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# High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs

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**Amphibians tend to exhibit conservative morphological evolution, and the application of molecular and bioacoustic tools in systematic studies have been effective at revealing morphologically 'cryptic' species within taxa that were previously considered to be a single species. We report molecular genetic findings on two forest-dwelling ranid frogs from localities across Southeast Asia, and show that sympatric evolutionary lineages of morphologically cryptic frogs are a common pattern. These findings imply that species diversity of Southeast Asian frogs remains significantly underestimated, and taken in concert with other molecular investigations, suggest there may not be any geographically widespread, forest-dwelling frog species in the region. Accurate assessments of diversity and distributions are needed to mitigate extinctions of evolutionary lineages in these threatened vertebrates.**

**Keywords:** biodiversity; speciation; conservation; amphibians

## 1. INTRODUCTION

Despite a worldwide decline in amphibian populations (Stuart *et al.* 2004), the number of recognized species of amphibians has increased dramatically in recent years (Glaw & Köhler 1998; Hanken 1999; Meegaskumbura *et al.* 2002; Köhler *et al.* 2005; Ron *et al.* 2006), making amphibians one of the vertebrate groups with the highest proportional rate of description of new species (Hanken 1999). Amphibians tend to exhibit conservative morphological evolution (Cherry *et al.* 1978), and the application of molecular genetic and bioacoustic tools in systematic studies have been particularly effective at revealing morphologically 'cryptic' species within taxa that were previously considered to be a single species (e.g. Bogart & Tandy 1976; Hillis *et al.* 1983; Highton 1989; Wynn & Heyer 2001; Gower *et al.* 2005).

We examined genetic variation across the geographic ranges of two Southeast Asian ranid frogs. *Odorrana livida* and *Rana chalconota* live in intact forest, along cascading streams (*livida*) or slow-moving streams and swamps (*chalconota*). Both frogs have been hypothesized to represent a complex of species across their ranges, but identifications of members of these complexes have been greatly confused due to their morphological similarity (Iskandar & Colijn 2000; Bain *et al.* 2003). *Odorrana livida* was originally described from Myanmar and has been reported from India to Vietnam (Bain *et al.* 2003). *Rana chalconota* was originally described from Java and has been reported from peninsular Thailand, peninsular Malaysia, Sumatra and Borneo (Boulenger 1920; Iskandar & Colijn 2000).

## 2. MATERIAL AND METHODS

We sampled frogs from across Southeast Asia that were identified in taxonomic studies on the basis of morphology as *O. livida* and *R. chalconota* (Boulenger 1920; Taylor 1962; Inger 1966; Inger & Chanard 1997; Bain *et al.* 2003). We sequenced and analysed 2150 aligned characters of mitochondrial (mt) DNA (partial COXIII, complete tRNA$^{\mathrm{Gly}}$, complete ND3, partial tRNA$^{\mathrm{Arg}}$, partial 16S, partial tRNA$^{\mathrm{Met}}$, complete ND2, and partial tRNA$^{\mathrm{Trp}}$ genes) from frogs morphologically resembling *O. livida* and 1082 aligned characters of mt DNA (partial COXIII, complete tRNA$^{\mathrm{Gly}}$, complete ND3, partial tRNA$^{\mathrm{Arg}}$, partial 16S genes) from frogs morphologically resembling *R. chalconota*. We also used historic DNA methods to obtain 600 and 361 nucleotide base pairs (bp) of the 16S gene, respectively, from the neotype museum specimen of *O. livida* (collected in 1887) and the syntype museum specimen of its junior synonym *O. chloronota* (collected prior to 1875). See electronic supplementary material for voucher information, sequencing protocols, GenBank accession numbers and methods of phylogenetic analyses.

## 3. RESULTS

Mitochondrial DNA sequences from both frog species complexes unexpectedly reveal two deeply divergent lineages occurring in sympatry (uncorrected pairwise sequence divergences of 9.95–16.12% between sympatric lineages of *O. livida* and 10.79–15.21% between sympatric lineages of *R. chalconota*) at 10 localities (figure 1), and phylogenetic analyses show that sympatric lineages are usually not each other's closest relatives (figure 2). Sorting voucher specimens *a posteriori* by mt DNA lineage illuminates at least one diagnostic morphological character (in body size, coloration in life, pattern on the back and rear of the thigh, presence/absence of spinules on the body, presence/absence of gular pouches and condition of the nuptial pads) in all but two lineages (the allopatric lineages A3 and A5; figure 2). We hypothesize that these mt DNA lineages represent distinct species on the basis of their sympatric occurrences and long evolutionary history of isolation, as inferred by deep genetic divergences and, in most cases, diagnostic morphological characters. Names can be assigned to eight of the 14 recovered clades by resurrecting old, junior synonyms (A3, B2, B7; figure 2), correctly applying commonly used names (A2, A4, B6; figure 2), and recognizing newly described species (A6–A7; figure 2), while the remaining six clades represent species undescribed to science (A1, A5, B1, B3, B4–B5; figure 2). True *O. livida* and *R. chalconota* were identified from an historical DNA sequence obtained from a type museum specimen preserved since 1887 (A2; figure 2) or from recent material collected at the type locality (B6; figure 2), and these two species actually occupy only small portions of their published geographic ranges.

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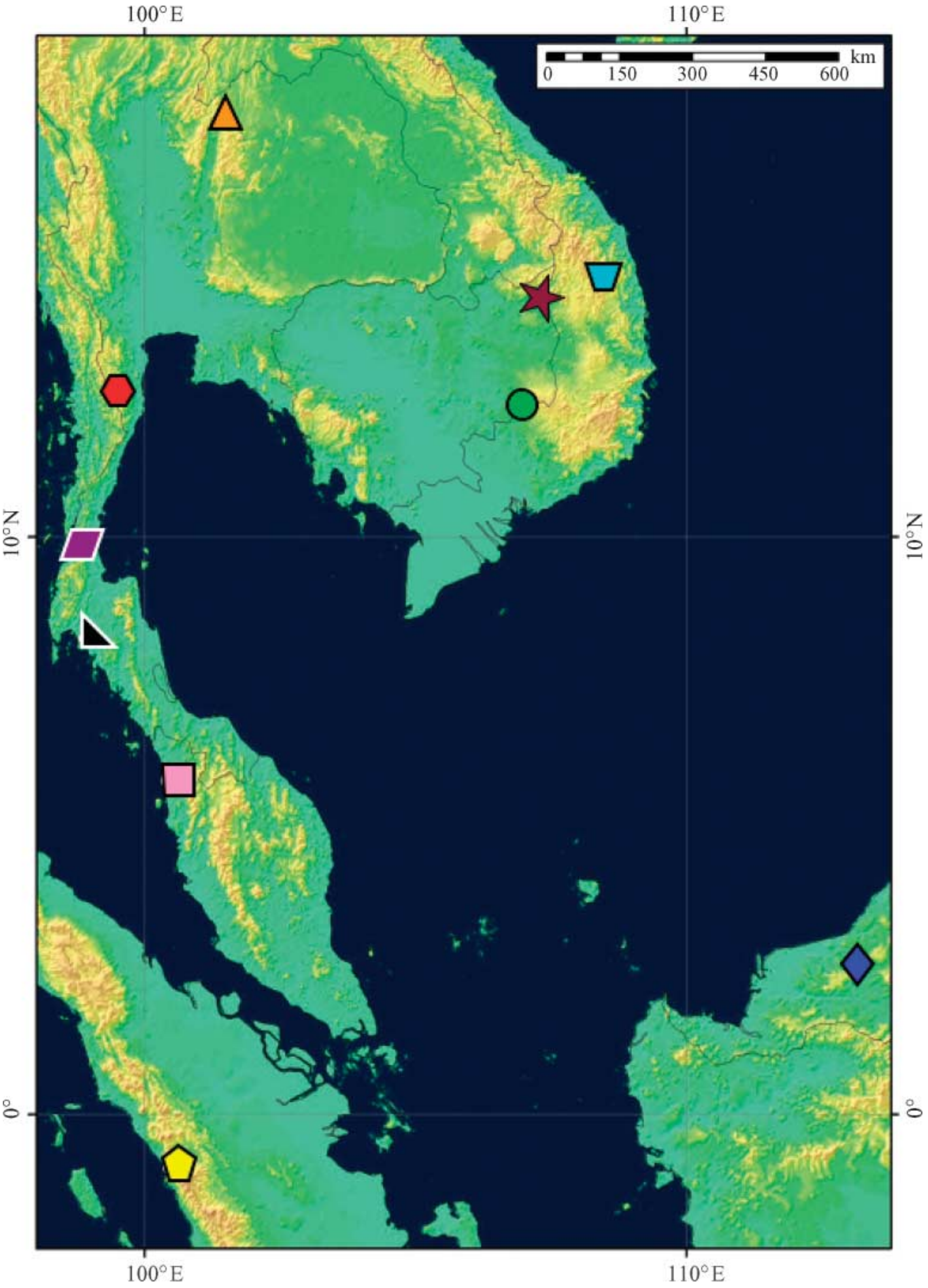

Figure 1. Map illustrating localities where two sympatric, morphologically 'cryptic' lineages of frogs were sampled in this study. Localities are Phu Luang, Loei Prov., Thailand (equilateral triangle); Khao Phanom Bencha, Krabi Prov., Thailand (right-angle triangle); Kaeng Krachan, Prachuap Kirikhan Prov., Thailand (hexagon); Namtok Ngao, Ranong Prov., Thailand (parallelogram); O'Rang, Mondolkiri Prov., Cambodia (circle); Ta Veng, Ratanakiri Prov., Cambodia (star); An Khe, Gia Lai Prov., Vietnam (trapezoid); Padang, West Sumatra (pentagon); Gunong Jerai, Peninsular Malaysia (square); Bukit Sarang, Sarawak (diamond).

## 4. DISCUSSION

Our findings of multiple cases of morphologically cryptic species in sympatry across the geographic ranges of two frog species complexes imply that amphibian species diversity remains significantly underestimated in Southeast Asia. Frogs that have been identified on the basis of morphology as *O. livida* and *R. chalconota* represent at least 14 species. We suggest that these species have been overlooked or confused due to their extreme morphological similarity compounded by an erroneous assumption that samples collected together are conspecific. Since sympatric lineages are usually not sister lineages, these frogs probably diversified by allopatric or parapatric speciation, with subsequent range shifts into sympatry. Many of the sympatric cryptic species were collected in syntopy, with individuals of different species found within centimetres of each other. It is not known how these morphologically similar species partition resources and maintain reproductive isolation, but investigations of microhabitat utilization (by adults and larvae) and vocalizations may offer insight into these topics.

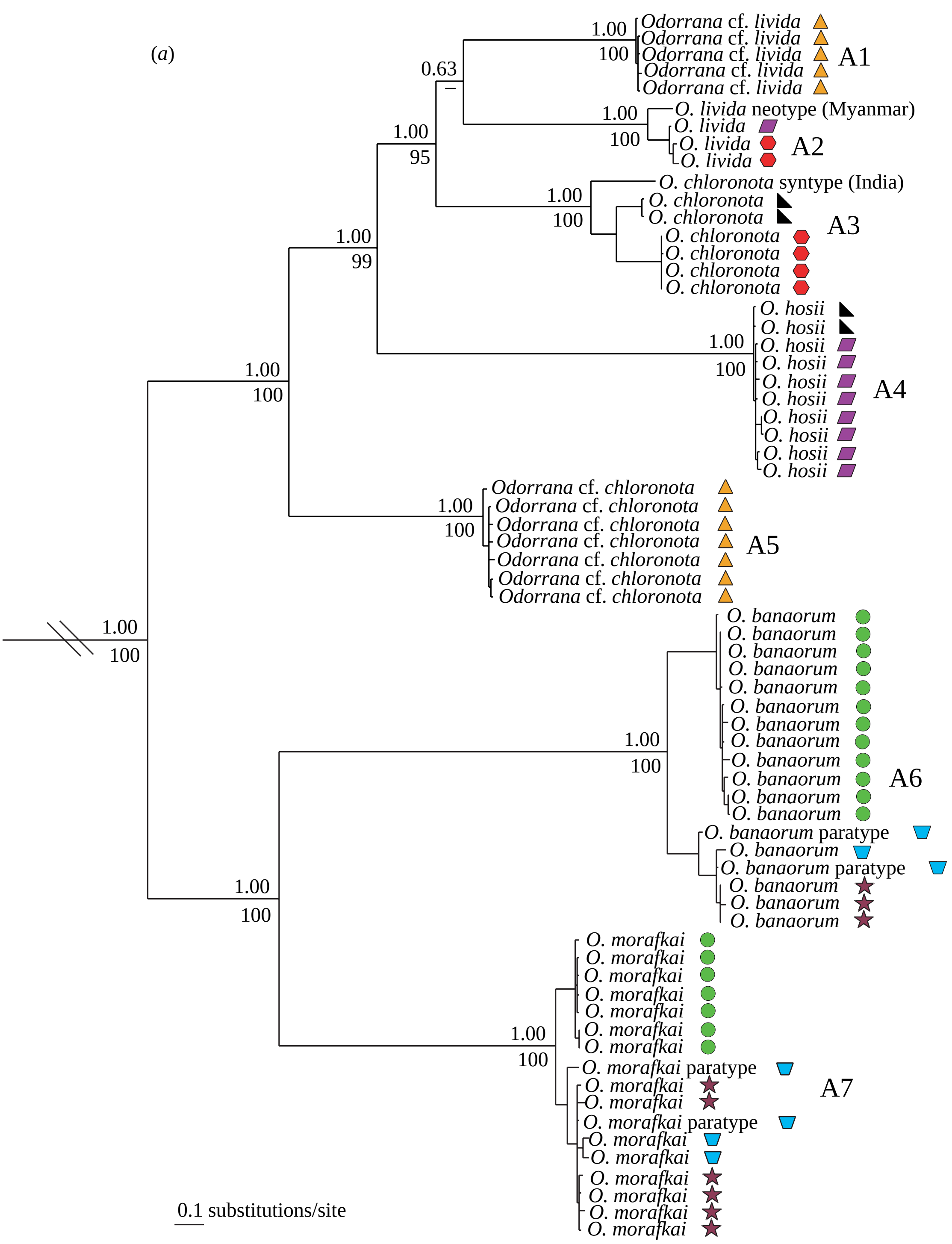

Figure 2. Fifty percent majority-rule consensus phylograms resulting from mixed-model Bayesian analyses of mitochondrial DNA from frogs morphologically resembling (*a*) *Odorrana livida* and (*b*) *Rana chalconota*. Trees were rooted with (*a*) *O.* cf. *chapaensis* and *O. bacboensis* and (*b*) *R. cubitalis* and *R. erythraea*. Numbers above and below nodes are Bayesian posterior probabilities and parsimony bootstrap values greater than 50, respectively. Maximum parsimony analyses recovered the same topologies except that *O. chloronota* and *O. livida* were hypothesized to be sister clades (bootstrap 58%). Symbols refer to localities shown in figure 1.

To date, every molecular genetic study that has broadly sampled populations across the range of a widespread frog species in Southeast Asia (southern China to Sulawesi) has uncovered genetic diversity interpreted by those authors as unrecognized species diversity (Toda *et al.* 1998; Emerson *et al.* 2000; Li *et al.* 2001*a*,*b*; Matsui *et al.* 2001; Veith *et al.* 2001; Brown & Guttman 2002; Bain *et al.* 2003; Evans *et al.* 2003; Matsui *et al.* 2005). Seven of these ten studies involved frog species that live only in intact forest. Most of the new species identified by molecular approaches are allopatrically or parapatrically distributed, but cases of morphologically cryptic frog species occurring in sympatry have been revealed in Vietnam (Bain *et al.* 2003), Peninsular Malaysia (Narins *et al.* 1998) and Java (Toda *et al.* 1998; Veith *et al.* 2001). These studies and our results suggest there may not be any geographically widespread, forest-dwelling frog species in Southeast

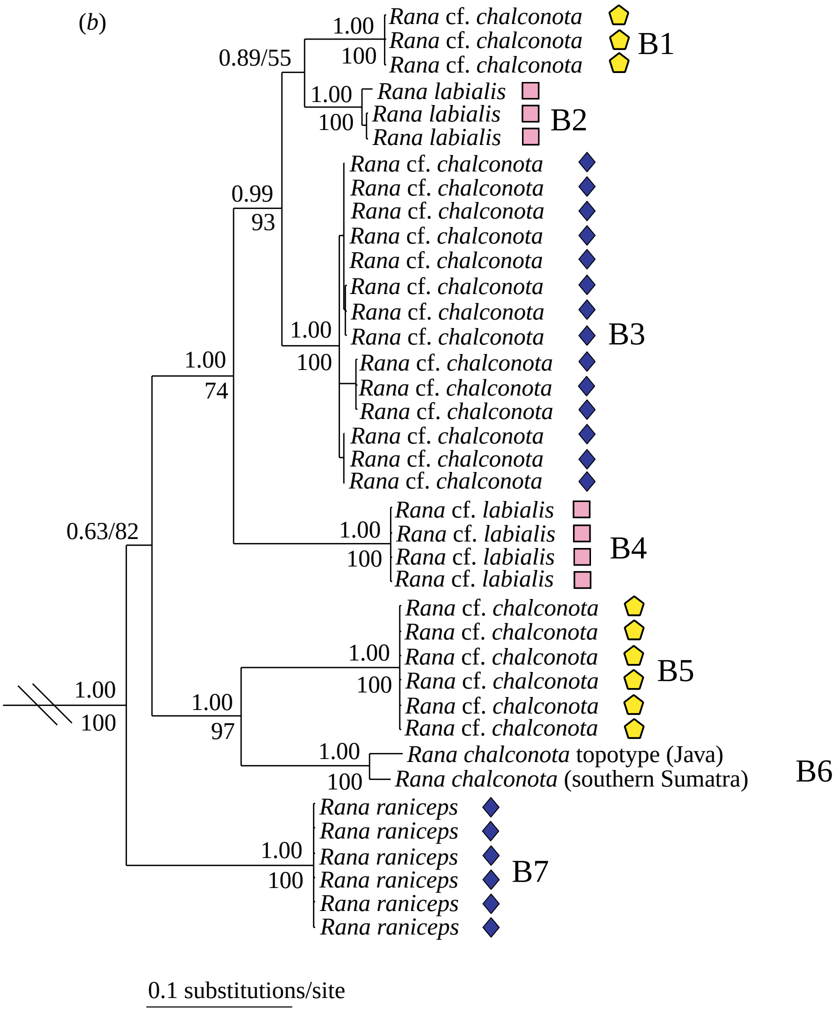

Figure 2. (*Continued*.)

Asia, and that sympatric evolutionary lineages of morphologically cryptic frogs are a common pattern in the region.

The process of defining species boundaries is more than academic. Southeast Asia has the highest relative rate of deforestation of any major tropical region (Sodhi *et al.* 2004), and understanding which species occur where is essential to conservation managers mitigating loss of biodiversity. Single, widespread 'species' actually represent multiple species having smaller geographic ranges, and consequently, greater vulnerability to extinction. It is apparent that biodiversity inventories of frogs based on morphology alone can be misleading, and that the conservative morphological evolution of amphibians obscures divergent evolutionary lineages that warrant recognition and protection. Tissue sampling should become routine in amphibian inventories so that molecular genetic tools can play a significant role in more realistically defining amphibian species diversity in coming years.

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