Molecular phylogeny and diversification of the genus *Odorrana* (Amphibia, Anura, Ranidae) inferred from two mitochondrial genes

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**ABSTRACT**

A diversity of hypotheses have been proposed for phylogenetic relationships and taxonomy within the genus *Odorrana*, and great progress has been made over the past several decades. However, there is still some controversy concerning relationships among *Odorrana* species. Here, we used many paratypes and topotypes and utilized 1.81 kb of mitochondrial sequence data to generate a phylogeny for approximately 4/5 of *Odorrana* species, and *Odorrana* haplotypes form a strongly supported monophyletic group relative to the other genera sampled. The deepest phylogenetic divergences within *Odorrana* separate 3 lineages whose interrelationships are not recovered with strong support. These lineages include the ancestral lineage of *O. chapaensis*, the ancestral lineage of a strongly supported clade comprising many western species, and the ancestral lineage of a strongly supported clade comprising all other *Odorrana* sampled. Within the latter clade, the first phylogenetic split separates *O. ishikawae* from a well-supported clade comprising its other species. These divergences likely occurred in the middle Miocene, approximately 12–15 million years ago. Separation of the ancestral lineage of *Odorrana* from its closest relative, *Babina* in our study, likely occurred in the early Miocene or possibly late Oligocene. Rates of lineage accumulation remained high from the middle Miocene through the Pleistocene.

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1. **Introduction**

The genus *Odorrana* (Fei et al., 1990), consisting of approximately 53 species, is endemic to East and Southeast Asia (Frost, 2013). The taxonomy and phylogeny of *Odorrana* have long attracted the interest of evolutionary biologists and have been investigated using both morphological and molecular data (Dubois, 1992; Fei et al., 1990, 2009, 2010; Ye and Fei, 2001; Bain et al., 2003; Chen et al., 2005; Jiang and Zhou, 2005; Frost et al., 2006; Matsui et al., 2005; Cai et al., 2007; Che et al., 2007; Stuart, 2008; Wiens et al., 2009; Kurabayashi et al., 2010; Pyron and Wiens, 2011). Although considerable progress has been made in the past two decades toward advancing our understanding of *Odorrana* phylogenetics and taxonomy, these studies have left unresolved issues and also created some new controversies. One controversy, for example, was the status of *Odorrana*. This genus was first recognized by Fei et al. (1990) with the type species *Odorrana margaretae*, while Dubois (1992) treated *Odorrana* as a subgenus of the genus *Rana* and erected a new subgenus *R. (Ebrana)* that included *R. (E.) ishikawae, R. (E.) ijimae, R. (E.) narina, R. (E.) swinhoana* and *R. (E.) livida* with the type species *R. (E.) narina*. Later, Frost et al. (2006) greatly expanded the genus *Huia* to include both *Odorrana* and *R. (Ebrana)* based only on the analysis of 4 species: *H. nasica, Amolops chapaensis, R. (E.) chloronota* and *O. grahami*. However, subsequent analyses of mtDNA data (Jiang and Zhou, 2005; Matsui et al., 2005; Cai et al., 2007), nuclear data (Stuart, 2008) and combinations of mtDNA and nuclear data (Che et al., 2007; Wiens et al., 2009; Pyron and Wiens, 2011) supported monophyly of *Odorrana* and rejected *R. (Ebrana)* and *Huia*. Additionally, the systematic status and phylogenetic position of some taxa such as *O. ishikawae* and *O. chapaensis* remain controversial (Fig. 1), despite a variety of studies using a diverse array of systematic markers (Matsui et al., 2005; Cai et al., 2007; Stuart, 2008; Wiens et al., 2009), even in complete mitogenomes (Kurabayashi et al., 2010) and large concatenations of data (Pyron and Wiens, 2011). Additional conflicts regarding the relationships

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and taxonomies exist within the genus Odorrana, where previous phylogenetic hypotheses contradicted one another (Fig. 1).

In this study, we sampled approximately 4/5 of the named species and two unnamed species with many paratypes and topotypes for Odorrana based on the analysis of 1.81 kb of molecular sequence data from two mitochondrial genes (12S and 16S rRNA) to present a molecular phylogeny of Odorrana. The resulting phylogeny was used to test the validity of Odorrana.
taxonomic sampling. In addition, Bayesian relaxed-clock estimation was performed to improve our understanding of the Odorrana radiation.

2. Materials and methods

2.1. Taxon sampling, DNA extraction, amplification, and sequencing

Tissue samples from a total of 36 species, including 28 Odorrana specimens and 8 outgroup species, were collected for DNA sequencing. Additional sequence data of 15 Odorrana species and two other ranids were obtained from GenBank (Table 1). Specimen data (species names, sampling localities, specimen voucher No. and GenBank Accession Numbers) are given in Table 1, and geological distributions of all sampling sites are presented in Fig. 2.

Total genomic DNA was extracted from thigh muscle or liver using a DNeasy Tissue Extraction Kit (Qiagen) or with a standard phenol/chloroform procedure followed by ethanol precipitation (Sambrook and Russell, 2006). Two fragments of 12S and 16S rRNA genes were amplified using Ex-Taq DNA polymerase (TaKaRa) under the following conditions: 35 cycles at 95 °C for 5 s min, 95 °C for 40 s, 47 °C–57 °C for 40–50 s, and 72 °C for 45–90 s followed by a 10-min extension at 72 °C. Primer information is given in Table S1.
2.2. Sequence alignment and phylogenetic analyses

Sequences from the 12S and 16S rRNA genes were separately aligned in Clustal X 1.81 (Thompson et al., 1997) with default parameters, and the software GBlocks (Castresana, 2000) was used under default settings to delete regions of ambiguous alignment (the alignment file is available in TreeBase http://purl.org/phylo/treebase/phylows/study/TB2: S14355). Further saturation testing was performed using DAMBE (Xia et al., 2003).

Phylogenies were built using maximum likelihood algorithms in MetaPIGA 2.0 (Helaers and Milinkovitch, 2010) and Bayesian inference (BI) in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) for each gene independently and for a combined dataset of 12S and 16S rRNA. Modeltest 3.7 (Posada and Crandall, 1998) was used to select the optimal models for each partition based on the Akaike Information Criterion (AIC). Maximum Likelihood analyses were performed using MetaPIGA 2.0 with 1000 replicate metaGA searches. The Bayesian analyses of the nucleotide matrix were performed using the GTR + I + G model. Four Markov chains were run for 20 million generations with sampling every 1000 generations. The stationarity of the likelihood scores of sampled trees was determined in Tracer 1.4 (Rambaut and Drummond, 2007). The first 10% of trees were removed as the “burn-in” stage followed by calculation of Bayesian posterior probabilities (PP) and the 50% majority-rule consensus of the post burn-in trees sampled at stationarity. Phylogenetic trees were deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/ TB2: S14355).

2.3. Molecular divergence estimates

Divergence time was estimated using a two-gene concatenated dataset with an uncorrelated lognormal model incorporated in BEAST 1.6.1 (Drummond and Rambaut, 2007). No reliable ranid fossil record is presently available to provide proper calibration within the genus Odorrana and therefore, 3 additional sequences of Rana bedriagae, R. cretensis, and R. lessonae from Lymberakis et al. (2007) and 2 of R. signata and R. chalconota from Bossuyt et al. (2006) were combined with our initial data and reanalyzed with the aim to obtain a useful calibration. Divergence age estimates were established in this study for R. cretensis and R. bedriagae (log-normal distribution with youngest of 5 MY and standard deviation [SD] of 0.159) based on geological data (5–5.5 MY) (Dermitzakis, 1990; Beerli et al., 1996). Divergence between R. signata and R. chalconota (25 MY, 1.264 SD) was estimated based on a multiple gene/calibration analysis (25–33 MY) (Bossuyt et al., 2006; Roelants et al., 2004). Analyses were executed with 20 million generations while sampling every 1000th tree. Three identical BEAST runs were conducted to ensure the stability and convergence of the MCMC chains. The results were combined using LogCombiner (Drummond and Rambaut, 2007) and examined using Tracer 1.4 (Rambaut and Drummond, 2007) to evaluate stationarity. The first 10% of trees were discarded as burn-in. Molecular rates for the two mtDNA genes were calculated using BEAST 1.6.1 (Drummond and Rambaut, 2007) and compared with similar estimates from other vertebrates to cross-validate our analysis of evolutionary dating.

3. Results and discussion

3.1. Sequence characteristics

Sequence statistics and average nucleotide composition for the two gene fragments and for the combined alignment are given in Table S2 in Appendix A. The total alignment for the dataset
included 1890 bp (12S = 789 bp; 16S = 1101 bp). Elimination of ambiguous sites produced 754 bp for the 12S dataset and 1061 bp for the 16S dataset. A total of 805 out of 1815 sites were variable in the combined dataset, with 646 being parsimony informative (35.6%). The average Ts/Tv ratio varied among genes and was 2.08 in the combined dataset (Table S2 in Appendix A). Saturation analysis did not show any kind of saturation (data not shown), and all substitutions of these two genes were therefore used for phylogenetic reconstructions.

### 3.2. Phylogenetic relationships of Odorrana

The topologies of the ML and BI trees inferred from the analysis of the combined dataset were identical, and both bootstrap support (BP) from ML and Bayesian posterior probability (PP) are represented on the BI tree (Fig. 3). Our results support the sister-group relationship of Babina and a clade containing all of the included Odorrana species, a finding consistent with several recent studies (Che et al., 2007; Kurabayashi et al., 2010; Pyron and Wiens, 2011). Seven major branches within Odorrana were identified and denoted A–G (Fig. 3). Odorrana chapaensis appears as the sister taxon to a clade comprising all other Odorrana (Clade I in Fig. 3), but Clade I is not well supported (PP = 0.79, BP = 39). The result is thus an unresolved three-way split between O. chapaensis and the two major subclades forming Clade I: Clade B (PP = 1.0, BP = 96) and Clade II (PP = 0.99, BP = 75) in Fig. 3.

In Clade B, subclades B1 and B2 were recovered with strong supports (PP = 1.0, BP = 100). In subclade B1, the relationships among O. anlungensis, O. yizhangensis, and O. lungshengensis could not be resolved with strong support. Clade B nonetheless rejects the alliance of O. anlungensis, O. yizhangensis and O. lungshengensis with the O. schmacheri group, a proposal based upon morphological data (Fei et al., 2009). Subclade B2 consisted of the remaining species from southwestern China, the type species of the genus Odorrana, O. margaretae and two species from Vietnam. Relationships within subclade B2 were well-resolved except for one node (Fig. 3). The first phylogenetic split within subclade B2 separates O. wuchuensis from a clade comprising the remaining species (PP = 1.0, BP = 100). Odorrana margaretae and O. kuangwuensis are grouped (PP = 0.99, BP = 80) as the sister taxon to a clade comprising O. grahami, O. junliensis, O. daorum, O. imongorum, O. andersonii and O. jingdongensis (PP = 1.0, BP = 100).

<table>
<thead>
<tr>
<th>Species</th>
<th>PP</th>
<th>BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. grahami</td>
<td>0.99</td>
<td>94</td>
</tr>
<tr>
<td>O. junliensis</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>O. daorum</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>O. imongorum</td>
<td>0.99</td>
<td>94</td>
</tr>
<tr>
<td>O. andersonii</td>
<td>0.99</td>
<td>94</td>
</tr>
<tr>
<td>O. jingdongensis</td>
<td>1.00</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 3. Phylogenetic trees reconstructed using BI and ML methods based on the concatenated dataset of 12S and 16S rRNA for species of Odorrana and related species. Integers associated with branches are bootstrap support values for ML inference whereas values of 1 or less are Bayesian posterior probabilities. Representative members are delimited by vertical lines to the right of the tree. Numbers in parentheses correspond to those of localities in Table 1 and Fig. 2. The topotypes analyzed in this study are shown by bold, and asterisk after taxon name indicates paratypes.
In Clade II, O. ishikawae from Amami Island is the sister taxon to a highly supported clade comprising the remaining species (PP = 1.0, BP = 98, Clade III in Fig. 3). This position of O. ishikawae among the Odorrana species was concordant with previous molecular analyses (Matsui et al., 2005; Kurabayashi et al., 2010; Pyron and Wiens, 2011) but contradicted with the results of Cai et al. (2007) as shown in Fig. 1C.

Clade III consisted of 9 species from southwestern to southeast China and O. bacoenssis (PP = 1.0, BP = 100, Clade D in Fig. 3) and a highly supported monophyletic group (Clade IV in Fig. 3). The basal split within Clade D separated one well supported clade O. tianmuui + O. huanggangensis (PP = 1.0, BP = 100, Fig. 3) from a more weakly supported clade comprising O. bacoenssis and the other 7 species from southwestern to southeast China (PP = 0.93, BP = 73, Fig. 3), within which interspecific relationships were well resolved. In addition, O. bacoenssis and O. tianmuui are sister taxa (PP = 1.0, BP = 100), and they have a close affinity with O. schmackeri, which contradicts prior result grouping O. tianmuui and O. livida (Cai et al., 2007; Pyron and Wiens, 2011) and the alliance of O. tianmuui with O. narina, O. tormota, O. nasica and O. versabilis (Kurabayashi et al., 2010).

Clade E included O. chloronota, O. graminea, O. hosii, O. leporipes, O. banaorum and O. morafkai (PP = 1.0, BP = 100), and interspecific relationships are well resolved. Clade E was the sister clade to Clade V (PP = 1.0, BP = 100) consisting of two well-supported monophyletic groups (Clade F and G in Fig. 3). In agreement with the findings of recent molecular analyses (Matsui et al., 2005; Stuart, 2008; Wiens et al., 2009; Pyron and Wiens, 2011), data here clearly placed the O. livida complex and O. hosii well-nested within the genus Odorrana.

Odorrana tormota, O. nasica, O. nasuta, O. exiliversabilis and O. versabilis formed a monophyletic group (Clade F in Fig. 3), which was resolved as the sister group of Clade G (PP = 1.0, BP = 100). In Clade F, O. tormota, the concave-eared frog, is the sister taxon to a highly supported clade comprising the remaining species (PP = 1.0, BP = 94). Odorrana nasica is the sister species to O. exiliversabilis, O. versabilis and O. nasuta; among the latter three species, the basal divergence separates O. exiliversabilis from O. versabilis and O. nasuta (PP = 1.0, BP = 99). The inclusion of O. tormota and O. nasica in the genus Odorrana was largely congruent with several previous molecular studies (Cai et al., 2007; Stuart, 2008; Wiens et al., 2009; Kurabayashi et al., 2010; Pyron and Wiens, 2011).

Clade G contained O. amamiensis, O. supranarinana, O. narina, O. utsunomiyaorum and O. svinhoana from 2 different localities in Taiwan, China (PP = 1.0, BP = 96), Odorrana amamiensis, O. utsunomiyaorum and O. narina formed a monophyletic group (PP = 1.0, BP = 100), within which O. amamiensis and O. narina were grouped as sister species (PP = 1.0, BP = 99), a finding concordant with several previous molecular studies (Matsui et al., 2005; Pyron and Wiens, 2011).

3.3. Divergence-time estimation

According to our estimates, we obtained an average rate for the two mtDNA genes of 0.746% per lineage per million years for all substitutions, and this rate is comparable to those (0.5–1%/million years) in other vertebrates (Caccone et al., 1997). The divergence dates inferred by the Bayesian relaxed clock analyses suggest that the genus Odorrana began to diversify 18.99 million years ago (95% HPD interval 14.90–23.23 MYA), during the Late Oligocene to Middle Miocene (Fig. S1 and Table S3 in Appendix A), which conflicted dramatically with the Late Eocene split approximately 38 MYA proposed by Wiens et al. (2005) based on the ranid crown-group calibration. The earliest split within Odorrana, between O. chapaensis and the remaining Odorrana species, is estimated at 14.44 MYA (11.26–18.01 MYA) during the Early to Middle Miocene (Fig. S1 and Table S3 in Appendix A). The divergence time between O. ishikawae and its sister clade is estimated to be 13.22 MYA (10.21–16.40 MYA), which accorded closely with the divergence at 12.6 (7.9–18.0) MYA predicted by Matsui et al. (2005). In addition, the origin of the other major clades (Clades B, D to G in Fig. 3) within Odorrana occurred in the Early to Late Miocene, and each of the major clades underwent a radiation from the Middle Miocene to the Pleistocene, with most divergences in the Plio-Pleistocene (Fig. S1 and Table S3 in Appendix A). The divergence times predicted in the present study were slightly older or younger than other studies in some lineages, but overall these findings were consistent with or overlapped in range the previous estimates (Matsui et al., 2005; Wiens et al., 2009). Climate change from greenhouse to icehouse, plate tectonic movements, and the uplift of mountain ranges might have played a key role in the Odorrana radiation (Matsui et al., 2005; Molnar, 2005; Metcalfe, 2006; Hall, 2009).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.07.023.

References
