of the global freshwater-crab species (Cumberlidge *et al.* 2009). Eighty percent of these species are considered to be at some risk of extinction, making urgent conservation actions imperative (Cumberlidge *et al.* 2009). Furthermore, Sri Lanka's freshwater crabs occur at all elevations throughout the island, and elevational variation in diversity and richness could be relevant to the national conservation planning process (Wiens *et al.* 2007).

Contemporary Sri Lanka is divided into four climatic and ecological zones, primarily defined by the average (annual) rainfall: the wet, intermediate, dry and arid zones (Fig. 1a), with different boundaries depending on the isohyets chosen by various authors (see references in Puvaneswaran & Smithson 1993). Yet, without ignoring the complex structure of the highlands (Erb 1984), Sri Lanka can also be divided into elevational zones based on a combination of elevation, slope and regional topographic discontinuities—*viz.*, lowlands (0–270 m), uplands (270–1,060 m) and highlands (910–2,420 m) (Vitanage 1970; Dahanayake 1982)—that are also linked to three periods of erosion (Waida 1945) and could possibly function as geographical barriers. Although arbitrary, such elevational zonation could serve as a proxy for several (often) correlated environmental gradients (Willig *et al.* 2003).

Fig. 1

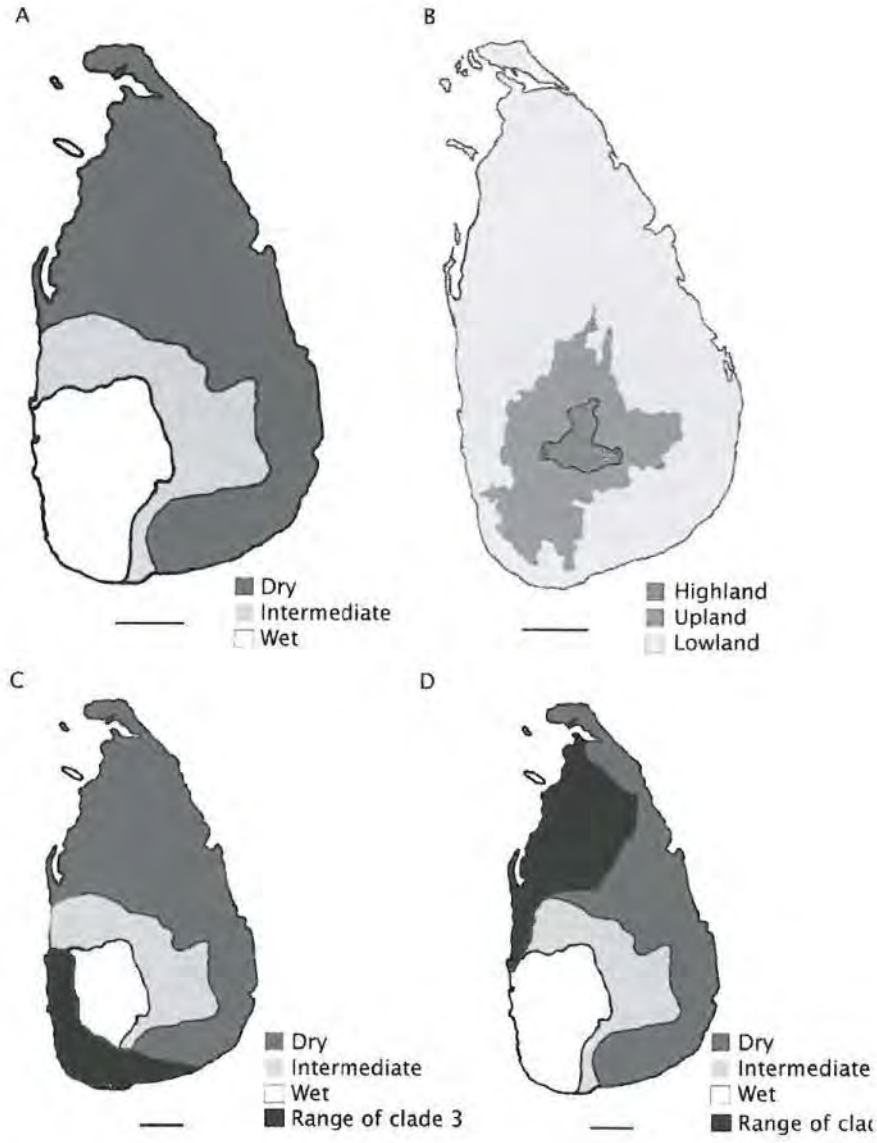
A
Dry
Intermediate
Wet
B
Highland
Upland
Lowland
C
Dry
Intermediate
Wet
Range of clade 3
D
Dry
Intermediate
Wet
Range of clac

Fig. 1 (previous page): Schematic maps of Sri Lanka representing: A. Ecological zones, mainly determined by annual rainfall, known as the dry (< 1,250 mm/yr), intermediate (between 1,250 and 1,900 mm/yr) and wet zones (>1,900 mm/yr). B. Elevational zones (Vitanage 1970); light grey: lowlands (0–270 m), dark grey: uplands (270–1060 m) and black: highlands (910–2,420 m).
C. Ecological zones, as explained in a. The extra black zonation represents the presence of clade 3. D. Ecological zones, as explained in a. The extra black zonation represents the presence of clade 4. This is the only clade that shows evidence for (albeit limited) dispersal of freshwater crabs to and from India. Scale bars: 50 km.

The highest species richness for all major groups of organisms reported to date occurs in the wet zone (WWF & IUCN 1995; plants: Wikramanayake *et al.* 2002; freshwater fish: Pethiyagoda 1991; amphibians: Manamendra-Arachchi & Pethiyagoda 2005, 2006; freshwater crabs: Bahir *et al.* 2005), which partly overlaps all three elevational zones. Although the species composition of fauna and flora seems to differ between these elevational zones (e.g., fish: Pethiyagoda 1991; agamid lizards: Biswas & Pawar 2006; pollen: Bonnefille *et al.* 1999; plants: Gunatilleke *et al.* 2005; snails: Naggs *et al.* 2005), variation of biodiversity across the three zones remains less well understood. Recent studies elsewhere, however, have shown that intermediate elevations often harbour the greatest species richness (Wiens *et al.* 2007; McCain 2005; Oomen & Schanker 2005). Besides species richness, other correlated indices—such as phylogenetic diversity (PD) —are also used to assess diversity. Some authors, however, have argued that species richness cannot predict PD. For example, Forest *et al.* (2007) argued that PD values can be unexpectedly higher or lower in certain regions, and suggested that species richness be decoupled from PD. In this study we use molecular phylogenetic analyses, elevational distribution data and dating estimates to assess the relative importance of species richness and PD (Faith 1994;

Vane-Wright *et al.* 1991) of freshwater crabs in the three major elevational zones (highlands, uplands, lowlands) of Sri Lanka.

The Old World true freshwater crabs are (sub)tropical, characterized by direct development, brood care and complete independence of the marine environment (Yeo *et al.* 2008; Cumberlidge & Ng 2009). They are currently classified in two superfamilies: the Potamoidea, with a distribution in Europe, Africa and Asia; and the Gecarcinucoidea, which occur in Asia and Africa (Bott 1969, 1970a,b) and which include all the Sri Lankan species. The classification within the Gecarcinucoidea, however, has not been stable (Ng *et al.* 2008). Although knowledge of phylogenetic relationships and evolutionary history of these groups is still scant, molecular phylogenetic analyses suggest that the Gecarcinucoidea comprise of the paraphyletic Gecarcinucidae (including the synonymous Sundathelphusidae), and the monophyletic Parathelphusidae (Bossuyt *et al.* 2004, Daniels *et al.* 2006, Klaus *et al.* 2006), with Klaus *et al.* (2006) arguing that the Gecarcinucidae and Parathelphusidae may be synonymous. Klaus *et al.* (2009) recently completed a detailed reappraisal of this superfamily, which formally regards the Parathelphusidae to be a junior synonym of Gecarcinucidae, and the Gecarcinucoidea to include only a single family, *viz.* the Gecarcinucidae.

Relatively few taxonomists have studied the freshwater crabs of the Indian peninsula and Sri Lanka. After the first two species descriptions by Kingsley (1880), only a handful of new species was described in the following decades (e.g., Alcock 1909, 1910). In the first extensive review of Sri Lankan freshwater crabs, Bott (1970a) recognized seven species classified in four genera and two families: Parathelphusidae and Sundathelphusidae. None of these genera was considered endemic to the island. In the course of the last decade, intensive exploration in Sri Lanka resulted in the discovery and description of several new genera and

species endemic to the island (Ng 1995; Bahir 1998, 1999; Ng & Tay 2001; Bahir & Ng 2005; Bahir & Yeo 2005). Based on a relatively small number of available morphological characters (Ng 1988), the 51 known species, including 50 island endemics, are currently classified into seven genera (*Oziotelphusa, Spiralothelphusa*, *Perbrinckia, Ceylonthelphusa*, *Mahatha*, *Clinothelphusa* and *Pastilla*) (Bahir & Ng 2005; Bahir & Yeo 2005) in the family Gecarcinucidae *sensu* Klaus *et al.* (2009). Earlier taxonomic and molecular phylogenetic analyses have shown that the first two genera are also represented in peninsular India, while the five other genera are members of a large, endemic insular radiation (Bossuyt *et al.* 2004). As such, this study aims to contribute also to the clarification of phylogenetic relationships within the Sri Lankan Gecarcinucidae.

The conservation status of the freshwater crabs of Sri Lanka has already been assessed in detail (Bahir *et al.* 2005) in the *IUCN Red List of Threatened Species* using the current IUCN categories and criteria (IUCN 2001) and was reviewed in the global context (Cumberlidge *et al.* 2009). These analyses demonstrate the immense conservation value, as well as dire threats, facing the Sri Lankan gecarcinucid fauna. With most of the species having ranges wholly or substantially outside the protected areas network, recovery plans for taxa at imminent risk of extinction will call not only for innovative management actions, but a process of triage whereby the species of greatest conservation value receive the most urgent attention. The present study is intended to help inform such a process.

5.2. Material and Methods

5.2.1 Data collection and choice of outgroup

We obtained 106 specimens from a broad range of micro-habitats (e.g., hill streams; lowland streams and rivers; stream-, reservoir- and

riverbanks; moist forest habitats such as bogs and phytothelms; and rice fields) at 66 localities in Sri Lanka and southern India. Our dataset is distilled from the comprehensive survey of Sri Lanka's aquatic carcinofauna that RP and PKLN commenced in 1992. This work was later joined MMB, who expanded on it in collaboration with DCJY, with field surveys being conducted in Sri Lanka and peninsular India until 2005. The survey effort, which was designed primarily to maximize the number of populations/species sampled, focused on sites at which the surveyors thought it likely that interesting crabs would be found. While the dataset reflects this extensive effort, not all the species recorded from the region are included in the sample analyzed here because in the earlier phase of the survey specimens were preserved in alcohol, with tissue for molecular analysis being accumulated only after 2000. The sites surveyed encompass a range of elevations in all three physiographic zones (from 0 to 2,100 m asl).

The ingroup consists of 96 specimens. As an outgroup, we used 10 gecarcinucid species from 10 different localities in India. Previous molecular studies in the same family (Bossuyt *et al.* 2004, Klaus *et al.* 2006, 2009) have shown that Indian members of the family of Gecarcinucidae (*Travancoriana*, *Barytelphusa*, *Gubernatoriana*, *Cylindrotelphusa*) are from separate clades that are sufficiently distant from the Sri Lankan taxa to be used for this purpose. A list of species represented in this study, together with their haplotypes, voucher numbers, geographic coordinates, and altitudes, is provided as supplementary information (S1).

5.2.2 *DNA extraction, amplification and sequencing*

Whole genomic DNA was extracted from muscle tissue of legs or claws using a standard phenol/chloroform procedure (Sambrook *et al.* 1989). Two mitochondrial DNA fragments were amplified: (i) a *ca* 1320 base pair (bp) region including a small part of the 12S rRNA gene fragment, the complete $tRNA^{Val}$ gene and part of the 16S rRNA gene fragment, and (ii) a *ca* 650 bp fragment of the Cytochrome *c* Oxidase subunit 1 gene (COI). The primers used for the former fragment are given elsewhere (Bossuyt *et al.* 2004). The primers used for the COI fragment are the invertebrate primers LCO1490 and HCO2198 (Folmer *et al.* 1994) and two newly designed primers PMT3 (5'-CTCTTCTCTACAAATYCATAAAGA-3') and PMT-4 (5'-CGAAAAATCAGAATAGRTGTTG-3'). PCR products were purified following the Qiagen agarose gel extraction protocol, cycle-sequenced on both strands and analyzed using an ABI 377 or ABI 370 automated sequencer (Applied Biosystems). The sequences have been deposited in GenBank under Accession Nos. GQ289586-GQ289613 and GQ289614-GQ289669. Additionally, sequences of the large fragment of several species used in previous studies (Bossuyt *et al.* 2004, Daniels et al. 2006) were downloaded from GenBank. Accession numbers are provided as supplementary information S2.

5.2.3 *Alignment and phylogenetic analysis*

All sequences of the 'unique haplotypes' were aligned using the software ClustalX_V1_81 (Thompson *et al.* 1997) for the COI fragment and ProAlign_version0.5a0 (Löytynoja & Milinkovitch 2003) for the large fragment. The latter method provides a statistical approach to multiple sequence alignment. A posterior probability is assigned to each aligned position. This value can be used as an efficient criterion for detecting and removing the most unreliably aligned sites. All sites with posterior

probability values < 90% were removed before further analysis. Minor corrections were made in MacClade v4.0 (Maddison & Maddison 2000). Transitions (T_i) and transversions (T_v) were plotted against uncorrected pairwise distances to evaluate mutational saturation. For specimens with identical sequences only one representative was selected for further analysis. Maximum parsimony (MP) analyses were performed using the program PAUP* 4.0 b10 (Swofford 1998). Heuristic searches were executed in 10,000 replicates, using tree bisection reconnection (TBR) branch swapping. Clade support was calculated using non-parametric bootstrapping (Felsenstein 1985) in 1,000 replicates (MPBS). We used Modeltest v3.7c (Posada & Crandall 1998) to identify the best fitting model of DNA-evolution. Maximum Likelihood (ML) searches were performed in PAUP* with 100 replicates of random taxon addition, TBR branch swapping, and estimated model parameter values obtained by recurrent ML estimation on a guide tree estimated by PHYML (Guindon & Gascuel 2003). Clade support under ML was calculated using non-parametric bootstrapping in 1,000 replicates (MLBS) using PHYML (Guindon & Gascuel 2003). Bayesian analyses were performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) using a locus-based data partitioned GTR + Γ + I – model, as this was identified as the best fitting evolutionary model. Two runs of four chains each were run simultaneously for 2 million generations. They were sampled every 200 generations and the first 2,000 trees discarded as the "burn-in". Hence, Bayesian posterior probabilities (BPP) were estimated as the 50% majority-rule consensus of the 8,000 last sampled trees.

5.2.4 Divergence time estimates

The hypothesis of the molecular clock was rejected using the likelihood ratio test (LRT; df=61; p=0.05; Felsenstein 1981). Consequently, posterior divergence times were estimated with the Bayesian multi-locus

relaxed molecular clock method implemented in Multidivtime (Thorne & Kishino 2002). The above method allows the molecular rate to vary throughout the tree in an autocorrelated manner, with closely related species sharing similar rates. Prior gamma distributions on three parameters of the relaxed clock model were assumed and specified through the mean and standard deviation of the root age (rttm and rttmsd), the root rate and the rate autocorrelation. Because rttm and reliable calibration points within Gecarcinucidae are unavailable, we firstly calibrated our tree with previously published mtDNA substitution rates. The estimated rate for crabs (*Sesarma*, Sesarmidae) for 16S and COI combined is 1.63% divergence sequence per million years (Schubart *et al.* 1998). The 12S in our alignment only constitutes about 45 bp: consequently the rate of evolution for 12S was not considered in these analyses. To our knowledge there is no published rate of evolution for $tRNA^{val}$ of freshwater crabs or any closely related group. Considering the short length of the fragment (~73 bp) and its immediate proximity to 16S, we included it as part of the '16S' data partition in further analyses. By dividing the median path length from root-to-tips for all ingroup taxa by this rate, we obtained a prior for the ingroup root age at 8.76 Ma (see Thorne & Kishino 2002; Sanderson 1997). Other parameters were set as recommended by the authors of the Multidivtime package (Thorne & Kishino 2002), although we allowed for larger standard deviations (e.g., confidence intervals of 90% for the rate of evolution and 50% for the ingroup root age). Monte Carlo Markov Chains were run for 1.1 million generations with sampling intervals of 100 generations and burn-in corresponding to the first 100,000 generations. All analyses were repeated to confirm successful convergence towards the proper distributions for divergence ages. Relative estimates, setting the prior for the ingroup root age at an arbitrary 1 and thereby using the median path length from root-to-tips for all ingroup taxa as the prior rate of evolution, gave the same proportional differences in 'time' estimates.

Secondly, we expanded our dataset of 63 sequences with 16S and COI sequences of 43 species from Genbank resulting from a recent study on the evolution of Afrotropical freshwater crabs (Daniels *et al.* 2006). This allows us to use the following calibration points (Daniels *et al.* 2006): (1) a fossil record of the potamid *Potamon* of 24 Ma, and (2) a fossil record of the potamonautid *Potamonautes niloticus* of 6 Ma sediments. We did not include the third calibration point corresponding to Seychelles-Africa split (see Daniels *et al.* 2006, Cumberlidge *et al.* 2008) because it is of doubtful accuracy. It is also known that poor fossil data can postdate divergence time (Hedges & Kumar 2004, Benton & Donoghue 2007). In the case of freshwater crabs the recent dynamics at all levels of taxonomic classification (from superfamily to species) might have an effect on earlier assignment of fossil records, especially records at genus level, such as calibration point 1. We therefore also ran the same dataset without calibration constraints with relative time scales (i.e., rttm set at an arbitrary 1) and tested for convergence. Additionally, we preferred to use our own dataset for further analyses and discussion because the 16S fragments of the additional 43 Genbank specimens are much shorter.

The date estimates and their lower and upper bounds are given in millions of years. The latter predicted interval is given in parentheses (lower bound – upper bound).

5.2.5 Patterns of species diversity and phylogenetic history

To test whether the evolution of clades is determined by the elevational geography of Sri Lanka we used our ML tree to reconstruct ancestral distributions (i.e., in this context, lowland, upland and highland) in MacClade v4.0 under the maximum-parsimony criterion. We recorded the geographical location and altitude for our specimens using GPS or inch-to-

the-mile topographic maps (see supplementary information S1), and extended the distribution range of the corresponding species with locality and elevational data from unpublished data (M.M.Bahir) and data available from the literature (Ng & Tay 2001; Bahir & Ng 2005; Bahir & Yeo 2005). By these means we could determine the present distribution for all ingroup taxa and tally them to one or more of the three elevational zones (lowland, upland, highland, *sensu* Vitanage 1970).

We used two quantitative measures to assess biodiversity: species richness and PD. We calculated species richness per predefined area as the percentage of all haplotypes that occur uniquely in that predefined zone. We also calculated species richness of every IUCN extinction-risk category and for each zone. To estimate the geographical distribution of PD in Sri Lanka we analyzed our phylogeny using the Phylogenetic Diversity Analyzer (PDA; Minh *et al.* 2006). The PD for a subset of taxa is the sum of the branch lengths of the minimal sub-tree that spans this set counting back to the root of the tree (Faith 1992, 2006; Vane-Wright *et al.* 1991). In this study the PD score for several predefined areas was computed. An area refers to the user-defined subset of taxa. PD scores were calculated based on the divergence time phylogeny (e.g., branch lengths reflect divergence times with a clock rate of 1.63% divergence sequence per million years, see above). Consequently, the PDA computes clade evolutionary history in millions of years (My) (Sechrest *et al.* 2002) within predefined areas (e.g., lowland, upland, highland). We tested (by conducting simulations of 10,000 trials) whether the PD scores observed in each elevational zone are significantly higher than expected for the same number of species randomly drawn from the tree. As with the analyses of the observed data, we included only species that occur in a single elevational zone and excluded species that occur in more than one zone.

To determine the vulnerability of the freshwater crabs within Sri Lanka and in the three elevational zones separately, we estimated PD scores for the IUCN (2001) Red List categories (Bahir *et al.* 2005): Critically Endangered (CR), Endangered (EN), Vulnerable (VU), Near Threatened (NT), or Least Concern (LC) or Data Deficient (DD). We dealt with DD taxa in two ways: including them all, or excluding them all (for a similar approach see Purvis & Hector 2000). For the first approach we considered DD as a separate category. For the second approach, we added the DD taxa occurring in a specific elevational zone to the respective category for which we were calculating the diversity indices. We tested for the observed PD significance per defined category for the island as a whole as well as for each zone. After the above categories had been related to the elevational zones, only species apparently endemic to that zone were considered. The Red List category for each species is recorded alongside each haplotype in Fig. 2.

5.3. Results

5.3.1 Sequence characteristics and phylogeny

The final topology compiles 63 'unique' haplotypes, 53 of which are ingroup taxa. All specimens used in this study are listed in S1 (supplementary information). Alignment resulted in a data matrix of 2,047 base pairs (bp). After exclusion of 583 bp due to ambiguities in the alignments, the total dataset consisted of 1,464 characters, 502 sites of which were parsimony-informative. The total dataset showed a maximum uncorrected pairwise divergence of 16.7% and is not saturated for transitions or transversions. Even after cloning, three specimens, *Perbrinckia integra*, '*P.* cf. *integra*' and '*P.* cf. *integra* 2' (clade 8 in Fig. 3) gave a single amplification product for the protein coding CO1 fragment that showed one or multiple frame shifts. For *P.* cf. *integra* the first part of

the fragment, most probably including a frameshift, was completely excluded from analysis due to ambiguity. The sequence of *P.* cf. *integra*2 contained a deletion of 4 nucleotides and *P. integra* contained two deletions of 4 and 43 nucleotides, respectively. These sequences were consequently excluded from further analysis.

The ML topology (-lnL = 15,177.41) is presented as a phylogeny in Fig. 3 and ultrametrically (using the previously published rate of substitution, see M&M) in Fig. 2. The MP analyses of the total dataset retrieved 10 optimal trees (tree length = 2,857). The consensus trees of the MP and of the Bayesian analysis were highly congruent with the topology of the ML analysis. They differed only at a few weakly supported nodes within clade 7. All analyses show a basal split between two well-supported clades (Fig. 3, clade 1 and 2): (i) the *Oziotelphusa-Spiralothelphusa* clade (*O.-S.* clade,); and (ii) all the other genera, all strictly Sri Lankan endemics. Within clade 1, the monophyletic genus *Spiralothelphusa* is nested within the paraphyletic *Oziotelphusa*. Within clade 2, phylogenetic relationships are incompatible with current classifications (see Ng & Tay 2001; Bahir & Ng 2005; Bahir & Yeo 2005) for several reasons. Firstly, crabs of the genus *Perbrinckia* are taxonomically delimited based on the ratio of the distal versus basal segment length of the male second pleopods. However, our analyses indicate that this genus is polyphyletic (Fig.1, clades 5 and 6), comprising two evolutionarily distinct clades that correspond to the 'smooth' (Fig. 3, clade 5) and 'rough' (Fig. 3, clade 6) carapace groups previously defined morphologically by Bahir & Ng (2005). Secondly, the semi-terrestrial crabs of the genus *Mahatha* fall into two groups, the *M. ornatipes* group and the *M. adonis-M. iora* group, that are both robustly supported. Thirdly, the genus *Ceylonthelphusa* comprises a number of well-supported clades, but the genus as a whole is clearly polyphyletic. Fourthly, *Ceylonthelphusa venusta* emerges as sister species of *Clinothelphusa kakoota* with high support.

5.3.2 *Divergence time estimates*

According to the divergence time estimates on our dataset, using 1.63% per million years as the rate of evolution, the ancestors of the contemporary freshwater crabs colonized Sri Lanka around 7.4 (4.6–11.4) Ma. Further, posterior age estimates relevant to explaining some major biogeographical patterns are the splitting of the *Oziotelphusa-Spiralothelphusa* clade around 5.5 (3.2–8.9) Ma and the splitting of the *Perbrinckia* clade (clade 5; Fig. 3) at 5.7 (3.4–9.1) Ma. Additionally, within the same time frame, we identified the group more or less defined as the upland clade (clade 9; Fig. 3), for which the suggested divergence age is 5.5 (3.3–8.7) Ma.

The analyses calibrated with the African calibration points (Daniels *et al.* 2006) yielded comparable divergence time estimates (for the Sri Lankan colonization 14.2 (10.3–19.2) Ma, and for the three clades (clade 1, 5 and 9) 8.3 (5.5–12.3) Ma, 8.0 (5.0–11.9) Ma and 8.9 (6.1–12.7) Ma, respectively.

5.3.3 *Patterns of species diversity and phylogenetic diversity*

The MP reconstruction of ancestral distributions indicates lowland ancestry and an early invasion of the three elevational zones, with little interchange (such as *Ceylonthelphusa kandambyi* and *Perbrinckia nana*) afterwards. The majority of the clades are restricted largely to one of the three elevational zones (Fig. 2). Even under DELTRAN-optimization (i.e. favoring most recent dispersal in case of ambiguity) the three major geographic clades (see Fig. 2: clades 1, 5 and 9) were established prior to 5.46 Ma.

The *Oziotelphusa-Spiralothelphusa* clade (clade 1) mainly occurs in the dry-zone, the largest of the island's climatic zones (Fig. 1b). The *P*-clade (clade 5), with rather restricted habitat ranges, occurs in the highlands, except for the more basal *P. crascens*, which is a lowland species. Clade 9 (*Mahatha-Ceylonthelphusa*-'rough'-*Perbrinckia* clade) dispersed throughout the uplands. A few species, especially within the genus *Mahatha,* occur in more than one area.

Within the lowland clade, only one dispersal event between elevational zones is reconstructed: *Oziotelphusa* sp. 1, has invaded the uplands. Clade 3 (Fig. 3) comprises only species from the southwestern and southern lowlands of Sri Lanka (Fig 1c). The species of *Oziotelphusa* and *Spiralothelphusa*, sampled in the Indian peninsula, all fall within clade 4 (Fig. 3). The Sri Lankan representatives within this latter group are restricted to the (semi)-arid part of the northern Sri Lankan lowlands (Fig. 1d), except for some specimens of *S. parvula* that occur in the wet lowlands of the island's southwest.

Of the 38 different putative species in our phylogenetic tree, 28 appear to be zone-restricted endemics (see columns in Fig. 2), i.e. they are restricted to a single elevational zone. As to species richness, unique lowland richness is 28.9 %, unique upland richness 28.9 % and unique highland richness 15.8% (Fig. 4). Phylogenetic diversity is 56.2 My for the lowland zone, 43.1 My for the upland zone and 21.5 My for the highland zone (Table 1). When species richness is used as a measure of biodiversity, the results show the highest proportion of zone-restricted endemic species to occur in the upland and lowland zones (contrary to Wiens *et al.* 2007 and references therein; Roberts *et al.* 2006). However, our PD estimates demonstrate that the evolutionary history of the Sri Lanka lowland freshwater crab community (assessed using the PDA) exceeds both upland and highland PD values (Fig. 4). When the same

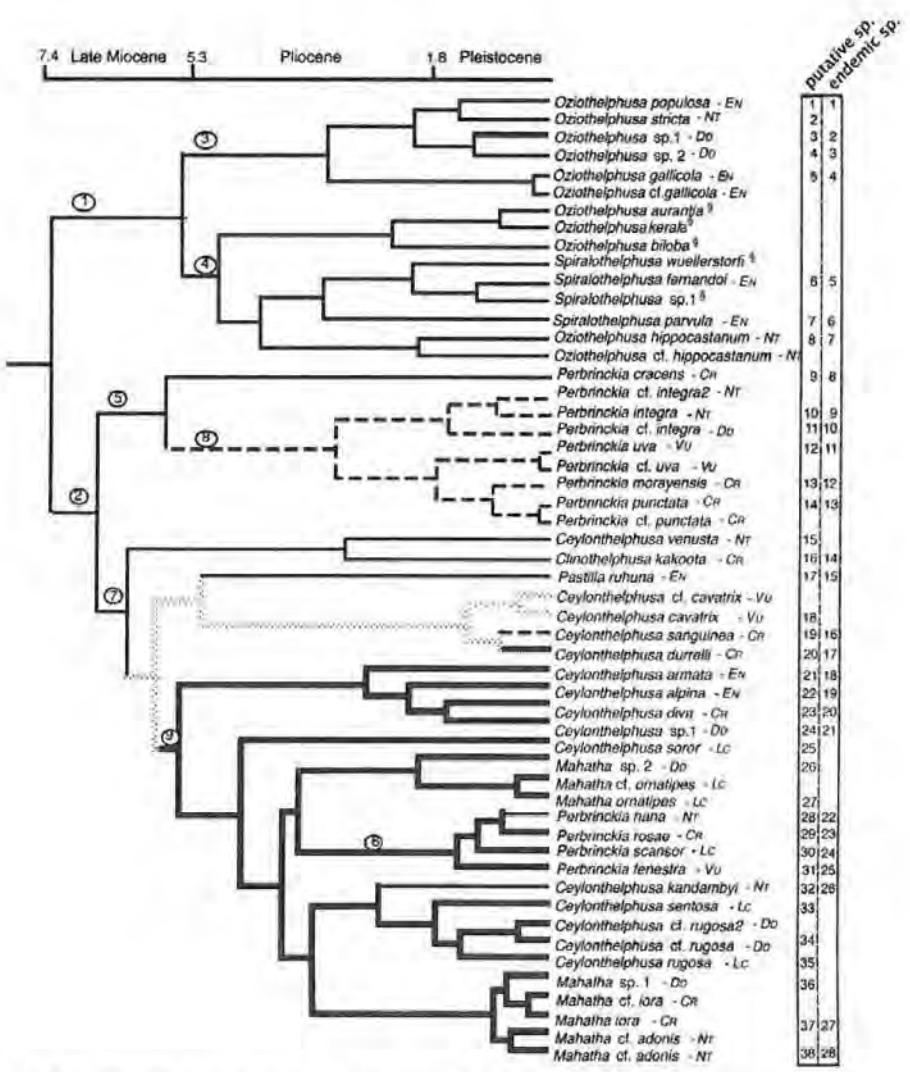

Fig. 2: The ML tree (see Fig. 3) converted to an ultrametric tree by estimating relative divergence ages (rttm = 8.757 My, rttmsd = 4.0 My, prior evolutionary rate is 1.63% My^{-1}; Schubart *et al.*, 1998) under a relaxed molecular clock. Branch colours indicate the different elevational zonations (mapped under maximum parsimony), whereby thin solid black branches represent presence in the lowlands, thin dashed black branches represent presence in the highlands and the broad black branches represent presence in the uplands. The stippled branches indicate equivocal ancestry. The Red List category is indicated alongside each putative species. The first and second columns represent, respectively, the number of putative species and the number of apparently zone-restricted endemic elevational species, which are used in the PD and species richness analyses.

number of taxa was randomly drawn from the tree, PD for the lowlands was significantly lower than observed (51.7, vs. 56.2 My; $p<0.05$), while PD for the upland and highland zones gave significantly higher results than observed (51.7 and 33.4 My, vs. 43.1 and 21.5 My, respectively; $p<0.05$). Performing similar PD computations on the alternative chronogram resulted in remarkably higher estimates (lowland: 269.5 My, upland: 224.2 My, highland: 91.9 My). The PD results for the species assessed to fall into the various IUCN Red List categories are provided in Table 1 for Sri Lanka as a whole and for each of the three elevational zones. Seven of the species included in the analysis have yet to be taxonomically validated; they were therefore not assigned to any Red List categories by Bahir *et al.* (2005), and are considered DD for this part of the analysis More than half of the Sri Lankan freshwater crab species included in this study are assessed as Vulnerable, Endangered or even Critically Endangered (Fig. 5). When we included the taxa currently regarded as DD, this threatened group increased considerably (from 9 to 16). The PD for this threatened group was 77 Ma (not shown in Table 1). However, all the results in Fig. 5, except for the species richness results, should be interpreted separately so as to be comparable; they cannot be treated cumulatively. Random sampling of the same number of taxa present in the different categories mentioned above shows no consistent lower PD values ($p<0.05$) than clustering Red List categories with the area constraint.

Table 1 (next page): Phylogenetic diversity (PD) measured as clade evolutionary history in million years and species richness (SR) in percentage (total number of putative species = 38 as indicated in Fig. 2). The freshwater crabs are grouped according to the IUCN Red List categories assessed for Sri Lanka as a whole and for each of the three elevational zones (lowland, upland, highland); a) data deficient taxa (DD) are treated as a separate category, b) data deficient taxa within each zone are regarded as taxa within the respective category (bold indicates $p<0.05$ or less). CR = Critically Endangered, VU = vulnerable, EN = endangered, LC = Least Concern, NT = Near Threatened, DD = Data Deficient.

Table 1

a)

Phylogenetic diversity (my)	Total	Data Deficient taxa (DD) as a separate category					
		CR	EN	VU	LC	NT	DD
Sri Lanka		43.8	35.5	20.1	21.3	40.8	35.7
LL	56.2	14.2	27.0	7.4		30.6	74.2
UL	43.1	22.7	10.1	7.4	21.3	7.4	27.8
HL	21.5	15.1		7.4		7.4	7.4
Species diversity							
Sri Lanka	38	9	7	3	5	7	7
Lowland	11	2	5	1	0	5	1
Upland	11	4	2	1	5	1	5
Highland	6	3	0	1	0	1	1

b)

Phylogenetic diversity (my)	Total	Data Deficient taxa (DD) included in each category				
		CR	EN	VU	LC	NT
Sri Lanka		64.2	60.5	48.5	48.9	58.0
Lowland	56.2	21.6	29.0	14.8	7.4	32.6
Upland	43.1	39.4	32.6	31.5	41.0	28.6
Highland	21.5	18.3	18.3	10.6	7.4	8.9
Species diversity						
Sri Lanka	38	16	14	10	12	14
Lowland	11	3	6	2	1	6
Upland	11	9	7	6	10	6
Highland	6	4	1	2	1	2

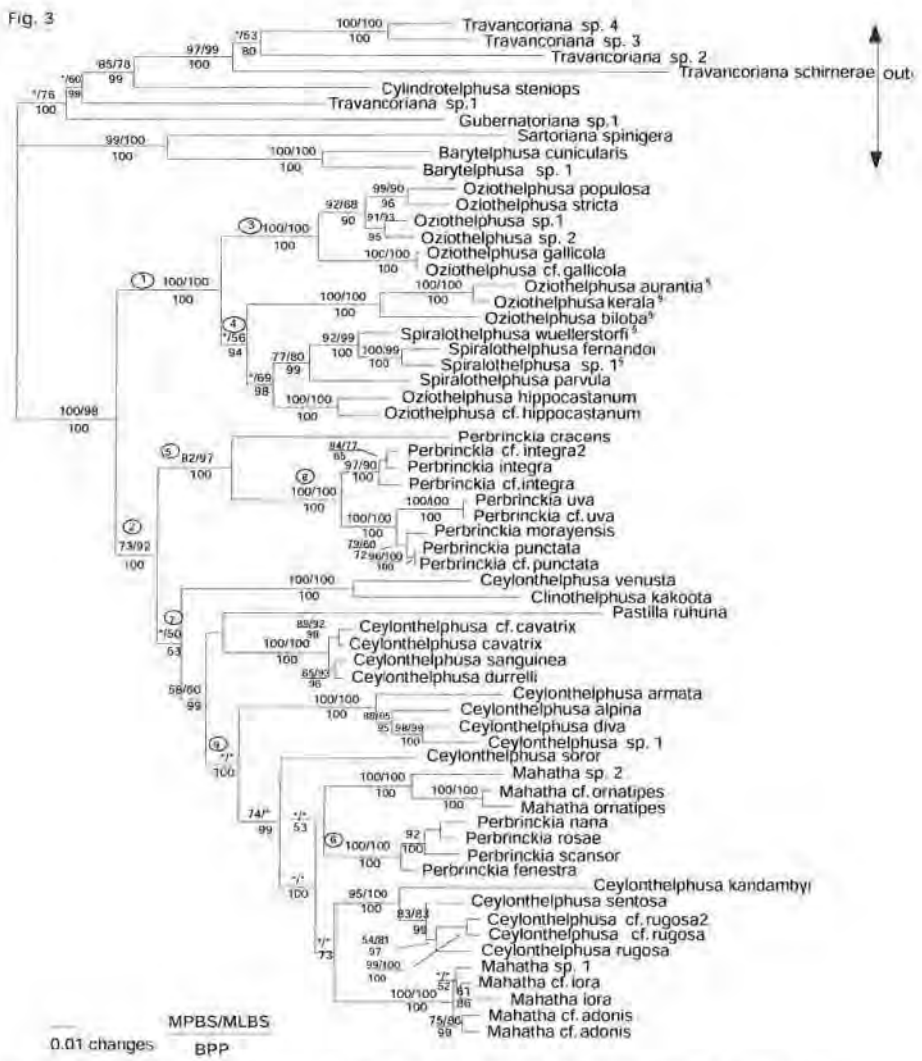

Fig. 3: The ML phylogram (-lnL = 15,177.40797) obtained from the analyses of the combined (total of 2047 bp consisting of the large fragment of about 1320 bp and COI) data set (n = 63 haplotypes) under a locus-based data partitioned GTR + Γ + I –model. Numbers above branches represent bootstrap values of Maximum Parsimony/Maximum Likelihood analyses. Values below branches represent Bayesian posterior probabilities (BPP). Values for the bootstraps or BPP of <50% are indicated with an asterisk. Numbers 1–9 refer to different clades mentioned in the text. Species sampled on the Indian subcontinent are indicated with §.

5.4. Discussion and conclusion

5.4.1 Molecular phylogeny and phylogenetic history

Sri Lanka's orography appears to have been relatively stable in the course of the past 10 million years, or at least since the Late Miocene (8 Ma). The different stages in the uplift of the Himalayas and Tibetan Plateau played an important role in the evolution of the South Asian monsoon (Prell and Kutzbach 1992; Zhisheng *et al.* 2001), leading to major changes in the climate of the Indian Ocean (Molnar *et al.* 1993). Coinciding with these events, significant changes in floral and faunal diversity too, occurred (Cerling *et al.* 1997), which are reflected in the results of the present study. Indeed, our dating estimates situate the first colonization of Sri Lanka by freshwater crabs in the late Miocene (around 7.42 Ma; Fig. 2). Furthermore, our results show that the initial colonization events were followed by simultaneous radiations within each of the elevational zones separately, with little interchange afterwards. These three radiation events started around the Miocene-Pliocene boundary (between 5.73 and 5.46 Ma), a period of global cooling, drying and of changing phytography. Our dating estimates converge with previous estimates obtained by Bossuyt *et al.* (2004). Although the alternative molecular dating approach (see Results, section 3.2) produces slightly older estimates, the patterns of diversification and phylogenetic diversity (see later in Discussion) allow for the same inferences.

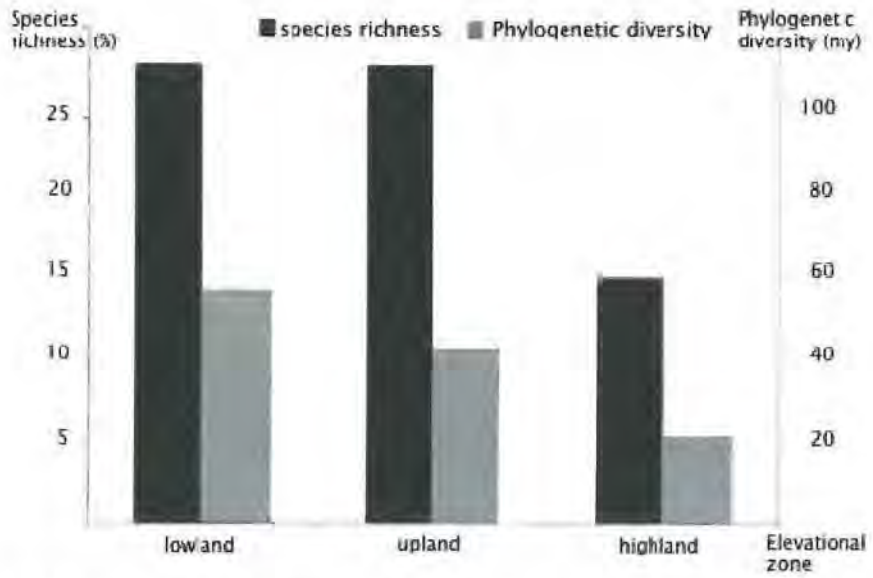

Fig 4: Species richness and phylogenetic diversity (PD) for the three elevational zones (lowland, upland and highland). The left vertical axis indicates percentage species richness; the right vertical axis indicates PD scores as clade evolutionary history (in million years).

Generally, species and PD are expected to be highest in intermediate elevational zones, both showing a hump-shaped elevational pattern (see Wiens *et al.* 2007). For freshwater crabs in Sri Lanka, our results show that species richness is highest in the intermediate and lower elevational zone (i.e. the lowlands and uplands). Moreover, several species could not be attributed to any one of these zones since they occur in more than a single zone. However, Sri Lankan lowland crabs show an unexpectedly high PD, implying that they have the richest evolutionary history among the island's carcinofauna. This clearly indicates that in biodiversity assessments, it cannot be taken for granted that species richness is a good surrogate for PD, as has been suggested by Brooks *et al.* (2006), or *vice versa*: we recommend that both parameters be considered in the

conservation-assessment process and the designation of protected areas and habitats.

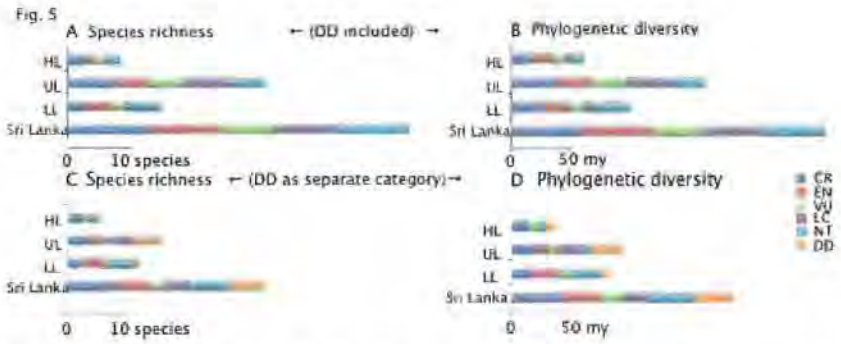

Fig 5: Species richness (SR) and Phylogenetic diversity (PD) according to IUCN Red List categories, for Sri Lanka as a whole and for the three different elevational zones separately (lowland, LL; upland, UL; highland, HL). A. Species richness for 'unique' haplotypes within each zone with DD taxa included as taxa belonging to the considered category (see text). B. PD scores for species 'endemic' to each zone with DD taxa included as taxa belonging to the considered category. C. Species richness for 'unique' haplotypes with DD taxa considered as a separate category. D. PD values for species 'endemic' to each zone with DD taxa considered as a separate category. The PD values (5b and 5d) and SR values (4a) are not to be accumulated (i.e. every category interpreted separately). CR = Critically Endangered, VU = vulnerable, EN = endangered, LC = Least Concern, NT = Near Threatened, DD = data deficient.

When Sri Lankan freshwater crabs are grouped according to their Red List category and PD and species richness calculated accordingly, several inferences can be made. Firstly, overall species richness and PD show similar trends for the different elevational zones (Fig 5). Secondly, over 50% of the freshwater crabs in this study are threatened (categories CR, EN and VU). This is equivalent to about 77 million years of evolutionary

freshwater-crab history. Thirdly, threatened species in each of the three elevational zones seem to experience a similar risk of population decline. Lastly, the upland or intermediate zone has the highest species richness and PD for Critically Endangered species, while the lowlands can be regarded as having the greatest evolutionary history and species richness for Endangered species. Although in the case of analyses that account for Red List categories PD and species richness results lead in general to the same conclusions, we nevertheless argue for the use of both diversity indices. For example, in an instance where there is only a single species in a given Red List category present in a given zone, the species richness will be low, but PD will reflect the occurrence of a possibly important species (endemic, ancient, rare) in that zone. It is more informative therefore, to assess both PD and species richness.

Currently, conservation measures in Sri Lanka are based purely on data relating to endemicity and species richness. As a result, these mostly aim at preserving upland and highland areas. However, the remaining natural habitats of both the wet lowland and upland areas are extremely fragmented, densely populated and liable to extreme anthropogenic stress (deforestation, plantations, rice fields, pollution, stream diversions). Our results, based on a combination of species richness and PD, indicate that the lowlands deserve greater attention. If this area were to be degraded further, whether from natural or anthropogenic causes, this could lead to the loss of about 56 Ma of freshwater-crab evolutionary history (Table 1). In addition, entire lineages that occur exclusively in the lowlands, such as the already endangered *Pastila ruhuna* and the Critically Endangered *Clinothelphusa kakoota*, would be vulnerable to extinction.

Conservation measures should strive to protect the broadest range of evolutionary diversity such as species that are rare, endemic (at various levels), threatened, or members of especially old lineages within groups of

organisms. Our results underline the necessity to include invertebrates in multi-taxonomic approaches to set conservation priorities in a hotspot region (Kremen *et al.* 2008).

While this study has important implications for conservation in Sri Lanka, it also lays a foundation for further work in this area. For instance, in our phylogenetic analysis, we used only mitochondrial loci. In other groups, studies with nuclear loci have already shown contradictory phylogenetic relationships (e.g., Brower *et al.* 1996; Shaw 2002; Galewski *et al.* 2006; Zink & Barrowclough 2008) and the future availability of more sequence data should diminish the influence of the priors (Holder & Lewis 2003; Huelsenbeck *et al.* 2002). Hence, future studies employing also nuclear loci, additional fossil records and morphological characters could investigate whether our results for the phylogenetic relationships, and consequently the dating estimates and evolutionary history calculations, still hold. Moreover, we acknowledge that the elevational zonation used is rather arbitrary. A future prospective could apply a more fine-tuned categorization to estimate PD in the different elevational zones and to the IUCN Red List categories. The pattern for the ancestral geographical distribution (i.e., ancestors occurring in lowlands, uplands and highlands) is clearly reflected in our phylogeny. However, in this study we do not make any inferences with regard to possible range shift of elevational zones over geological time.

5.4.2 Molecular phylogenetic relationships

All the freshwater brachyuran crabs of Sri Lanka belong to the Gecarcinucidae as defined by Klaus *et al.* (2009). As yet, however, no other genus from the Indian peninsula, not even in the fossil record, is known from Sri Lanka, except for members of two lowland genera (*Oziotelphusa* and *Spiralothelphusa*). This suggests that there has never

been a successful colonization of the island by highland Indian species (or *vice versa*) during the sea level low-stands that are known to have occurred frequently in the past (Bossuyt *et al.* 2004). Indeed, as pointed out also by Bossuyt *et al.* (2004), the remarkable diversity of many groups of terrestrial fauna in Sri Lanka appears to be the result of autochthonous insular speciation, especially in the mountains and moist south-western quarter of the island, from a relatively small number of colonizers from the mainland.

According to the present taxonomy, seven freshwater-crab genera occur in Sri Lanka, including two non-endemic genera, *Oziotelphusa* and *Spiralothelphusa*, which also have representatives in southern India. The apparently monophyletic *Spiralothelphusa* is nested within a paraphyletic *Oziotelphusa* and together they form a monophyletic clade (*Oziotelphusa-Spiralothelphusa*). The remaining five genera, all endemic to Sri Lanka, form the sister group to the *Oziotelphusa-Spiralothelphusa* clade. However, apart from the monotypic genera *Clinotelphusa* and *Pastilla*, all these endemic genera are polyphyletic. These results suggest that the generic classification of the Sri Lankan Gecarcinucidae is badly in need of revision, including a critical re-evaluation of the limited suite of morphological characters that has been used up to now. This matter is now being investigated in more taxonomically-orientated studies.

5.5 References

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Supplementary material

S1: Table 1. List of species, including haplotypes, family, voucher number, coordinates, ranges and elevational zone. Altitudinal ranges cover the geographical elevations at which the haplotype was collected and the different location/altitudes retrieved from the literature (see Material and Methods).

S2: List of species with their accession numbers

Supplementary Table 1:

	Family	Genus	Species	Voucher number	Country	Coordinates	Elevation (m)	Altitudinal range (m)	Elevational Zones
H1	Parathelphusidae	Pastilla	ruhuna	WHT10813	Sri Lanka	06°04′N, 080°13′E	10	10-50	LL
H1	Parathelphusidae	Pastilla	ruhuna	WHT10237	Sri Lanka	06°04′N, 080°13′E	10	10-50	LL
H1	Parathelphusidae	Pastilla	ruhuna	WHT10236	Sri Lanka	06°04′N, 080°13′E	10	10-50	LL
H2	Parathelphusidae	Ceylonthelphusa	soror	WHT10274	Sri Lanka	07°47′N, 080°41′E	810	150-2140	LL/UL/HL
H2	Parathelphusidae	Ceylonthelphusa	soror	WHT10269	Sri Lanka	07°47′N, 080°41′E	810	150-2140	LL/UL/HL
H2	Parathelphusidae	Ceylonthelphusa	soror	WHT10279	Sri Lanka	07°47′N, 080°41′E	810	150-2140	LL/UL/HL
H3	Parathelphusidae	Ceylonthelphusa	kandambyi	WHT10788	Sri Lanka	06°10′N, 080°21′E		80-150	LL
H4	Parathelphusidae	Ceylonthelphusa	rugosa	WHT10347	Sri Lanka	06°52′N, 080°37′E	1300	45-1714	LL/UL/HL
H4	Parathelphusidae	Ceylonthelphusa	rugosa	WHT10281	Sri Lanka	06°56′N, 080°30′E	900	45-1714	LL/UL/HL
H5	Parathelphusidae	Ceylonthelphusa	sentosa	WHT10272	Sri Lanka	06°15′N, 080°21′E	165	50-350	LL/UL
H5	Parathelphusidae	Ceylonthelphusa	sentosa		Sri Lanka	06°22′N, 080°28′E	350	50-350	LL/UL
H6	Parathelphusidae	Ceylonthelphusa	rugosa	WHT10778	Sri Lanka	07°17′N, 080°35′E	620	45-1714	LL/UL/HL
H6	Parathelphusidae	Ceylonthelphusa	rugosa	WHT10779	Sri Lanka	07°34′N, 080°45′E	450	45-1714	LL/UL/HL
H6	Parathelphusidae	Ceylonthelphusa	rugosa	WHT10348	Sri Lanka	06°52′N, 080°37′E	1300	45-1714	LL/UL/HL
H6	Parathelphusidae	Ceylonthelphusa	rugosa	WHT10785	Sri	07°16′N,	1000	45-1714	LL/UL/HL

					Lanka	080°37′E			
H7	Parathelphusidae	Ceylonthelphusa	cf. rugosa	WHT10340	Sri Lanka	06°29′N, 080°36′E	500	500	UL
H8	Parathelphusidae	Mahatha	sp. 2	WHT10326	Sri Lanka	06°04′N, 080°12′E	15	15-1220	LL/UL/HL
H9	Parathelphusidae	Mahatha	cf. ornatipes	WHT10280	Sri Lanka	06°56′N, 080°30′E	900	15-1220	LL/UL/HL
H10	Parathelphusidae	Mahatha	cf. ornatipes	WHT10810	Sri Lanka	06°55′N, 080°17′E	60	15-1220	LL/UL/HL
H11	Parathelphusidae	Ceylonthelphusa	scansor	WHT10774	Sri Lanka	07°16′N, 080°37′E	950	150-960	UL
H11	Parathelphusidae	Ceylonthelphusa	scansor	WHT10783	Sri Lanka	07°16′N, 080°37′E	950	150-960	UL
H12	Parathelphusidae	Perbrinckia	nana	WHT10289	Sri Lanka	06°15′N, 080°21′E	165	100-165	LL
H13	Parathelphusidae	Perbrinckia	rosae	WHT10295	Sri Lanka	06°24′N, 080°36′E	1060	1060	UL
H14	Parathelphusidae	Mahatha	iora	WHT10189	Sri Lanka	06°53′N, 081°02′E	900	900	UL
H14	Parathelphusidae	Mahatha	iora	WHT10190	Sri Lanka	06°53′N, 081°02′E	900	900	UL
H15	Parathelphusidae	Mahatha	sp.1	WHT10368	Sri Lanka	07°34′N, 080°41′E	745	745	UL
H16	Parathelphusidae	Mahatha	adonis	WHT10374	Sri Lanka	06°54′N, 081°08′E	750	150-750	LL/UL
H17	Parathelphusidae	Mahatha	cf. iora	WHT10192	Sri Lanka	07°01′N, 081°04′E	610	610	UL
H17	Parathelphusidae	Mahatha	cf. iora	WHT10193	Sri Lanka	07°01′N, 081°04′E	610	610	UL
H18	Parathelphusidae	Ceylonthelphusa	alpina	WHT10774	Sri Lanka	07°17′N, 080°35′E	620	620-1000	UL
H18	Parathelphusidae	Ceylonthelphusa	alpina	WHT10775	Sri Lanka	07°17′N, 080°35′E	620	620-1000	UL
H18	Parathelphusidae	Ceylonthelphusa	alpina	WHT10776	Sri Lanka	07°17′N, 080°35′E	620	620-1000	UL
H19	Parathelphusidae	Ceylonthelphusa	alpina	WHT10786	Sri Lanka	07°16′N, 080°37′E	1000	620-1000	UL

H20	Parathelphusidae	Ceylonthelphusa	diva	WHT10367	Sri Lanka	07°34'N, 080°42'E	745	745	UL
H21	Parathelphusidae	Ceylonthelphusa	armata	WHT10267	Sri Lanka	07°15'N, 080°28'E	305	305	UL
H22	Parathelphusidae	Ceylonthelphusa	cf. cavatrix	WHT10361	Sri Lanka	07°33'N, 080°44'E	1100	450-1100	UL/HL
H22	Parathelphusidae	Ceylonthelphusa	cf. cavatrix	WHT10365	Sri Lanka	07°34'N, 080°42'E	850	450-1100	UL/HL
H22	Parathelphusidae	Ceylonthelphusa	cf. cavatrix	WHT10354	Sri Lanka	07°33'N, 080°44'E	1100	450-1100	UL/HL
H23	Parathelphusidae	Ceylonthelphusa	cavatrix	WHT10352	Sri Lanka	07°33'N, 080°44'E	1100	450-1100	UL/HL
H23	Parathelphusidae	Ceylonthelphusa	cavatrix	WHT10362	Sri Lanka	07°33'N, 080°44'E	1100	450-1100	UL/HL
H23	Parathelphusidae	Ceylonthelphusa	cavatrix	WHT10403	Sri Lanka	07°34'N, 080°45'E	450	450-1100	UL/HL
H24	Parathelphusidae	Ceylonthelphusa	sanguinea	WHT10357	Sri Lanka	07°32'N, 80°44'E	1220	1220	HL
H25	Parathelphusidae	Ceylonthelphusa	durrelli	WHT10209	Sri Lanka	07°22'N, 80°50'E	1000m	1000	UL
H26	Parathelphusidae	Clinothelphusa	kakoota	WHT10812	Sri Lanka	06 52 N, 80 09E	156	100-275	LL
H27	Parathelphusidae	Ceylonthelphusa	venusta	WHT10268	Sri Lanka	06°15'N, 080°21'E	165	150-460	LL/UL
H27	Parathelphusidae	Ceylonthelphusa	venusta	WHT10041	Sri Lanka	06°15'N, 080°21'E	165	150-460	LL/UL
H27	Parathelphusidae	Ceylonthelphusa	venusta	WHT10817	Sri Lanka	06°15'N, 080°21'E	165	150-460	LL/UL
H28	Parathelphusidae	Oziothelphusa	sp. 2	WHT10336	Sri Lanka	06°25'N, 080°56'E	73m	73	LL
H28	Parathelphusidae	Oziothelphusa	sp. 2	WHT10337	Sri Lanka	06°25'N, 080°56'E	73m	73	LL
H28	Parathelphusidae	Oziothelphusa	sp. 2	WHT10335	Sri Lanka	06°25'N, 080°56'E	73m	73	LL
H29	Parathelphusidae	Oziothelphusa	sp. 1	WHT10789	Sri Lanka	06°21'N, 080°29'E	365	365	UL
H30	Parathelphusidae	Oziothelphusa	gallicola	WHT10270	Sri	06°03'N,	10	2-15	LL

					Lanka	080°12′E			
H31	Parathelphusidae	Oziothelphusa	gallicola	WHT10815	Sri Lanka	06°03′N, 080°12′E	10	2-15	LL
H32	Parathelphusidae	Oziothelphusa	stricta	WHT10780	Sri Lanka	07°34′N, 80°45′E	450	20-600	LL/UL
H32	Parathelphusidae	Oziothelphusa	stricta	WHT10338	Sri Lanka	06°13′N, 081°18′E	20	20-600	LL/UL
H32	Parathelphusidae	Oziothelphusa	stricta	WHT10339	Sri Lanka	06°13′N, 081°18′E	20	20-600	LL/UL
H33	Parathelphusidae	Oziothelphusa	populosa	WHT10325	Sri Lanka	06°54′N, 079°51′E	5	1-33	LL
H34	Parathelphusidae	Oziothelphusa	biloba	ZRC 2003. 0246	India	010°21′N, 076°18′E	6	5-6	LL
H34	Parathelphusidae	Oziothelphusa	biloba	ZRC 2003. 0247	India	010°21′N, 076°21′E	5	5-6	LL
H34	Parathelphusidae	Oziothelphusa	biloba	WHT10733	India	010°21′N, 076°18′E	5	5-6	LL
H34	Parathelphusidae	Oziothelphusa	biloba	WHT10735	India	010°21′N, 076°18′E	5	5-6	LL
H35	Parathelphusidae	Oziothelphusa	kerala	WHT10746	India	08°56′N, 076°50′E	56	56	LL
H35	Parathelphusidae	Oziothelphusa	kerala	WHT10723	India	08°54′N, 077°32′E	120	120	LL
H35	Parathelphusidae	Oziothelphusa	kerala	WHT10722	India	08°54′N, 077°32′E	120	120	LL
H36	Parathelphusidae	Oziothelphusa	aurantia	WHT10795	India	app. 13° 4' N, 080° 15' E	2	2	LL
H37	Parathelphusidae	Oziothelphusa	hippocastanum	WHT10859	Sri Lanka	07°50′N, 079°50′E	6	2-70	LL
H38	Parathelphusidae	Oziothelphusa	hippocastanum	WHT10377	Sri Lanka	08°16′N, 080°13′E	70	2-70	LL
H38	Parathelphusidae	Oziothelphusa	hippocastanum	WHT10382	Sri Lanka	08°16′N, 080°13′E	70	2-70	LL
H39	Parathelphusidae	Spiralothelphusa	parvula	WHT10852	Sri Lanka	06°52′N, 079°53′E	5	5-150	LL
H40	Parathelphusidae	Spiralothelphusa	wuellerstorfi	WHT10793	India	app. 13° 4' N, 080° 15' E	2	2	LL

H41	Parathelphusidae	Spiralothelphusa	fernandoi	WHT10732	India	10°03'N, 076°31'E	60	4-30	LL
H41	Parathelphusidae	Spiralothelphusa	fernandoi	WHT10854	Sri Lanka	07°50'N, 079°50'E	6	4-30	LL
H42	Parathelphusidae	Spiralothelphusa	sp. 1	WHT10726	India	10°21'N, 076°18'E	6	6	LL
H42	Parathelphusidae	Spiralothelphusa	sp. 1	WHT10725	India	10°21'N, 076°18'E	6	6	LL
H43	Parathelphusidae	Perbrinckia	cracens	WHT10809	Sri Lanka	06°55N, 080° 17E	60	60-100	LL
H44	Parathelphusidae	Perbrinckia	uva	WHT10375	Sri Lanka	07°05'N, 081°09'E	1300	1300-1900	HL
H45	Parathelphusidae	Perbrinckia	morayensis	WHT10214	Sri Lanka	06°50'N, 080°39'E	1650	1370-1650	HL
H45	Parathelphusidae	Perbrinckia	morayensis	WHT10215	Sri Lanka	06°50'N, 080°39'E	1650	1370-1650	HL
H45	Parathelphusidae	Perbrinckia	morayensis	WHT10333	Sri Lanka	06°48'N, 080°30'E	1980	1370-1650	HL
H45	Parathelphusidae	Perbrinckia	morayensis	WHT10313	Sri Lanka	06°50'N, 080°39'E	1650	1370-1650	HL
H46	Parathelphusidae	Perbrinckia	punctata	WHT10258	Sri Lanka	06°51'N, 080°49'E	1860	1800-2150	HL
H47	Parathelphusidae	Perbrinckia	punctata	WHT10806	Sri Lanka	06°51'N, 080°49'E	1860	1800-2150	HL
H48	Parathelphusidae	Perbrinckia	cf. integra	WHT10327	Sri Lanka	06°56'N, 080°46'E	1750	1060-2140	HL
H48	Parathelphusidae	Perbrinckia	cf. integra	WHT10328	Sri Lanka	06°56'N, 080°46'E	1750	1060-2140	HL
H48	Parathelphusidae	Perbrinckia	cf. integra	WHT10254	Sri Lanka	06°59'N, 080°45'E	1980	1060-2140	HL
H48	Parathelphusidae	Perbrinckia	cf. integra	WHT10256	Sri Lanka	06°59'N, 080°45'E	1980	1060-2140	HL
H48	Parathelphusidae	Perbrinckia	cf. integra	WHT10802	Sri Lanka	07°00'N, 080°53'E	1400	1060-2140	HL
H49	Parathelphusidae	Perbrinckia	integra	WHT10286	Sri Lanka	07°00'N, 080°40'E	1830	1060-2140	HL
H49	Parathelphusidae	Perbrinckia	integra	WHT10791	Sri	06°51'N,	1555	1060-2140	HL

					Lanka	080°41'E			
H49	Parathelphusidae	Perbrinckia	integra	WHT10334	Sri Lanka	06°48'N, 080°30'E	1980	1060-2140	HL
H49	Parathelphusidae	Perbrinckia	integra?	WHT10288	Sri Lanka	06°52'N, 080°40'E	1450	1060-2140	HL
H50	Parathelphusidae	Perbrinckia	fenestra	WHT10245	Sri Lanka	06°47'N, 080°23'E	480	480	UL
H50	Parathelphusidae	Perbrinckia	fenestra	WHT10245	Sri Lanka	06°47'N, 080°23'E	480	480	UL
H51	Parathelphusidae	Perbrinckia	cf. uva	WHT10376	Sri Lanka	07°05'N, 081°09'E	1300	1300-1900	HL
H52	Parathelphusidae	Perbrinckia	cf. integra2	WHT10253	Sri Lanka	06°59'N, 080°45'E	1980	1980	HL
H53	Parathelphusidae	Mahatha	cf. adonis	WHT10782	Sri Lanka	07°34'N, 080°45'E	450	150-450	LL/UL
OUT	Gecarcinucidae	Barytelphusa	cunicularis	WHT10532	India	11°10'N, 076°19'E	52	nn	nn
OUT	Gecarcinucidae	Cylindrotelphusa	steniops	WHT10565	India	08°39'N, 090°03'E	100	nn	nn
OUT	Gecarcinucidae	Gubernatoria	sp. 1	WHT10703	India	08°46'N, 077°06'E	975	nn	nn
OUT	Gecarcinucidae	Sartoriana	spinigera		India	nn	nn	nn	nn
OUT	Gecarcinucidae	Travancoriana	sp. 4	WHT10678	India	Munnar-Pollachchi Rd, Kerala	nn	nn	nn
OUT	Gecarcinucidae	Travancoriana	sp. 3	WHT10696	India	Kumerli-Munnar Rd, Kerala	nn	nn	nn
OUT	Gecarcinucidae	Travancoriana	sp. 2	WHT10635	India	Ponmudi, Kerala	nn	nn	nn
OUT	Gecarcinucidae	Travancoriana	schirnerae	WHT10612	India	Mettupalayam-Ooti Rd, Tamil Nadu	nn	nn	nn
OUT	Gecarcinucidae	Travancoriana	sp. 1	WHT10606	India	Ponmudi, Kerala	nn	nn	nn
OUT	Gecarcinucidae	Barytelphusa	sp. 1	WHT10522	India	Chathankodu, Kerala	nn	nn	nn

S2: List of accession numbers and ID for all species used in the different analyses of this study.

Genus	Species	ID	extended 16S	COI
Mahatha	*cf. adonis*	2001MahAd	GQ289586	GQ289614
Oziothelphusa	*stricta*	2006OziNn	AY708086	GQ289615
Ceylonthelphusa	*alpina*	2007CeyNn	AY708051	GQ289616
Ceylonthelphusa	*scansor*	2008CeySca	AY708052	GQ289617
Oziothelphusa	*sp. 2*	2012OziNn	AY708053	GQ289618
Perbrinckia	*nana*	2013PerNan	AY708054	GQ289619
Mahatha	*iora*	2016MahIor	AY708055	GQ289620
Ceylonthelphusa	*sanguinea*	2017CeySa	GQ982590	GQ289621
Ceylonthelphusa	*rugosa*	2018CeyRug	AY708056	GQ289622
Ceylonthelphusa	*alpina*	2019CeyAl	GQ289587	GQ289623
Ceylonthelphusa	*cavatrix*	2021CeyCav	AY708057	GQ289624
Perbrinckia	*morrayensis*	2022PerMor	AY708058	GQ289625
Ceylonthelphusa	*diva*	2033CeyDi	GQ289588	GQ289626
Mahatha	*adonis*	2035MahAdo	AY708059	GQ289627
Ceylonthelphusa	*cf. rugosa*	2036CeyN	GQ289589	GQ982585
Ceylonthelphusa	*cf. cavatrix*	2039CeyCa	GQ289590	GQ982586
Mahatha	*sp.*	2042CeyDi	GQ289591	GQ289628
Perbrinckia	*uva*	2056PerUv	GQ289592	GQ289629
Perbrinckia	*cf. uva*	2058PerUv	GQ289593	GQ289630
Oziothelphusa	*populosa*	2070OziCey	AY708060	GQ289631
Oziothelphusa	*kerala*	2073OziNn1	AY708062	GQ982587
Travancoriana	*sp.4*	2074TraNn2	AY708063	GQ289632
Spiralothelphusa	*sp. 1*	2077SpiNn3	AY708064	GQ289633
Barytelphusa	*cunicularis*	2084BarCun	AY708065	GQ289634

Travancoriana	*schirnerae*	2086TraSch	AY708066	GQ289635
Travancoriana		2094TraNn3	AY708068	GQ289636
Barytelphusa	*sp.1*	2098BarNn1	AY708071	GQ289637
Travancoriana	*sp.1*	2099TraNn6	AY708072	GQ289638
Cylindrotelphusa	*steniops*	2100CylSte	AY708073	GQ289639
Mahatha	*iora*	2102MahIor	AY708074	GQ289640
Ceylonthelphusa	*armata*	2106CeyArm	AY708075	GQ289641
Perbrinckia	*fenestra*	2108PerFe	AY708076	GQ289642
Ceylonthelphusa	*rugosa*	2109CeyRu	GQ289594	GQ289643
Perbrinckia	*cf. integra*	2119PerN	GQ289595	frameshift!
Mahatha	*sp. 2*	2120MahOrn	AY708078	GQ289644
Ceylonthelphusa	*soror*	2122CeySor	AY708079	GQ289645
Perbrinckia	*cf. integra2*	2126PerN	GQ289596	frameshift!
Oziothelphusa	*gallicola*	2128OziGa	GQ289597	GQ289647
Perbrinckia	*punctata*	2132PerPu	GQ289598	GQ289648
Ceylonthelphusa	*sentosa*	2134CeySen	AY708081	GQ289649
Mahatha	*cf ornatipes*	2139MahOr	GQ289599	GQ289650
Pastilla	*ruhuna*	2140PasRuh	AY708082	GQ289651
Oziothelphusa	*sp. 1*	2143OziNn	AY708083	GQ289652
Ceylonthelphusa	*kandambyi*	2151CeyKan	AY708084	GQ289653
Oziothelphusa	*biloba*	2154OziBi	GQ289600	GQ289654
Gubernatoria	*sp.*	2158NewGen	AY708087	GQ289655
Travancoriana		2174TraNn	AY708088	GQ289656
Oziothelphusa	*hippocastanum*	2176OziHi	AY708089	GQ982588
Perbrinckia	*rosae*	2183PerRo	GQ289601	GQ289657

Perbrinckia	*integra*	2186PerIn	GQ289602	frameshift!
Spiralothelphusa	*wuellerstorfi*	2188SpiWue	AY708090	GQ289658
Oziothelphusa	*aurantia*	2190OziAu	GQ289603	GQ289659
Ceylonthelphusa	*durrelli*	2192Ceydu	AY708091	GQ289660
Sartorinana	*spinigera*	2196SSpi	GQ289604	GQ289661
Perbrinckia	*punctata*	2207PerPu	GQ289605	GQ289662
Perbrinckia	*cracens*	2208PerCr	GQ289606	GQ982589
Mahatha	*cf. ornatipes*	2209MahOr	GQ289607	GQ289663
Clinothelphusa	*kakoota*	2211CliKa	GQ289608	GQ289664
Oziothelphusa	*gallicola*	2214OziGa	GQ289609	GQ289665
Ceylonthelphusa	*venusta*	2215Ceyve	GQ289610	GQ289666
Spiralothelphusa	*fernandoi*	2218SpiFe	GQ289611	GQ289667
Oziothelphusa	*hippocastanum*	2219OziHi	GQ289612	GQ289668
Spiralothelphusa	*parvula*	2221SpiPa	GQ289613	GQ289669

Sequences from Daniels et al, 2006		**16S**	**COI**
Barytelphusa	*sp.3*	AY919092	AY919116
Barytelphusa	*sp.1*	AY919091	AY919115
Barytelphusa	*sp.2*	AY919082	AY919111
Barytelphusa	*jacquemontii*	AY919088	AY919110
Barytelphusa	*cunicularis*	AY919087	AY919113
Ceylonthelphusa	*rugosa*	AY803560	AY803587
Deckenia	*imitatrix*	AY803544	AY803576
Gecarcinucus	*jacquemonti*	AY919086	AY919108
Geothelphusa	*albogilva*	AY803559	AY803586
Gubernatoriana	*gubernatoris*	AY919089	AY919112

Hydrothelphusa	*agilis*	AY803546	AY803578
Hydrothelphusa	*madagascariensis*	AY803549	AY803580
Hydrothelphusa	*goudoti*	AY803548	AY803579
Isolapotamon	*consobrinum*	AY803557	N/a
Marojejy	*longimerus*	AY803552	AY803582
Parathelphusa	*sp.*	AY803561	AY803588
Potamon	*fluviatilis*	AY803554	AY803584
Potamonautes	*obesus*	AY803537	AY803591
Potamonautes	*rayboldi*	AY803540	AY803573
Potamonautes	*platynotus*	AY803539	AY803572
Potamonautes	*niloticus*	AY803536	N/a
Potamonautes	*obesus*	AY803537	AY803570
Potamonautes	*lirrangensis*	AY803534	AY803568
Potamonautes	*emini*	AY803533	N/a
Pricothelphusa	*limula*	AY803565	AY803591
Rouxana	*sp.2*	AY803563	AY803589
Sartoriana	*spinigera*	AY803566	AY803592
Sayamia	*sexpunctata*	AY803564	AY803590
Skelosophusa	*eumeces*	AY803553	AY803583
Socotra	*pseudocardisoma*	AY803555	AY803585
Sudanonautes	*floweri*	AY803541	AY803574
Sudanonautes	*aubryi*	AY803542	AY803575

Daniels SR, Cumberlidge N, Pérez-Losada M, Marijnissen SAE, Crandall KA (2006) Evolution of Afrotropical freshwater crab lineages obscured by morphological convergence. Molecular Phylogenetics and Evolution ***40****, 227–235.*

06

Conclusions, future considerations and challenges

CONCLUSIONS, FUTURE CHALLENGES AND CONSIDERATIONS

The freshwater crabs have not been lavished with a lot of attention in the biological sciences, although they are important in the food chain of several animals and human populations, and they could be used as bio-indicators or serve as invertebrate representative in biodiversity.

This study has given more insight in several evolutionary aspects of freshwater crabs, using molecular tools to answer questions regarding bogeographical patterns and biodiversity issues with their implications for conservation.
In summary the conclusions are follows:
The contemporary distribution of the Old World freshwater crabs can best be explained through a post-Gondwanan diversification that started about the mid Eocenen. Nevertheless, this most plausible biogeographical pattern requires several oceanic dispersals to explain the distribution (chapter three).
Furthermore, the almost complete inventory of the freshwater crabs of Sri Lanka allowed us to focus further on the fauna of an island that is part of the Western Ghats/Sri Lanka hotspot, one of the 34 recognized biodiversity hotspots (Mittermeier et al., 2004; chapter 4 and 5). The superficial similarity between biotic groups of both landmasses has made people believe that much exchange between both landmasses occurred in the past (mainly the Pleistocene). Yet, we demonstrated (chapter four) that the Sri Lankan fauna shows high levels of endemism and that faunal exchanges between the mainland of the Indian subcontinent and Sri Lanka occurred less then previously expected. Moreover, these exchanges occurred before both landmasses were connected through a land bridge on several occasions during the ice ages.
This high level of endemism on Sri Lanka and the genetic difference with the Indian mainland species should trigger conservationists to consider Sri Lanka as a separate biodiversity hotspot. For the freshwater crabs, being part of this unique Sri Lankan fauna, we discovered an almost simultanous dispersal of three clades into three elevational zones. Moreover, we discovered that species

richness is highest in the lowlands and uplands, but phylogenetic diversity (Faith 1992, 2006; Sechrest *et al.*, 2002) is, unexpectedly, higher in the lowlands than in the uplands and highlands. It shows that PD and species richness pertain to different issues in biological diversity. The same indices for species in the IUCN Red List categories indicate that more than 50% of the freshwater crab species, or more than 72 my of evolutionary history, is threatened with extinction. We conclude that both measures, PD and species richness, should be considered in setting priorities for conservation (chapter 5).

Evidently, as in all fields of sciences, the results are the source of several new questions that demand future research. In the following paragraph I highlight some ideas for prospective research.

To assess biodiversity, biogeography and conservation in an evolutionary context, a first prerequisite is the inference of the correct phylogenetic relationships of the organisms. The recent increase in molecular studies is providing more material for discussion, but compared to other groups, such as mammals, birds and amphibians, the available datasets remain less extensive, especially for nuclear sequences. Apparently adding more taxa has less influence on the correct phylogenetic relationships than adding different loci (mitochondrial and nuclear) and longer sequences (Holder & Lewis, 2003). Within the freshwater crabs, the existing DNA-data definitely needs extension with more markers, e.g., including EST's, two- and three-dimensional macromolecular structures, or even whole genomic sequences. Within Brachyuran molecular phylogenetic studies, a first step would already involve the development of 'good' primers (Schubart, 2009), for both traditional and new molecular markers.

Moreover, the molecular data for the New World freshwater crabs, Pseudothelphusidae and Trichodactylidae, are very scant. There is a consensus that the New World Trichodactylidae and the other true freshwater crab families within the Potamoidea sensu Cumberlidge & Ng, 2009, together do not form a monophyletic group. However, most evidence is gathered from morphological

studies (Rodriguez, 1992; Sternberg et al., 1999). The latest proposal (Cumberlidge & Ng, 2009), to consider the New World Pseudothelphusidae and the Old World freshwater crabs as a monophyletic group, is questioned in chapter three. I show the consistent presence of *Pachygrapsus marmoratus,* an intertidal marine crab, between the New World Pseudothelphusidae and Old World freshwater crabs, which raise questions on the widely believed monophyly of this group (see Cumberlidge & Ng, 2009 and references therein). To resolve this question, more sampling is needed, especially on the New World freshwater crabs and marine Brachyura. With a broad and complete brachyuran sampling, the closest sistergroup(s) to the freshwater crabs could be reliably identified. This question will be most efficiently be resolved through collaboration with other research groups, which attempt to resolve the decapod and brachyuran phylogeny.

In the quest to elucidate the evolutionary patterns, not only the 'correct' phylogenetic relationships within an accurate time frame are important, but also the availability of appropriate analytical computing tools to infer e.g. the most likely biogeographical scenarios are becoming increasingly important. Until recently, the most optimal ancestral routes between vicariance and dispersal were detected mainly with maximum parsimony based analysis programs, such as DIVA 1.1 (Ronquist, 1997). Biogeographers are increasingly implementing the probabilistic Dispersal-Extinction-Cladogenesis (DEC) model as implemented in the Lagrange software 1.0 (Ree et al., 2005, Ree & Smith, 2008). This program uses a phylogenetic hypothesis with divergence time estimates, and combines it with dispersal capacities and extinction rates. Different biogeographical scenarios are assigned with a likelihood value. In this study a first step has been taken, which only opened the gate towards a more intensive exploration.

To reveal the global biogeographical patterns for freshwater crabs (including New World freshwater crabs) within a temporal framework would call for the following summarized future research 1) provide enough molecular data on Pseudothelphusidae and Trichodactylidae (see above); 2) extension of the

molecular dataset with more varied DNA sequences (mitochondrial and nuclear) and possibly morphological data; 3) reliable identification of the closest brachyuran sister group; 4) and ideally the discovery of more fossil evidence for the different freshwater crab groups.

The ever-growing complex and computationally faster modeling and statistical tools to infer phylogenies, time estimates and ancestral range reconstructions will similarly increase the opportunities to understand and resolve the above questions with even more confidence.

From a different perspective, new insights in the biology and physiology of the freshwater crabs, such as their capacity to cross marine environments, could definitely back up some inferences. There are for instance only a handful of publications that actually performed in-depth physiological research with respect to the saltwater tolerance of freshwater crabs, i.e., tests on various degrees in salinity and periods of exposure. Additional research is needed in this field. Just as the various adaptations in the freshwater environment from strict freshwater, to semi-terrestrial to almost entirely terrestrial conditions, one might expect various levels of tolerance to saltwater. Some lowland freshwater crabs on Sri Lanka have been observed in brackish waters (personal observations; Bahir, unpublished data).

Within this study the role of climate has not been extensively explored. For instance the global climatological optima Eocene occurred about 55 mya and again in Late Oligocene (33-34 mya) (Zachos et al., 2001, Liu et al., 2009), which apparently boosted the diversity of life on Earth globally (e.g., Schram, 1986; Mercer and Roth, 2003; Knapp et al., 2005; Vieites et al., 2007, Moreau et al., 2006; Teeling et al., 2005; Merckx et al., 2008). This framework could be an interesting prospect for future studies within freshwater crabs. As far as I know, in-depth research on relating freshwater crab phylogenies to the historical climatic changes, with respect to geographical shifts over the past Tertiary has not been performed. Adding extra distributional data to the data of chapter three should enable preliminary predictions.

Climate has caused major shift in biological diversity over the past, but also in this epoch climate plays an important role. Climate change is an important issue on the political agenda and probably already has a major influence on the biological diversity. The society realizes biological diversity is declining rapidly (whether caused by natural and/or anthropological causes), and realizes the necessity to conserve the worlds natural richness. However, conservation is expensive, and to save at least part of the valuable biodiversity, specific areas for protection are selected worldwide. Commonly, species richness is being used as a measure to rank these areas, such as biodiversity hotspots, but also measures, such as endemism (endemism hotspots), risk of threat, ecological importance or attractiveness have been used. Recently the use of evolutionary heritage is gaining more advocates. Phylogenetic diversity estimates this evolutionary history in the tree of life and can/should be used as a complementary conservation ranking measure (see chapter 5). It can be used to set new priorities in conservation actions. It can for instance provide evidence that (important) groups of fauna occur in regions that are currently not part of protected areas.

This study also calls for more detailed future research on Sri Lanka. Expecially regarding the importance of phylogenetic diversity within a more fine-tuned categorization (e.g., combining elevation to more specific ecological and environmental factors) to estimate PD in the different elevational zones and related to the IUCN Red List categories. Phylogenetics have more potential for conservation biology, but too many conservationists still ignore using this valuable information.

Moreover, our results underline the necessity to include invertebrates in multi-taxonomic approaches to set conservation priorities in a hotspot region (Kremen *et al.* 2008), and urge the scientific community to gather more knowledge on the less studied groups, which are often invertebrates.

Finally our phylogenies clearly show that current classification is greatly in need of revision, and the value or weight, of some frequently used diagnostic morphological characters should be reassessed. Within the monophyletic Sri

Lankan group the first proposals are taking shape. After the initial major inventory and re-assessment (Ng & Tay, 2001; Bahir & Ng, 2005; Bahir & Yeo, 2005), several species will most likely be placed in different genera. The Old World phylogeny could contribute towards the re-appraisal of several morphological diagnostic characters used for deeper taxonomical levels (family, superfamily). Over the past years much work in this field has been done...but there is need for more. However, one can only truly understand and assess the correctness of the mathematical (molecular) phylogeny, if one also understands the morphology and biology of the organism making up the tree. This latter knowledge contributes to evaluate the presence of ambiguous sequences and relationships within molecular phylogenies. Moreover, there are still many new freshwater crabs discovered, which need to be described and identified. Both fields can facilitate each other to reach the correct phylogenetic relationships.

These are only a handful of opportunities to continue and elaborate on the research performed in my dissertation. Nevertheless, they already provide food and thought for many research years to come, not only for me...

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Appendices

APPENDIX 1:

Evaluation of the effect of base-composition in mitochondrial DNA in phylogenetic analyses

The nucleotide composition of mitochondrial genomes varies among animal taxa, but most vertebrates have a relatively even distribution. In many mitochondrial genes of invertebrates, the base composition is highly biased (Miller et al., 2004, Garcià-Machado et al., 1999), with, e.g., honeybees having 84.9% AT (Crozier & Crozier, 1993) compared to humans having a 55.6% AT composition (Anderson et al., 1981). The freshwater crab sequences used in this study also show this trend, with AT composition accounting for up to 78%.

To explore how base composition might influence phylogenetic inference and therefore our analyses, we performed a simulation test. We used the Seq-Gen software (Rambaut & Grassly, 1997), which simulates evolution under a model of substitution, from an initial sequence.

From a dataset of 25 species of freshwater crabs from the Indian subcontinent, I selected seven sequences (six ingroup and one outgroup) of the mtDNA fragment of approximately 1,300 bp (including a portion of 12S rRNA, the complete tRNA-Val and portion of 16S rRNA). I performed a new likelihood-based analysis on this latter dataset.

In Fig. 1 I present a schematic overview of the simulation procedure. Seq-Gen generated randomly 6 initial datasets having the following base compositions, 50, 60, 70, 80, 90 and 100% AT-richness. Starting from each of those six datasets Seq-Gen subsequently simulated the evolution of the nucleotide sequences under the Hasegawa, Kishino and Yano (HKY)-substitution model using the parameters retrieved from the above phylogenetic analysis. Every one of these different base compositional datasets was simulated under three specific situations for the rates of substitution. The first represents an ultrametric tree with equal branch lengths, thus equal rates of substitution (Fig. 2. tree A); the second represents a tree with different, but rather expected, differences in branch length (fig.2. tree B); and a third series reflects a topology with long and short branch lengths (fig. 2. tree C), which might be problematic for MP (see long branch attraction in material and methods). Since Seq-Gen

generates datasets randomly and models evolutionary processes stochastically, I performed 100 independent runs of each combination.

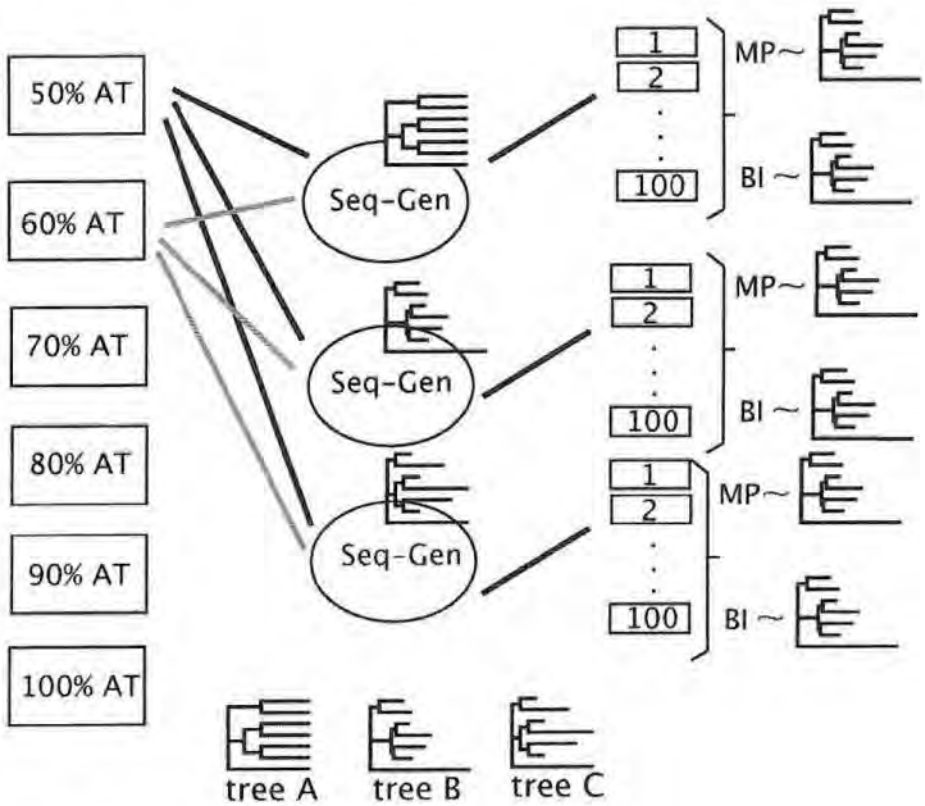

Fig.1: Scheme to outline the simulation performed

I wanted to evaluate the influence of the different AT compositions in these three different situations regarding branch lengths (tree A, B, C; fig 2) on the performance of two different methods to infer the correct phylogenetic relationships, i.e., The Maximum Parsimony (MP) method and the Bayesian approach (BI).

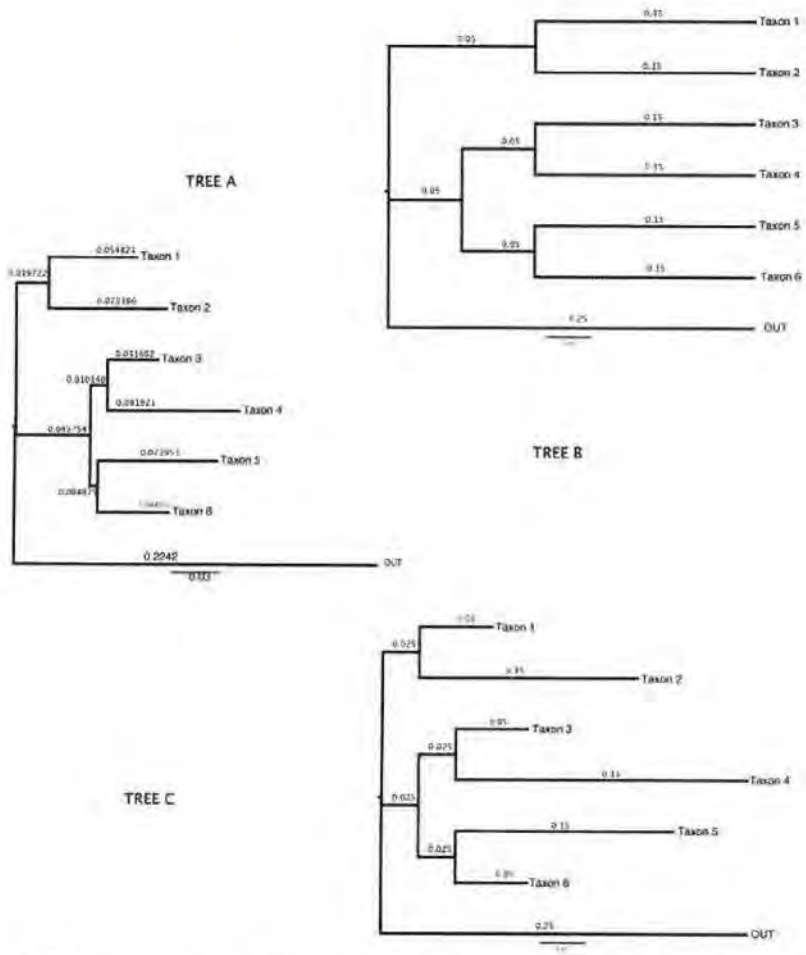

Fig. 2: Three different topologies: tree A reflects an ultrametric tree with equal branch lengths; tree B reflects a tree with different, but expected, branch lengths; tree C reflects a topology with long and short branch lengths (see 1.3 base composition in RNA codes).

This combination was then used to test the performance of two different statistical phylogenetic methods, i.e., Maximum Parsimony (MP) and Bayesian inferred (BI) phylogenetic analysis.

	Maximum Parsimony			Bayesian Inference		
%AT	**TREE A**	**TREE B**	**TREE C**	**TREE A**	**TREE B**	**TREE C**
50	75	22,4	27	93	65	87
60	65,8	24	14,2	91	57	86
70	70,9	29,8	18,9	91	55	83
80	67,5	17,7	19,4	90	47	75
90	70,2	23,5	23,5	83	49	68
100	76,3	27,4	22,7	84	46	54

Table 1: the percentage that the simulations under MP and BI inference retrieved the correct tree for the different compositional datasets and the different branch lengths.

MP analyses were performed using the program PAUP* 4.0 b10 (Swofford 1998). Heuristic searches were executed in 100 replicates, using tree bisection reconnection (TBR) branch swapping procedures to search for the optimal tree.
For the BI the MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) software performed one run of 4 chains for 200,000 generations, which were sampled every 200 generations and allowed a burn-in of 100,000 generations. Hence, the last 500 trees were used to infer a 50% consensus tree.

Per combination we calculated the percentage of 'correct' retrieved topologies (compared to the expected topologies) for both statistical approaches (MP and BI). Table 1 and Fig 3 reflect the performance for the various combinations tested; expressed as the number of times the observed topologies reflected the expected topologies (in %).

These results are presented in Table 1 and Fig. 2.

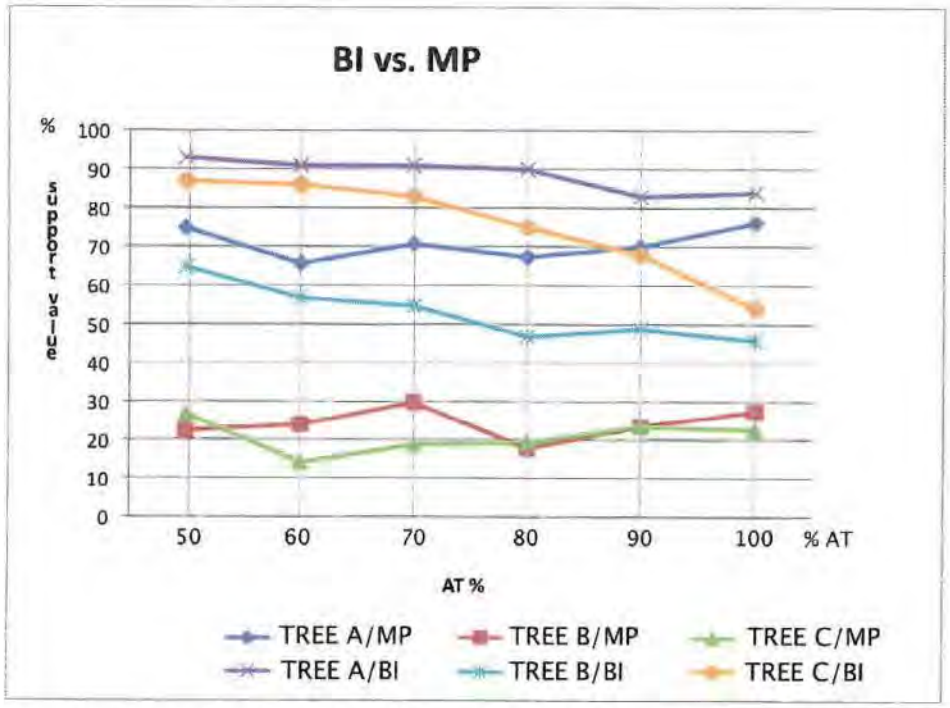

Fig. 3: Support values for MP and BI performed for 6 different base compositions, each for three different rates of substitution in the branch lengths.

We conclude that the percentage AT in the base composition only influences the performance of MP analyses slightly. The performance of BI tends to decrease as the percentage AT increases. We conclude that MP performs relatively well when a phylogeny with equal substitution rates has to be inferred, but has problems inferring the expected tree, when rates of substitution are unequal. Bayesian inference performs relatively well for all constrained phylogenies (tree A, B and C). Actually the BI outperforms MP in all three cases. This test was initially performed to test the influence on our own sequences and never considered to be an in-depth study for peer-review, but - considering that real data are often far from ideal - we preferred to present the BI or ML trees in the different chapters.

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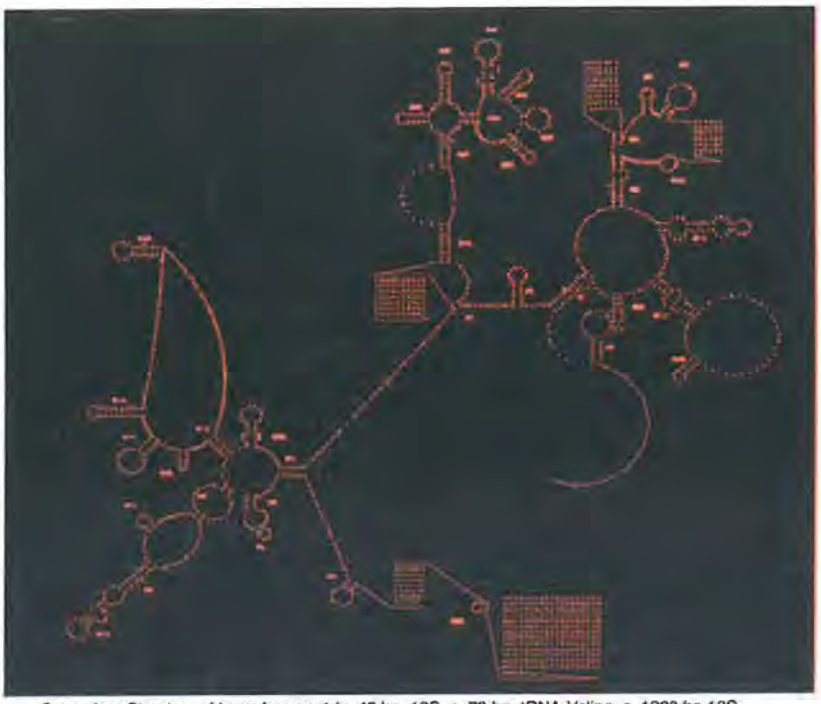
APPENDIX 2:

The effect of compensatory DNA substitution in rRNA stem regions

Most evolutionary programs assume evolutionary independent characters in a mitochondrial sequence, if not specifically indicated. Mutation rates are affected by many factors, such as the chromosomal position, GC content, nearest neighbor bases, transitions being more frequent than transversions, etc. The stem (double stranded) and loop (single stranded) regions of the secondary structure of rRNA have different selection pressures (Liò & Goldman, 1998). If stem-bases evolve as pairs (Orti, et al., 1996), then the above assumption is violated.

I tested whether non-independent evolution of sites in DNA from RNA encoding genes influences our phylogenetic inferences. To deal with this question on the effect of compensatory substitutions, I first constructed the secondary structure of a large fragment[1] ~1,300 bp (including a portion of 12S rRNA, the complete tRNA-Val and portion of 16S rRNA) as shown in Fig. 3[2]. We selected the base positions that are part of the 'stems', because most probably substitutions in stems are not strictly independent.
A dataset of the sequences of 25 species was then aligned with clustalX (Thompson et al., 1997) and manually corrected.

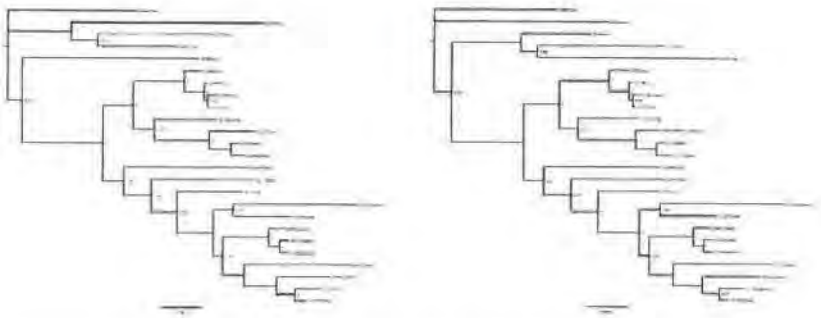
[1] The long 16S fragment we have used in every chapter
[2] I am grateful to J. Wuyts from Ghent University, Belgium for assisting me with this



Secondary Structure of large fragment (c. 45 bp. 12S, c. 73 bp. tRNA-Valine, c. 1200 bp 16S of *Oziotelphusa* species

To infer the most probable phylogeny for both the dependent (i.e. paired sets for stem positions) and the independent (i.e. no couples) data set, I performed simultaneous analyses. For these statistical analyses I used the MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) software. For the independent data set the analysis was run under a GTR+I+Γ model of substitution. The dependent dataset was run under the same model of substitution for the loop regions and under the 'doublet' model[3] for the stem regions. Both analyses were run with four chains for 1,000,000 generations. These were sampled every 100 generations and allowed a burn-in of 200,000 generations. Hence, the last 8,000 trees were used to infer a 50% consensus tree.

[3] a model that avoids overestimation of the confidence in the best tree (originally formulated by Schoniger & von Haeseler, 1994 – see Bayes manual)



Bayesian inferred topologies of non-dependent (left) and dependent (right) evolutionary sites

Subsequently the different topologies were visually compared. I conclude that secondary structure has no visual influence on the phylogenetic inferences and there is no need for compensation, such as the need for different 'weighting' to overcompensate for the interdependency (see also Dixon & Hillis, 1993). Again here I like to emphasize that this was merely performed as a test case for my own data in the beginning of this study and not to be extrapolated to other data. The influence of the secondary structure of rRNA and protein coding RNA is a large research field on its own.

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Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19**:1572-1574.

APPENDIX 3[1]*:*

RESPONSE to letter of Helgen & Groves in *Science* (below)

Authors: Franky Bossuyt,[1] Madhava Meegaskumbura,[2,3] **Natalie Beenaerts**,[1] David J. Gower,[4] Rohan Pethiyagoda,[3] Kim Roelants,[1] An Mannaert,[1] Mark Wilkinson,[4] Mohomed M. Bahir,[3] Kelum Manamendra-Arachchi,[3] Peter K. L. Ng,[5] Christopher J. Schneider[2], Oommen V. Oommen[6] and Michel C. Milinkovitch[7]

Helgen and Groves point about conservation is well taken. Yet, the major significance of our study is that it reaches beyond the recognition of a high degree of species endemism. Indeed, we have demonstrated that several Sri Lankan taxa are not only assemblages of endemics, but sometimes constitute distinct branches of the tree of life. Such higher level evolutionary history is also evident in ranid frogs (*Lankanectes*) (*1*), agamid lizards (*Ceratophora*) (*2*), and snails (*3*). The island may therefore be considered a significant reservoir of ancient lineages and clade evolutionary history (*4*).

From a conservationist's point of view, this is significant because radiations of tens of species are found exclusively on Sri Lanka. Because some members of these evolutionary lineages can be readily viewed in gardens (e.g., *Philautus* treefrogs) or in roadside torrents (e.g., parathelphusid freshwater crabs), they are ideal catalysts for stimulating environmental awareness.

With few possible exceptions (mice and shrews), mammals and birds do not reach this clade-level endemism on Sri Lanka. Therefore conservation managers could treat the clades of animals and plants as the island's major natural treasure, instead of selecting a single mammal or bird as a flagship species. This strategy will reinforce the fact that not only selected sites, but the island's habitats as a whole deserve protection.

It is in that perspective noteworthy that Sri Lanka's diversity is largely restricted to the formerly rain-forested south-western 'wet zone', where only ~750 km^2 of

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[1]Published as: Bossuyt, F., M. Meegaskumbura, Beenaerts, N., D. J. Gower, R. Pethiyagoda, K. Roelants, A. Mannaert, M. Wilkinson, M. M. Bahir, K. Manamendra-Arachchi, S. P. Loader, P. K. L. Ng, S. C. J., and M. C. Milinkovitch (2005). Biodiversity in Sri Lanka and Western Ghats. *Science*, **308**: 199.

(highly fragmented) natural forest now survives. At ~700 persons km^{-1}, human population density here is one of the highest of all Global Biodiversity Hotspots (5). The threats to the unique biodiversity we uncover, and the challenges to its conservation, are therefore formidable and demand urgent international scientific attention.

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LETTERS

Helgen, K. M. & Groves, C. P. (2005). Biodiversity in Sri Lanka and Western Ghats. *Science*, **308**: 199.

We read with interest the Report "Local endemism within the Western Ghats-Sri Lanka biodiversity hotspot" by F. Bossuyt et al. (15 Oct. 2004, p. 479), which documents patterns of diversification in selected vertebrate and invertebrate lineages from Sri Lanka and the Western Ghats region of western India. Although these two areas have long been united as a single biogeographic unit (1), and more recently as a biodiversity "hotspot" (2), Bossuyt et al. highlight the distinctive faunal histories of the two regions and caution against treating them as a single unit for conservation purposes. We would like to add two comments, which support and extend their results.

First, the respective bird and mammal faunas of Sri Lanka and the Western Ghats are distinct in many ways: There are marked differences in the regions' restricted-range mammal assemblages [the Western Ghats support at least 15

endemic mammal species; Sri Lanka supports at least 13 endemic species, and because they share few restricted-range birds, they are treated as separate "Endemic Bird Areas" (3)]. This is significant because it is birds and mammals that tend to act as "flagship species" for conservation.

Second, trenchant faunal differentiation is evident within both areas, especially in different climatic zones within Sri Lanka (4, 5), and the two regions can be subdivided into multiple "ecoregions" (6). There may sometimes be stronger faunal differentiation between wet, dry, and cloud forest zones within Sri Lanka than between that island's dry zone and the dry country of South India [e.g., (4)]. Lists of mammals restricted to Sri Lanka, the Western Ghats, or the hotspot as a whole are given in (7-10). Those apparently restricted to high-altitude cloud forest zones (marked with an asterisk) comprise all endemic genera, half of Sri Lankan endemics, one-third of Western Ghats endemics, and about one-third of mammal species endemic to the hotspot as a whole.

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Sri Lanka: *Crocidura miya, *Solisorex pearsoni, Suncus fellowesgordoni,

Suncus zeylanicus, Loris tardigradus, Macaca sinica, Trachypithecus vetulus, *Mus fernandoni, Mus mayori, *Rattus montanus, *Srilankamys ohiensis, *Vandeleuria nolthenii, Paradoxurus zeylonensis.

Shared exclusively: Crocidura horsfieldii, *Feroculus cf. feroculus, *Suncus montanus, Ratufa macroura, Petinomys fuscocapillus, Funambulus layardi, Funambulus sublineatus, Herpestes fuscus, Herpestes viticollis.

Western Ghats: Paraechinus nudiventris, Suncus dayi, *Latidens salimalii, Macaca silenus, Trachypithecus johnii, Funambulus tristriatus, *Mus famulus, *Vandeleuria nilagirica, Rattus ranjiniae, *Rattus satarae, Platacanthomys lasiurus, Martes gwatkinsi, Paradoxurus jerdoni, Viverra civettina, *Nilgiritragus hylocrius.

Endemic mammalian genera: Sri Lanka: *Solisorex, *Srilankamys; Western Ghats: *Latidens, *Platacanthomys, *Nilgiritragus; shared exclusively: *Feroculus.