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Phylogenetic diversity of Sri Lankan freshwater crabs and its implications for conservation

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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.

Keywords: elevational zone, Gecarcinucidae, Parathelphusidae, phylogenetic diversity, Red List

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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 (270–1060 m) and highlands (910–2420 m) (Vitanage 1970; Dahanayake 1982) – that are also linked to

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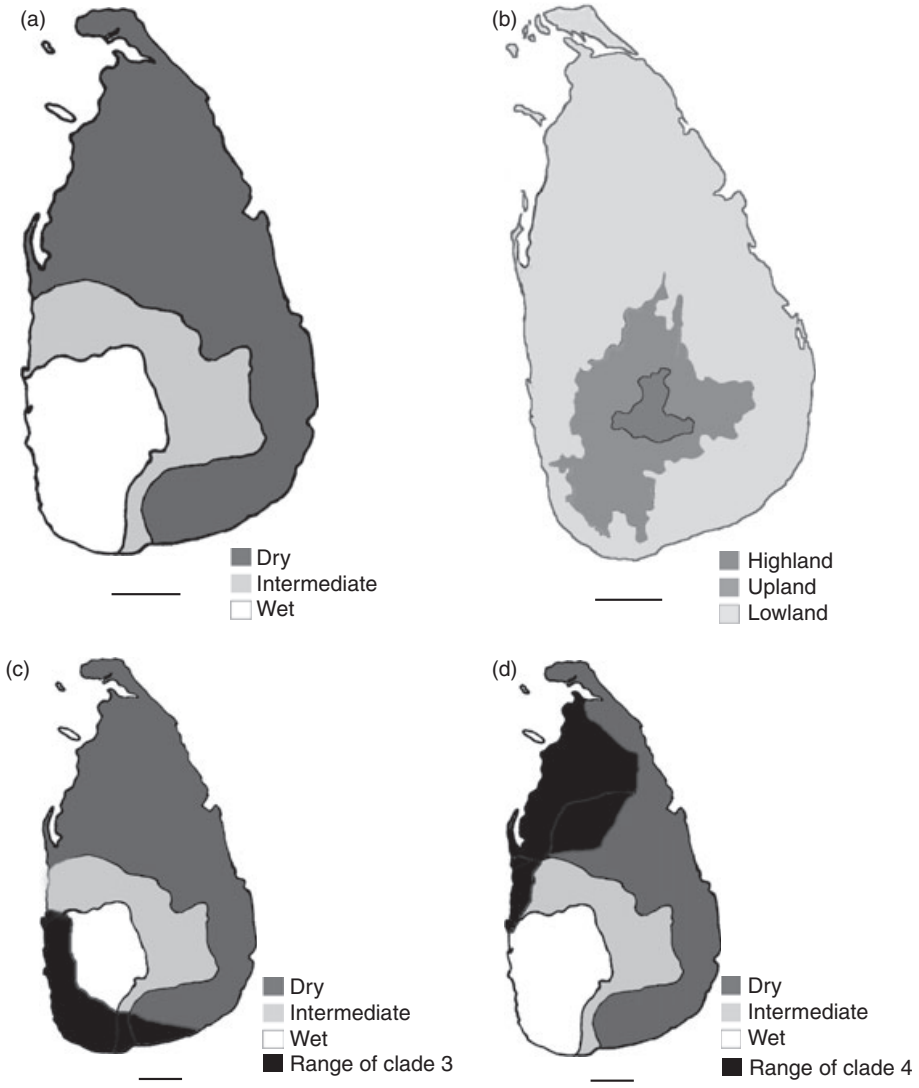

Fig. 1 Schematic maps of Sri Lanka representing: (a) Ecological zones, mainly determined by annual rainfall, known as the dry (<1250 mm/year), intermediate (between 1250 and 1900 mm/year) and wet zones (>1900 mm/year). (b) Elevational zones (Vitanage 1970); light grey: lowlands (0–270 m), dark grey: uplands (270–1060 m) and black: highlands (910–2420 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.

three periods of erosion (Wadia 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).

The highest species richness for all major groups of organisms reported to date occurs in the wet zone (World Wide Fund for Nature (WWF), The World Conservation Union (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 (McCain 2005; Oomen & Shanker 2005; Wiens *et al.* 2007). 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,

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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 (Vane-Wright *et al.* 1991; Faith 1994) 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 traditionally classified in two superfamilies: the Potamoidea, with a distribution in Europe, Africa and Asia; and the Gecarcinucoidea, which occur in Asia and Africa (Bott 1970a,b) and which include all the Sri Lankan species; for an alternative classification see Klaus *et al.* (2009). 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 (International Union for the Conservation of Nature and Natural Resources) 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.

Materials and methods

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 data set is distilled from the comprehensive survey of Sri Lanka's aquatic carcinofauna that RP and PKLN commenced in 1992. This work was later joined by 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 data set reflects this extensive effort, not all the species recorded from the region are included in the sample analysed here because in the earlier phase of the survey specimens were preserved in formalin, 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 2100 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)

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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 Table S1 (Supporting information).

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 *c.* 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 *c.* 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′-CTCTTCTCTACAAATY-CATAAAGA-3′) and PMT-4 (5′-CGAAAAATCAGAA-TAGRTGTTG-3′). PCR products were purified following the Qiagen agarose gel extraction protocol, cycle-sequenced on both strands and analysed 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. In addition, 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 Table S4 (Supporting information).

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 version 4.0 (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 1000 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 1000 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 2000 trees discarded as the 'burn-in'. Hence, Bayesian posterior probabilities (BPP) were estimated as the 50% majority-rule consensus of the 8000 last sampled trees.

Divergence time estimates

The hypothesis of the molecular clock was rejected using the likelihood ratio test (LRT; d.f. = 61; $P = 0.05$; Felsenstein 1981). Consequently, posterior divergence times were estimated with the Bayesian multilocus 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

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(~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 Sanderson 1997; Thorne & Kishino 2002). 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 data set 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): (i) a fossil record of the potamid *Potamon* of 24 Ma, and (ii) 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 data set 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 data set 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).

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 version 4.0 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 Table 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 analysed 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 (Vane-Wright *et al.* 1991; Faith 1992, 2006). 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 (Myr) (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 (International Union for the Conservation of Nature and Natural Resources) (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

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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

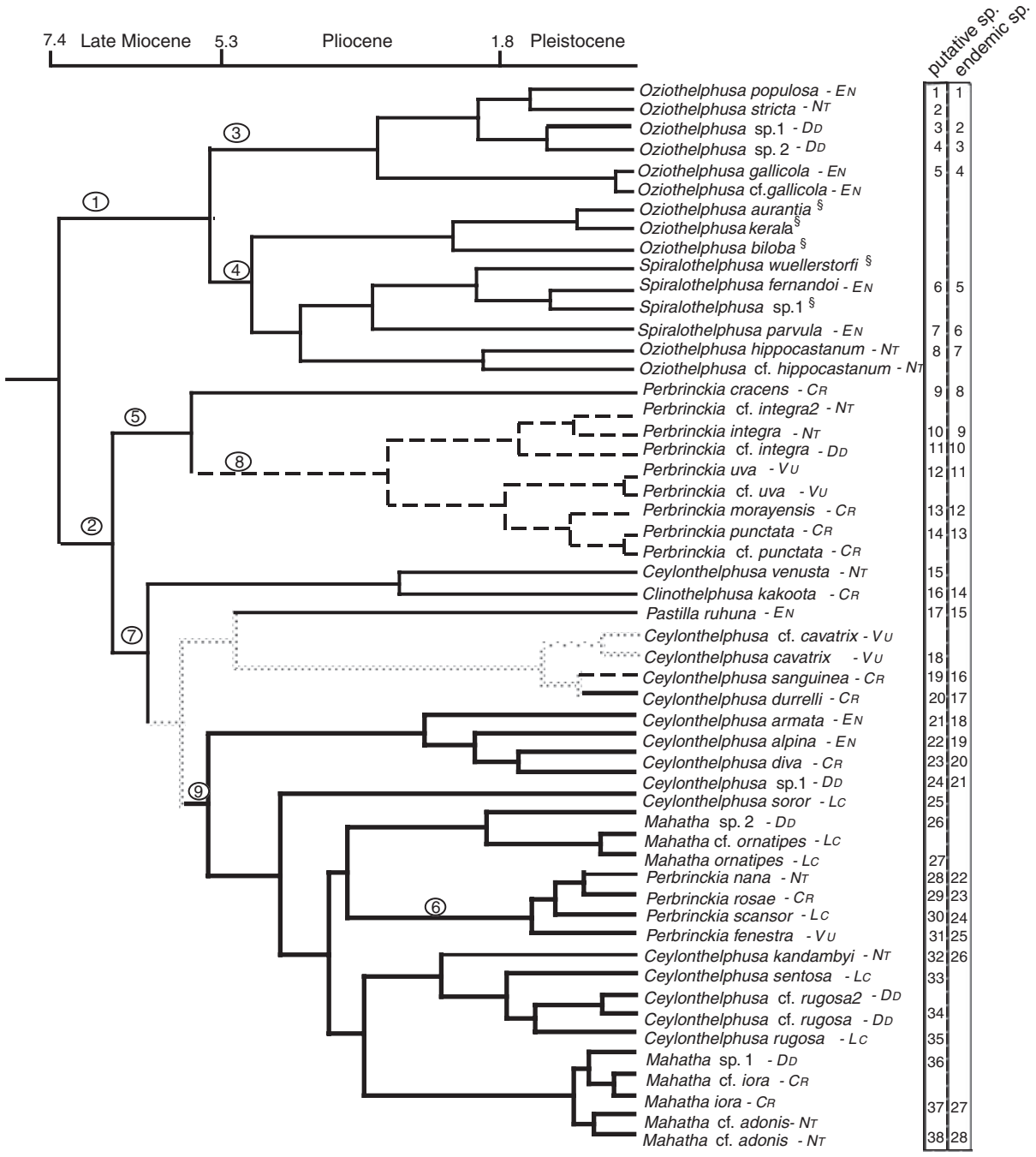

Fig. 2 The ML tree (see Fig. 3) converted to an ultrametric tree by estimating relative divergence ages (rttm = 8.757 Myr, rttmsd = 4.0 Myr, prior evolutionary rate is 1.63% Myr^{-1}; Schubart *et al.* 1998) under a relaxed molecular clock (time in Myr before present). Branch colours indicate the different 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. Numbers 1–9 refer to different studies mentioned in the text. Species sampled in the Indian subcontinent are indicated with §.

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Red List category for each species is recorded alongside each haplotype in Fig. 2.

Results

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 Table S1. Alignment resulted in a data matrix of 2047 base pairs (bp). After exclusion of 583 bp due to ambiguities in the alignments, the total data set consisted of 1464 characters, 502 sites of which were parsimony-informative (the alignment is provided as Table S2, Supporting information). The total data set 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 (alignment with positions of frame shift is added as Table S3, Supporting information). 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 data set retrieved 10 optimal trees (tree length = 2857). 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. First, 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). Second, 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. Third, the genus *Ceylonthelphusa* comprises a number of well-supported clades, but the genus as a whole is clearly polyphyletic. Fourth, *Ceylonthelphusa venusta* emerges as sister species of *Clinothelphusa kakoota* with high support.

Divergence time estimates

According to the divergence time estimates on our data set, 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).

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. favouring 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*

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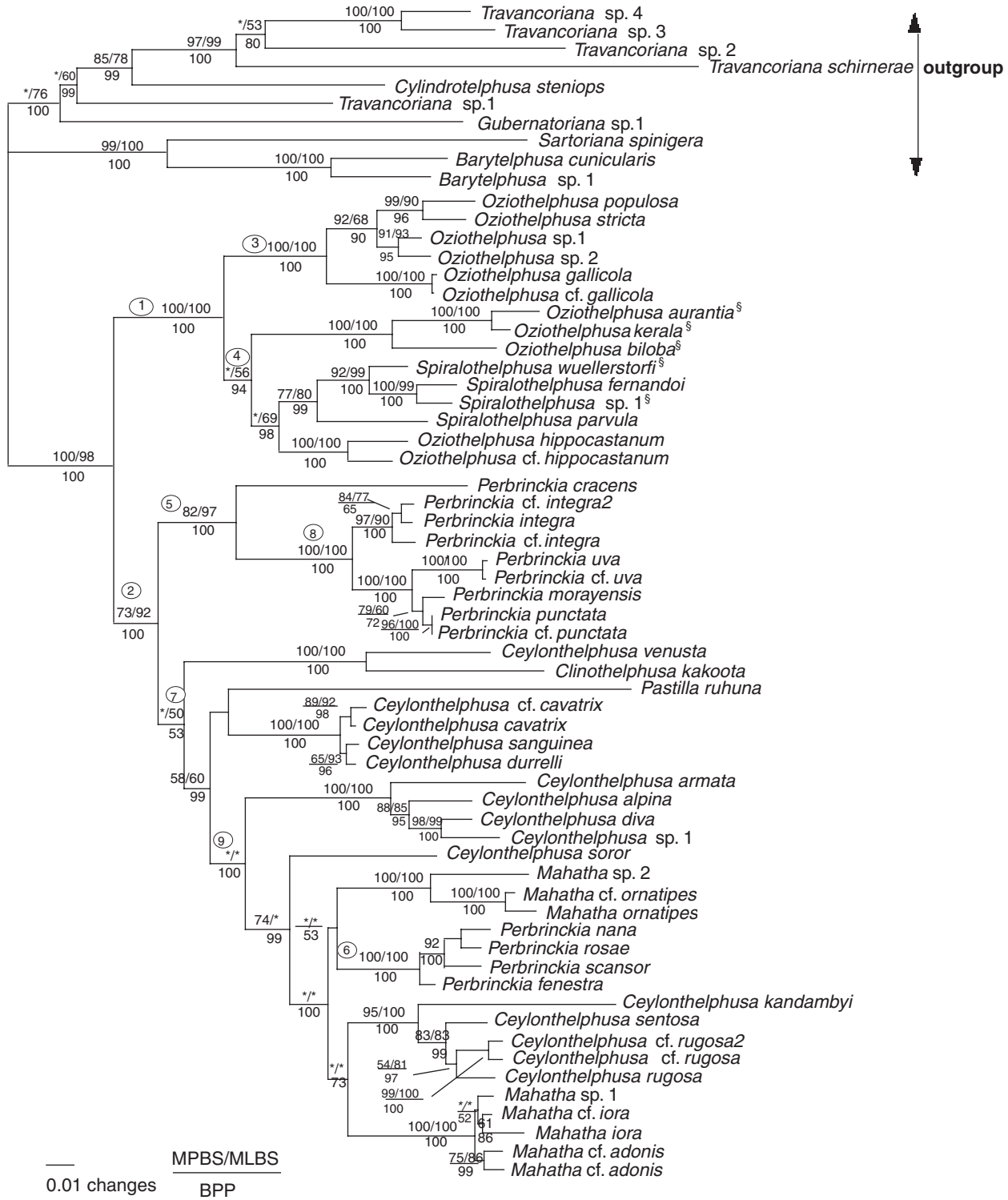

Fig. 3 The ML phylogram (−lnL = 15 177.40797) obtained from the analyses of the combined (total of 2047 bp consisting of the large fragment of about 1320 bp and COI) data set (n = 63 haplotypes) under a locus-based data partitioned GTR+ Γ + I–model. Numbers above branches represent bootstrap values of 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 §.

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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 Myr for the lowland zone, 43.1 Myr for the upland zone and 21.5 Myr 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 number of taxa was randomly drawn from the tree, PD for the lowlands was significantly lower than observed (51.7, vs. 56.2 Myr; $P < 0.05$), while PD for the upland and highland zones gave significantly higher results than observed (51.7 and 33.4 Myr, vs. 43.1 and 21.5 Myr, respectively; $P < 0.05$). Performing similar PD computations on the alternative chronogram resulted in remarkably higher estimates (lowland: 269.5 Myr, upland: 224.2 Myr, highland: 91.9 Myr).

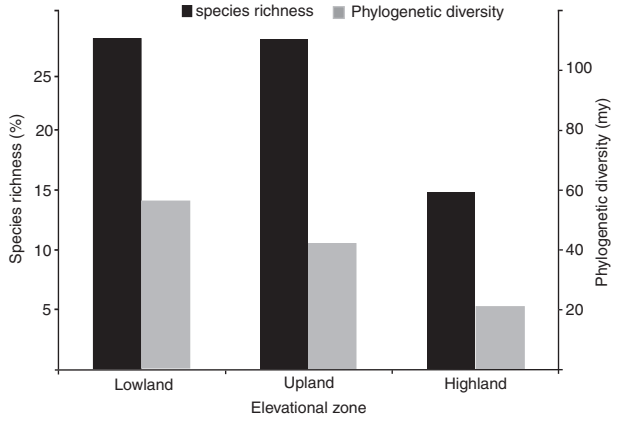

Fig. 4 Species richness and phylogenetic diversity (PD) for the three elevational zones (lowland, upland and highland). The left vertical axis indicates percentage species richness; the right vertical axis indicates PD scores as clade evolutionary history (in million years).

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.

Discussion and conclusion

Molecular phylogeny and phylogenetic history

Sri Lanka's orography appears to have been relatively stable in the course of the past 10 Myr, or at least since the Late Miocene (8 Myr). 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 & 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, substantial 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) produces slightly older estimates, the patterns of diversification and phylogenetic diversity (see later in Discussion) allow for the same inferences.

Generally, species richness and PD are expected to be highest in intermediate elevational zones, both showing

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Table 1 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)

		DD taxa as a separate category					
Phylogenetic diversity	Total	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 richness							
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

		DD taxa included in each category				
Phylogenetic diversity	Total	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 richness						
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

CR, Critically Endangered; VU, vulnerable; EN, endangered; LC, Least Concern; NT, Near Threatened; DD, Data Deficient.

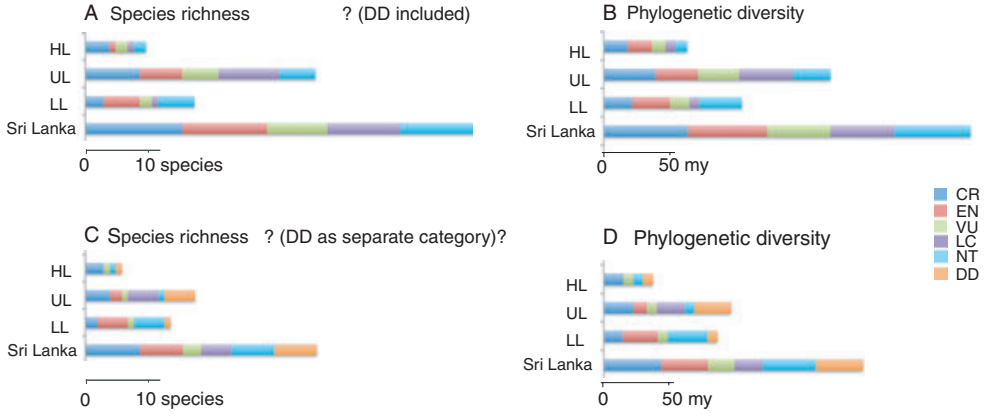

Fig. 5 Species richness (SR) and Phylogenetic diversity (PD) according to IUCN Red List categories, for Sri Lanka as a whole and for the three different elevational zones separately (lowland, LL; upland, UL; highland, HL). A. Species richness for 'unique' haplotypes within each zone with DD taxa included 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.

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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.

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. First, overall species richness and PD show similar trends for the different elevational zones (Fig 5). Second, over 50% of the freshwater crabs in this study are threatened (categories CR, EN and VU). This is equivalent to about 77 Myr of evolutionary freshwater-crab history. Third, threatened species in each of the three elevational zones seem to experience a similar risk of population decline. Finally, 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 (Huelsenbeck *et al.* 2002; Holder & Lewis 2003). 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.

Molecular phylogenic 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

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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.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 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 Materials and Methods)

Table S2 The nexus-file of the alignment with the positions of the bp to be excluded (ambiguous bp) in further analyses, indicated as 'badaligned'. The two fragments are indicated as COI and the large mitochondrial fragment, indicated here as 16S, but actually representing ~45 bp of 12S, ~73 bp of tRNA valine and ~1200 bp of 16S

Table S3 The MacClade files for 1) the CO1-alignment of the 63 DNA fragments considered for further alignment; 2) the protein code for the above alignment converted under the '*Drosophila* mtDNA code'. These files are added to demonstrate the three excluded sequences, '*Perbrinckia integra*, *P.* cf. *integra* and *P. integra* 2', which correspond, respectively, to PerIn2186, 2126PerN and 2119PerN. Even after cloning, these three specimens (clade 8 in Fig. 3) gave a single amplification product for COI that showed one or multiple frame shifts, which is not possible in protein coding genes. For *P.* cf. *integra* the first part of the fragment, most probably including a frameshift, was completely excluded for analysis due to ambiguity (positions 0–49). The sequence of *P.* cf. *integra*2 contained a deletion of 4 nucleotides (positions 46–49) and *P. integra* contained two deletions of, respectively, 4 and 43 nucleotides (positions 46–49 and 172–217). These sequences were consequently excluded from further analysis

Table S4 List of species with their accession numbers

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