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Multiple invasions of the Ryukyu Archipelago by Oriental frogs of the subgenus *Odorrana* with phylogenetic reassessment of the related subgenera of the genus *Rana*

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Abstract

The genus *Rana*, notably diversified in Oriental regions from China to Southeast Asia, includes a group of cascade frogs assigned to subgenera *Odorrana* and *Eburana*. Among them, *R. ishikawae* and the *R. narina* complex represent the northernmost members occurring from Taiwan to the Ryukyu Archipelago of Japan. Relationships of these frogs with the continental members, as well as the history of their invasions to islands, have been unclear. The taxonomic status of *Odorrana* and related genera varies among authors and no phylogenetic reassessment has been done. Using partial sequences of mitochondrial 12S and 16S rRNA genes, we estimated phylogenetic relationships among 17 species of the section *Hylarana* including *Odorrana* and *Eburana*, and related species from the Ryukyus, Taiwan, China, Thailand, Malaysia, and Indonesia. We estimate that (1) *Odorrana* is monophyletic and encompasses species of *Eburana* and *R. hosii*, which is now placed in *Chalcorana*, (2) the ancestor of *R. ishikawae* separated from other *Rana* in the middle to late Miocene prior to its entry to the Ryukyu Archipelago, (3) the ancestor of the *R. narina* complex later diversified in continental Asia, and invaded the Ryukyu Archipelago through Taiwan, (4) the *R. narina* complex attained its current distribution within the Ryukyus through niche segregations, and (5) vicariance of *R. hosii* between Malay Peninsula and Borneo occurred much later than the divergence events in the *R. narina* complex. Current subgeneric classification of *Rana*, at least of Southeast Asian members, requires full reassessment in the light of phylogenetic relationships.

Keywords: Phylogeny; Speciation; Classification; Molecular clock; Biogeography; Oriental cascade frogs; *Rana*; *Odorrana*

1. Introduction

Since the review of South Asian, Papuan, Melanesian, and Australian species by Boulenger (1920), taxonomy of the genus *Rana*, as well as many other genera of the Raninae in these and other regions, has experienced considerable change, and the contents of this genus have been reduced; current authors tend to split several groups, such as *Limnonectes* and *Fejervarya*, as distinct genera from *Rana* (Duellman, 1993; Frost, 2004). Yet, the genus *Rana* (sensu stricto: Frost, 2004), after removing such genera, is still large, including more than 240 species worldwide (Frost, 2004).

Rana (sensu stricto) is most divergent in the Oriental region (Duellman, 1999), and one of its centers of diversification ranges from China to Southeast Asia (Inger, 1999; Zhao, 1999). Some Chinese authors further limited the content of *Rana* and recognized many other distinct genera (Fei et al., 1990), while others regard these genera as mere subgenera of *Rana* (Dubois, 1992; Frost, 2004). One of the reasons for such taxonomic disagreements

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could be ascribed to the fact that these genera and/or subgenera were proposed solely on the basis of a small set of characters (Inger, 1996, 1999).

Among *Rana* (sensu stricto) occurring from China to Southeast Asia, there is a group of cascade frogs that includes *R. livida* (or *R. chloronota*) with a green dorsum, long hindlimbs, and well-developed disks on tips of fingers and toes (Bain et al., 2003). Because most of these frogs emit a bad odor, Chinese authors called them stink frogs (Wei et al., 1993) and combined them to establish a distinct genus *Odorrana* (Fei et al., 1990). On the other hand, Dubois (1992) studied species from wider regions of Asia and made *Odorrana* a subgenus of *Rana* (see comparison of the two systems of classification in Zhao, 1994). From minor morphological differences, he split *R. livida* from *Odorrana* and placed it in *Eburana*, another subgenus of *Rana* newly designated to encompass several species including those from the Ryukyus and Taiwan (Dubois, 1992). This action, however, was not supported by some authors (Matsui, 1994; Matsui et al., 1995).

The Ryukyu Archipelago is situated in the northernmost extremity of the distributional range of *Eburana* and *Odorrana*. How and when the members of *Eburana* and/or *Odorrana*, now centered in tropical regions, entered this most peripheral, subtropical insular region are biogeographically interesting problems. In particular, restriction of *R. ishikawae* to the middle Ryukyus has been a mystery (Ota, 1998).

We reassess validities of genera and subgenera that were proposed on the basis of insufficient phenotypic information. In elucidating phylogenetic relationships among amphibians, especially those lacking morphologically useful information, genetic information is very useful (e.g., Tanaka et al., 1996). In this study, we clarify phylogenetic relationships of *Odorrana*, *Eburana*, and several related genera/subgenera from Japan, China, Thailand, Malaysia, and Indonesia from an analysis of partial sequences of mt DNA. Based on the results obtained, we evaluate the history of speciation and evolution of these frogs with special attention to the northern peripheral members related to *R. livida*.

2. Materials and methods

2.1. Sampling design

We obtained partial sequences of 12S rRNA and 16S rRNA from 1 to 2 specimens for each of 13 species representing five subgenera of the subsection *Hylarana* (eight species of *Eburana*, two species of *Chalcorana*, one species each of *Odorrana*, *Hylarana*, and *Nasirana*) and four species representing three subgenera of the subsection *Hydrophylax* (two species of *Sylvirana*, and one species each of *Humerana* and *Pulchrana*: Table 1, Fig. 1).

Because the generic or subgeneric allocations of species of *Rana* is controversial (Fei et al., 1990; Dubois, 1992), we tentatively follow, with a few exceptions, Dubois' (1992) taxonomy. We selected outgroups representing *Staurois* (another genus of the tribe Ranini; *Staurois latopalmatus*), *Paa* (another tribe of Raninae; *Paa quadrana*), *Fejervarya* (another subfamily of Ranidae; sequence of *Fejervarya limnocharis* from GenBank as AY158705), and *Buergeria* [a member of Rhacophoridae, which is sometimes treated as a subfamily of Ranidae (Dubois, 1992); sequence of *Buergeria buergeri* from GenBank as AB127977]. All trees were rooted with these four taxa, because the sister groups of the subsections *Hydrophylax* and *Hylarana* (Dubois, 1992) are uncertain.

2.2. Preparation of DNA, PCR, and DNA sequencing

Tissues were obtained from either frozen or ethanol-preserved specimens. DNA was extracted using standard phenol–chloroform extraction procedures (Hillis et al., 1996). PCR was used to amplify fragments of the mitochondrial 12S rRNA and 16S rRNA genes. The primers and cycling procedures were:

(i) 12S:12Sh (light chain; 5′-AAA GGT TTG GTC CTA GCC TT-3′) of Cannatella et al. (1998) and H1548 (heavy chain; 5′-TAC CAT GTT ACG ACT TTC CTC TTC T-3′) made in the present study amplified a ca. 860 bp section of the mitochondrial 12S ribosomal RNA gene. Amplification was performed in a 25-μl volume reaction, with Blend Taq (TOYOBO). PCR cycling procedure was as follows. Initial denaturation step: 5 min at 94 °C; 25 cycles: denaturation 1 min at 94 °C, primer annealing for 1 min at 45 °C, extension for 3 min at 72 °C.

(ii) 16S:16L-1 (light chain; 5′-CTG ACC GTG CAA AGG TAG CGT AAT CAC T-3′) and 16H1 (heavy chain; 5′-CTC CGG TCT GAA CTC AGA TCA CGT AGG) of Hedges (1994) amplified a ca. 460 bp section of the mitochondrial 16S ribosomal RNA gene; PCR cycling procedure was the same as for 12S except that primer annealing temperature was 50 °C.

PCR products were purified using the PEG purification procedures and labeled with fluorescent-dye labeled terminators (ABI Prism Big Dye Terminators v.3.1 cycle sequencing kits). The labeled PCR products were ethanol-precipitated following the manufacturer's protocol. The sequencing products were run on ABI 3100 automatic sequencer. All samples were sequenced in both directions.

Sequences were aligned automatically using the clustal option of the BioEdit software (Hall, 1999). Initial

Table 1
Samples used in this study, and GenBank accession numbers

Species	Locality	Voucher	GenBank Accession No. (12S;16S)
Subsection *Hylarana*			
1. *Rana* (*Eburana*) *ishikawae*	Amamioshima, Ryukyu	KUHE 29548	AB200920; AB200944
2. *Rana* (*Eburana*) *ishikawae*	Okinawajima, Ryukyu	KUHE 10069	AB200921; AB200945
3. *Rana* (*Eburana*) *amamiensis*	Amamioshima, Ryukyu	KUHE 29529	AB200922; AB200946
4. *Rana* (*Eburana*) *amamiensis*	Tokunoshima, Ryukyu	KUHE 24635	AB200923; AB200947
5. *Rana* (*Eburana*) *narina*	Okinawajima, Ryukyu	KUHE 12788	AB200924; AB200948
6. *Rana* (*Eburana*) *supranarina*	Ishigakijima, Ryukyu	KUHE 24141	AB200925; AB200949
7. *Rana* (*Eburana*) *supranarina*	Iriomotejima, Ryukyu	KUHE 12898	AB200926; AB200950
8. *Rana* (*Eburana*) *utsunomiyaorum*	Ishigakijima, Ryukyu	KUHE 24144	AB200927; AB200951
9. *Rana* (*Eburana*) *utsunomiyaorum*	Iriomotejima, Ryukyu	KUHE 12896	AB200928; AB200952
10. *Rana* (*Eburana*) *swinhoana*	Taiwan	KUHE 34731	AB200929; AB200953
11. *Rana* (*Eburana*) *chloronota*	Hong Kong	KUZ 30216	AB200930; AB200954
12. *Rana* (*Eburana*) *livida*	Thailand	KUHE 23362	AB200931; AB200955
13. *Rana* (*Chalcorana*) *chalconota*	Thailand	KUHE 23936	AB200932; AB200956
14. *Rana* (*Chalcorana*) *hosii*	Malay Peninsula	KUHE 34491	AB200933; AB200957
15. *Rana* (*Chalcorana*) *hosii*	Sabah, Borneo	BOR 09097	AB200934; AB200958
16. *Rana* (*Odorrana*) *schmackeri*	Guangxi, China	KUHE 33623	AB200935; AB200959
17. *Rana* (*Hylarana*) *erythraea*	Sabah, Borneo	BOR 08125	AB200936; AB200960
18. *Rana* (*Nasirana*) *alticola*	Thailand	KUHE 19530	AB200937; AB200961
Subsection *Hydrophylax*			
19. *Rana* (*Humerana*) *miopus*	Malay Peninsula	KUHE 15247	AB200938; AB200962
20. *Rana* (*Pulchrana*) *signata*	Sabah, Borneo	BOR 22024	AB200939; AB200963
21. *Rana* (*Sylvirana*) *nigrovittata*	Thailand	KUHE 19781	AB200940; AB200964
22. *Rana* (*Sylvirana*) *nicobariensis*	Sumatra, Indonesia	KUHE 23598	AB200941; AB200965
Outgroup			
23. *Staurois latopalmatus*	Sabah, Borneo	BOR 08098	AB200942; AB200966
24. *Paa quadrana*	Yunnan, China	C91	AB200943; AB200967
Fejervarya limnocharis	China		AY158705; AY158705
Buergeria buergeri	Japan		AB127977; AB127977

Classification follows Dubois (1992). BOR: BORNEENSIS Collection, University Malaysia Sabah; C: Kumming Institute of Zoology; KUHE: Graduate School of Human and Environmental Studies, Kyoto University; KUZ: Department of Zoology, Faculty of Science, Kyoto University.

ClustalX alignments of 12S and 16S were inspected by eye and adjusted slightly to maintain elements of secondary structure, as suggested by Kjer (1995). We consistently obtained lengths, referring to the aligned sequences including gaps, of 1283 bp [847 bp (12S) and 436bp (16S)] for all samples.

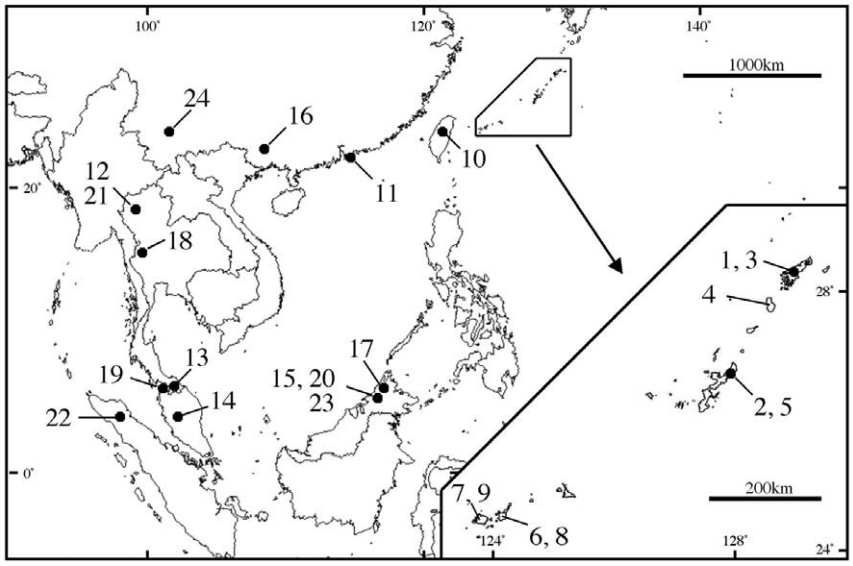

Fig. 1. A map of East to Southeast Asia, showing sampling localities of the section *Hylarana* and related species. For the locality number, refer to Table 1.

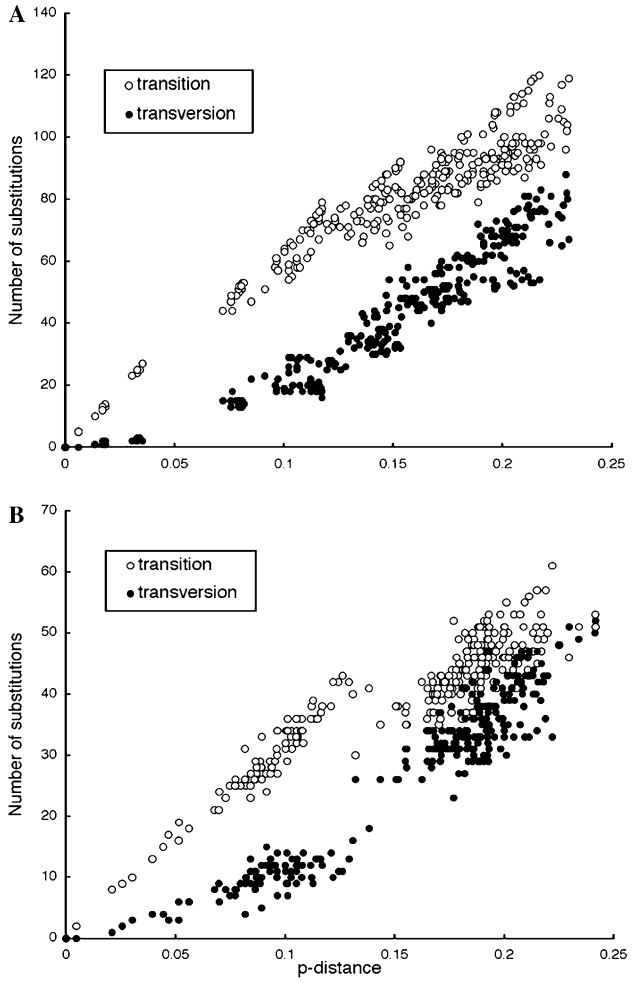

Fig. 2. Plots of uncorrected *p*-distance against the number of transitions and transversions. Fragments of 12S (A) and 16S (B) rRNA are plotted separately.

Partial sequences of the 12S rRNA (equivalent to half of our 12S fragment) and 16S rRNA (equivalent to our 16S fragment) for five Chinese species of *Odorrana* were accessible through GenBank. We aligned them to the homologous sequences of our samples to resolve relationships between *Odorrana* and *Eburana*.

To test for saturation in base substitutions we plotted uncorrected *p* distance against the number of transitions and transversions, and confirmed that 12S and 16S reach, if any, only slight saturations (Fig. 2).

2.3. Phylogenetic analysis

Prior to phylogenetic analysis we performed some tests to survey the properties of our data. To assess the amount of phylogenetic signal we generated 100,000 random trees and calculated the skewness (g1) of the resulting tree-length distribution with PAUP 4.0b (Swofford, 2002). We used the program MODELTEST 3.06 to examine goodness-of-fit of nested substitution models (for ingroup taxa only). MODELTEST 3.06 was also used to derive best-fit estimates of base-pair frequencies, the proportion of invariant sites, and the gamma shape parameter.

The data were then subjected to four different methods of phylogenetic reconstruction: (i) neighbor joining (NJ) using the Kimura's two-parameter substitution model (Kimura, 1980); (ii) maximum parsimony (MP) with gaps treated as missing data, equal weighting for transitions and transversions, heuristic search with the TBR branch-swapping algorithm, and 100 random-addition replicates; (iii) maximum-likelihood (ML) analysis based on the substitution model and phylogenetic parameters identified as optimal by MODELTEST 3.06; and (iv) Bayesian inference (Huelsenbeck et al., 2001; Rannala and Yang, 1996) using the same substitution model as ML, running four simultaneous Metropolis-coupled Monte–Carlo Markov chains for 1,000,000 generations. We sampled a tree every 100 generations and calculated a consensus topology for 9001 trees by omitting the first 1000 trees (burn-in).

With the exception of the Bayesian approach, which was performed by MrBayes (Huelsenbeck and Ronquist, 2001), all analyses were done with PAUP4.0b. Robustness of NJ, MP, and ML tree topologies was tested by bootstrap analyses (Felsenstein, 1985), with 2000 replicates for NJ and MP, and 100 replicates for ML (Hedges, 1992). Only bootstrap values 70% or greater indicate sufficiently resolved topologies (Huelsenbeck and Hillis, 1993), and those between 50 and 70% were regarded as tendencies. For the Bayesian method, we used posterior probabilities as indicators for confidence of nodes. Because these represent the true probabilities of the clades (Rannala and Yang, 1996), posterior probabilities 95% or greater were considered significant (Leaché and Reeder, 2002).

2.4. Divergence times

To estimate the age of each clade, we first tested for substitution-rate constancy between the sister groups using PHYLTEST software (Kumar, 1996; Takezaki et al., 1995), but ultrametric evolution was rejected in several comparisons ($p < 0.05$). We thus transformed branch length on an MP tree according to the Penalized likelihood method (PL: Sanderson, 2002) using TN algorithm (a truncated newton method with bound constraints) to estimate the age. Since no reliable information was available regarding absolute ages of internal nodes, we attempted to obtain an approximate calibration for our data utilizing the calibration of Beerli et al. (1996) for European water frogs. At first, we confirmed a rate constancy in 810 bp of 12S and 16S sequences between some of the species (data from GenBank) used by Beerli et al. (1996) and our ingroup, using *F. limnocharis* as an outgroup. Because the resultant rate constancy was not

rejected ($p > 0.05$), we then applied the estimation of Beerli et al. (1996) to the equation: time-of-separation = $0.198 + 8.189 \cdot D^*_{Nei} \cdot TrN$, with $1D^*_{Nei} = 8.14$ MY (Veith et al., 2003).

Following this equation, we estimated the age of a particular node from TrN distances among descendent taxa for the use of the PL chronogram (Sanderson, 2002). This calibration produced different estimate depending on the node chosen as a calibration point. We, thus, chose all available calibration points and took their range as an approximate estimate.

3. Results

3.1. Sequence and tree statistics

Sequence statistics for the two gene fragments and for the combined alignment are given in Table 2. All but one of the multiple samples of a taxon showed slightly different sequences. A total of 611 out of 1283 bp were variable in the combined alignment, with 468 being parsimony informative. The average ti/tv ratio varied among genes and was 2.13 in the combined dataset when considering only ingroup taxa.

The best substitution model derived from MODELTEST was the general time reversible model (GTR; Rodriguez et al., 1990) with a gamma shape parameter estimated as 0.634, and a proportion of invariant sites estimated as 0.324.

We got two equally most parsimonious trees that required 2105 evolutionary steps with a consistency index CI = 0.458 and retention index RI = 0.552. For 100,000 random trees we calculated the skewness g1 = 0.526, which shows that our data containing enough phylogenetic signal (Hillis and Huelsenbeck, 1992).

3.2. Phylogenetic relationships and divergence times

All phylogenetic analyses produced essentially the same topologies (only the ML tree is shown in Fig. 3). The following relationships were indicated by the four analyses with bootstrap *p*-values and Bayesian posterior probabilities mentioned above as statistically reliable:

Table 2
Alignment statistics for fragments of the 12S and 16S; number of base bairs (bp), number of variable sites (vs), number of parsimony informative sites (pi), the transition–transversion ratio given for ingroups only (ti/tv)

	bp	vs	pi	ti/tv
12S rRNA	847	414	313	2.419
16S rRNA	436	197	155	1.955
Combined	1283	611	468	2.132

(i) Monophyly of Raninae with respect to the outgroup Rhacophoridae and Dicroglossinae (support: 100, 99, 100, and 100%: NJ, MP and ML bootstrap values, and Bayesian posterior probability, respectively).
(ii) Monophyly of Ranini with respect to Paini (support: 82, 89, 97, and 100%).
(iii) Monophyly of the genus *Rana* with respect to *Staurois* (support: 94, 89, 76, and 100%).
(iv) A basal bifurcation of the section *Hylarana* into a monophyletic group comprising *Eburana*, *Odorrana*, and *R.* (*Chalcorana*) *hosii* (support: 100, 100, 98, and 100%) and another group comprising species of subgenera *Nasirana*, *Humerana*, *Sylvirana*, *Pulchrana*, *Hylarana*, and *R.* (*C.*) *chalconota* (support: 73, 65, 69, and 74%). Relationships within the latter are not well clarified.
(v) Monophyly of the subgenus *Chalcorana* is not supported. Monophyly of the subgenus *Sylvirana* [*R.* (*S.*) *nigrovittata* and *R.* (*S.*) *nicobariensis*] and of *R.* (*Hylarana*) *erythraea* and *R.* (*Humerana*) *miopus* is only weakly supported (support: 70, 58, 60, and < 50%, and 64, < 50, 58, and 95%, respectively).
(vi) Populations of *R.* (*E.*) *ishikawae* from two islands of the Ryukyu Archipelago form a monophyletic group (support: 100%) outside the clade comprising *R.* (*C.*) *hosii*, subgenus *Odorrana* and the remaining species of *Eburana*. *Rana* (*E.*) *ishikawae* therefore does not have a sister-group relationship with the other frogs from the Ryukyus (= the *R.* (*E.*) *narina* complex).
(vii) *Rana* (*O.*) *schmackeri* is the sister taxon to the species of the subgenus *Eburana*, other than *R.* (*E.*) *ishikawae*, and *R.* (*C.*) *hosii* (support: 56, 85, 89, and 100%).
(viii) Monophyletic grouping of the subgenus *Eburana*, other than *R.* (*E.*) *ishikawae*, and *R.* (*C.*) *hosii* is supported, although not strongly (support: 56, 51, 78, and 99%).
(ix) *Rana* (*E.*) *chloronota*, *R.* (*C.*) *hosii* and *R.* (*E.*) *livida* form a clade (support: 100%) within which interspecific relationships are not well resolved.
(x) Populations of *R.* (*C.*) *hosii* from Borneo and the Malay Peninsula form a monophyletic group (support: 100%).
(xi) The *R.* (*E.*) *narina* complex from the Ryukyus and Taiwan forms a monophyletic group (support: 99, 95, 97, and 100%).
(xii) *Rana* (*E.*) *swinhoana* and *R.* (*E.*) *utsunomiyaorum* are sister taxa (support: 100%).
(xiii) *Rana* (*E.*) *supranarina*, *R.* (*E.*) *narina* and *R.* (*E.*) *amamiensis* form a clade (support: 100%) within which *R.* (*E.*) *narina* and *R.* (*E.*) *amamiensis* are grouped as sister species (support: 100, 99, 99, and 100%).

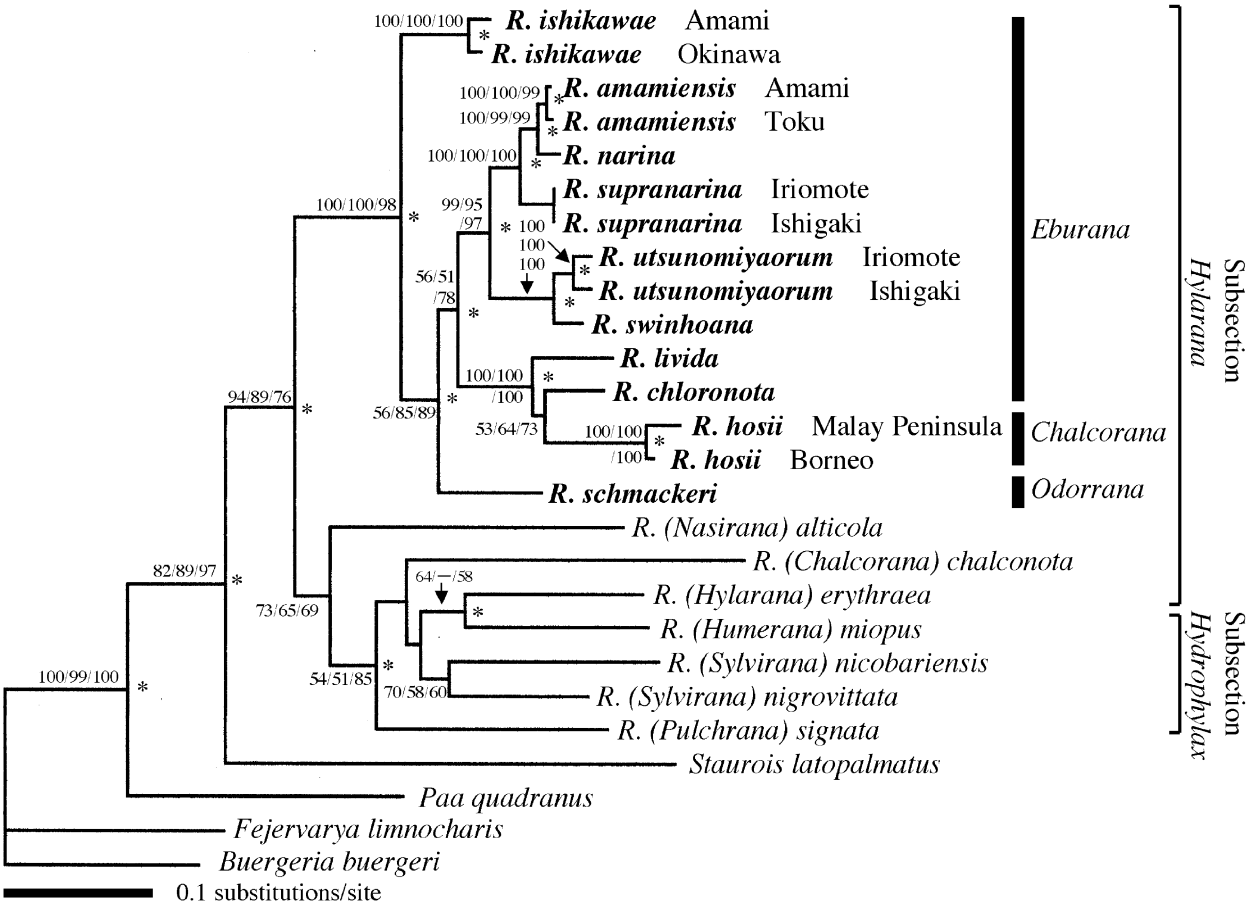

Fig. 3. Maximum-likelihood tree of 1283 bp of 12S and 16S for species of Oriental frogs. Bootstrap supports are given for NJ (2000 replicates), MP (2000), and ML (100) inference. Nodes with asterisks indicate significant support (>95%) by Bayesian inference.

Ages of nodes estimated from TrN distances among descendent taxa are shown in Table 3.

4. Discussion

4.1. Problems of current taxonomy of Rana

Dubois (1992) recognized two tribes (Paini, Ranini) in Raninae, one of the seven subfamilies he placed in Ranidae. He split Ranini into six genera, of which *Rana* is the largest and was further split into nine sections. Some of these sections have already been studied phylogenetically using molecular techniques [e.g., sections *Amerana* (Macey et al., 2001); *Rana* (Tanaka et al., 1996; Veith et al., 2003); *Pelophylax* (Austin et al., 2003); *Pseudorana* (Matsui et al., 2001)], species studied therein were mostly from North America, Europe, and East Asia. Many species in other sections or from the other regions of the world have been studied fragmentarily, chiefly as outgroups in studies of other frog groups.

Phylogenetic studies using molecular techniques are lacking for species from Southeast Asia. Subgenera examined in this study (*Chalcorana*, *Hylarana*, *Nasirana*, and *Odorrana*) belong to the phylogenetically understudied *Hylarana* section of Dubois (1992).

The section *Hylarana* is further divided into two subsections, *Hylarana* and *Hydrophylax*. The subsection *Hylarana* includes *Chalcorana*. *Clinotarsus*, *Eburana*, *Glandirana*, *Hylarana*, *Nasirana*, *Odorrana*, *Pterorana*, *Sanguirana*, and *Tylerana*, while *Hydrophylax* includes *Amnirana*, *Hydrophylax*, *Papurana*, *Humerana*, *Pulchrana*, and *Sylvirana* (Dubois, 1992). However, validity of this classification system has never been reassessed, although Matsui (1994) and Inger (1996) dispute the positions of several species.

4.2. Phylogenetics and taxonomy

Dubois (1992) once placed Paini as a tribe of Raninae, but he recently moved it to Dicroglossinae, another subfamily of Ranidae (Dubois, 2003). In our result, *Paa quadrana*, a member of Paini, was not sister taxon to *Fejervarya limnocharis*, a member of Dicroglossinae, but was sister taxon to a ranine clade as represented by *Staurois* and *Rana*. Thus, Dubois's older taxonomy (Dubois, 1992) was more consistent with our data. However, in many other respects, our results conflicted with the older

Table 3
TrN distance, relative age calculated by PL, and absolute age (mean followed by minimum and maximum values in parenthesis) calculated by Veith et al.'s (2003) equation between each clade

	D^*_{TrN}	Relative age	Isolation [Myr]
R. ishikawae vs. other *Odorrana* sensu lato	0.122	0.41	12.6 (7.9–18.0)
R. ishikawae (Amami) vs. *R. ishikawae* (Okinawa)	0.020	0.07	2.3 (1.5–3.2)
R. schmackeri vs. *the R. narina* complex + The *R. livida* complex + *R. hosii*	0.117	0.33	10.2 (6.4–14.5)
The *R. livida* complex + *R. hosii* vs. the *R. narina* complex	0.131	0.28	8.7 (5.4–12.3)
Between two large clades of the *R. narina* complex	0.084	0.21	6.6 (4.1–9.3)
R. supranarina vs. *R. amamiensis* + *R. narina*	0.040	0.11	3.5 (2.3–5.0)
R. amamiensis vs. *R. narina*	0.021	0.05	1.7 (1.1–2.4)
R. amamiensis (Amami) vs. *R. amamiensis* (Toku)	0.006	0.01	0.5 (0.4–0.6)
R. supranarina (Iriomote) vs. *R. supranarina* (Ishigaki)	0	0	0
R. swinhoana vs. *R. utsunomiyaorum*	0.039	0.09	2.9 (1.9–4.1)
R. utsunomiyaorum (Iriomote) vs.*R. utsunomiyaorum* (Ishigaki)	0.022	0.05	1.7 (1.1–2.4)
R. livida vs. *R. chloronota* + *R. hosii*	0.097	0.19	6.0 (3.8–8.4)
R. chloronota vs. *R. hosii*	0.091	0.14	4.4 (2.8–6.3)
R. hosii (Malay Peninsula) vs. *R. hosii* (Borneo)	0.023	0.04	1.4 (0.9–1.9)

taxonomy proposed by Dubois (1992). *Odorrana* (sense Fei et al., 1990) is monophyletic and includes all species currently treated as *Eburana* and one species (*R. hosii*) now included in *Chalcorana*.

Rana (*Eburana*) *ishikawae* was placed by Dubois (1992) in the subgenus *Eburana* together with the *R.* (*E.*) *narina* complex and *R.* (*E.*) *livida*. Matsui (1994), however, doubted to include *R.* (*E.*) *ishikawae* and the *R.* (*E.*) *narina* complex in the same subgenus. In our result, *R.* (*E.*) *ishikawae* falls outside the clade formed by *Eburana*, *Odorrana* and a part of *Chalcorana*. These relationships suggest the possibility of establishing a new subgenus for *R.* (*E.*) *ishikawae*, but action would further complicate classification.

The idea that *R.* (*E.*) *amamiensis* and *R.* (*E.*) *narina* are sister species has been supported both from allozyme (Matsui, 1994) and mt cyt *b* (Matsui et al., unpublished data) studies, and results of these studies agree in that they are very close in terms of genetic distance. The sister–taxon relationship of *R.* (*E.*) *supranarina* and the clade consisting of *R.* (*E.*) *amamiensis* and *R.* (*E.*) *narina* also conforms to the results of mt cyt *b* analyses (Matsui et al., unpublished data) and partially of allozyme analyses (Matsui, 1994). Similarly, the result that *R.* (*E.*) *utsunomiyaorum* from the Yaeyama Group was sister–taxon to *R.* (*E.*) *swinhoana* from Taiwan is supported by mt cyt *b* and allozyme analyses (Matsui, 1994; Matsui et al., unpublished data). Presence of some genetic diversification in *R.* (*E.*) *utsunomiyaorum* between the two islands of the Yaeyama Group is also concordant with results of previous studies (Matsui, 1994; Matsui et al., unpublished data).

Dubois' (1992) subgenus *Chalcorana* is even more problematical. He placed nine species, including *R.* (*C.*) *chalconota* and *R.* (*C.*) *hosii*, in this subgenus, but our result did not support his classification. Actually, *R.* (*C.*) *chalconota* and *R.* (*C.*) *hosii* differ in eggs and larvae. *Rana* (*C.*) *chalconota* lays pigmented eggs and has a larval dental formula of 4-5/3 (Inger, 1966), whereas in *R.* (*C.*) *hosii*, eggs are pigmentless and larvae have a dental formula of 5-6/4 (Manthy, 1994). According to Fei (1999), Chinese *R.* (*O.*) *livida* (should be *R. chloronota*, see Bain et al., 2003) is same as *R.* (*C.*) *hosii* in egg color and larval dental formula. These lines of evidence also support separation of *R.* (*C.*) *hosii* from the subgenus *Chalcorana* (type species = *R.* (*C.*) *chalconota*). All of these results indicate severe deficiency in Dubois' (1992) subgeneric designation and suggest reassessment of his subsection *Hylarana*.

Because we could obtain only one species of Chinese *Odorrana*, we combined the GenBank data of 12S (AF205562, AF205563, AF205564, AF205565, and AF 315128) and 16S rRNA (AF315156, AF315157, AF315158, AF315159, and AF315160) of five Chinese species, *R.* (*E.*) *livida*, *R.* (*O.*) *schmackeri*, *R.* (*O.*) *hejiangensis*, *R.* (*O.*) *margaretae* (all from Sichuan: Jiang and Zhou, 2001), and *R.* (*O.*) *grahami*, with our own data and analyzed phylogenetic relationships (Fig. 4). The results show that all species of *Odorrana*, *Eburana*, and *R.* (*C.*) *hosii* form monophyletic group (Fig. 4). A clade comprising *R.* (*O.*) *grahami* and *R.* (*O.*) *margaretae* diverged first from the others, in which *R.* (*E.*) *ishikawae* and a clade of all the remaining species were separated. The latter clade was separated into two subclades, the one consisting of *R.* (*E.*) *livida*, *R.* (*E.*) *chloronota* and *R.* (*C.*) *hosii*, and the other of the *R.* (*E.*) *narina* complex and of *Rana* (*O.*) *hejiangensis* and *R.* (*O.*) *schmackeri*.

Sequences of *R.* (*O.*) *schmackeri* differed between the Guangxi (our data) and Sichuan (AF205563, AF315158) samples, and the Guangxi sample lies outside a clade comprising the Sichuan sample and *R.* (*O.*) *hejiangensis*. Fei (1999) split Chinese *Odorrana* into four groups, but the phylogenetic tree obtained supported close relationships only for *R.* (*O.*) *schmackeri* and *R.* (*O.*) *hejiangensis*.

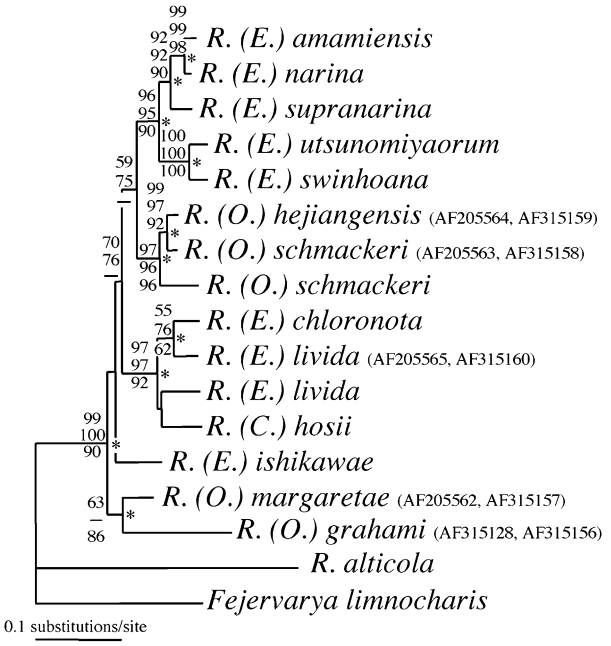

Fig. 4. Maximum-likelihood tree of 842 bp of 12S and 16S for species of *Rana* (*Odorrana*). Bootstrap supports are given for NJ (2000 replicates), MP (2000), and ML (100) inference. Nodes with asterisks indicate significant support (>95%) by Bayesian inference.

In conclusion, *Eburana*, *Odorrana*, and a part of *Chalcorana* [*R.* (*C.*) *hosii*] form a monophyletic group, and Dubois' (1992) proposal of *Eburana* and *Odorrana* as distinct subgenera is rejected because monophyly was not supported for either of them (Fig. 4). Instead, Fei et al.'s (1990) idea of including species of *Eburana* in *Odorrana* is supported, canceling its distinct generic status. Fei et al. (1990) once placed *R. narina* from Taiwan (actually *R. swinhoana*: see Matsui, 1994) in their distinct genus *Pelophylax*, but this erroneous treatment was corrected later, and *R. swinhoana* was moved to *Odorrana* (Fei, 1999). We also remove *R. hosii* from *Chalcorana* of Dubois (1992) and place in *Odorrana*. Furthermore, our results conflict with Dubois' (1992) subsection *Hylarana* because *R.* (*N.*) *alticola*, *R.* (*H.*) *erythraea*, and *R.* (*C.*) *chalconota* did not group with the other taxa of *Hylarana* but with the subsection *Hydrophylax*. Greater sampling of species is needed for further evaluation of these subsections.

4.3. Historical biogeography of the species in the northern peripheral region

Within subgenus *Odorrana*, the ancestor of *R.* (*O.*) *ishikawae* seems to have reached its northernmost range, the area of the present Ryukyu Archipelago, in the early history of this subgenus from the middle to late Miocene (18.0–7.9 MYBP: Table 3). Some authors consider formation of the Ryukyu Archipelago to have been initiated by its eastward drifting during the late Miocene to the middle Pliocene as a result of crustal thinning in the western part of the East China Sea (e.g., Kimura, 2000). Our estimation supports this geological scenario.

Rana (*Odorrana*) *ishikawae* has been considered a relict species like the Anderson's alligator newt, *Tylototriton* (*Echinotriton*) *andersoni* (Utsunomiya and Matsui, 2003), because it is isolated in the Amami and Okinawa groups of the middle Ryukyus (Maeda and Matsui, 1999; Ota, 1998). The present results support such an idea. A fossil frog identified as *R.* (*O.*) *ishikawae* has been recorded much later, from the lower Pleistocene (1.4 MYBP) of Tanegashima Island, north of Amamioshima (Otsuka and Kuwayama, 2000). This fossil suggests that the range of *R.* (*O.*) *ishikawae* once extended further north, although identity of the fossil still needs verification.

A common ancestral lineage of the *R.* (*O.*) *narina* complex is estimated to have invaded the Ryukyus through Taiwan in the late Miocene (12.3–5.4 MYBP), when these regions were still connected with the Chinese continent (Kimura, 2000). Namely, the invasion of *Rana* (*Odorrana*) into its northernmost range of distribution is considered to have occurred twice.

The *R.* (*O.*) *narina* complex is estimated to have acquired its present distribution within the Ryukyu Archipelago through interspecific, ecological interactions. From the pattern of divergence in the phylogenetic tree, the ancestor of the *R.* (*O.*) *narina* complex, first widely distributed from the present Taiwan to the central Ryukyus, was split into the northern and southern stocks in the late Miocene to the early Pliocene (9.3–4.1 MYBP). The southern stock diverged into *R.* (*O.*) *swinhoana* in Taiwan and *R.* (*O.*) *utsunomiyaorum* in the Yaeyama group by the end of the Pliocene.

Invasion of the ancestor of *R.* (*O.*) *supranarina* into the Yaeyama group is considered to have occurred much later, because the species is little diverged genetically between the two islands of the southern Ryukyus. In contrast, *R.* (*O.*) *utsunomiyaorum* shows much greater genetic divergence between the two islands, probably as a result of isolation since the beginning of the Pleistocene. Divergence between *R.* (*O.*) *supranarina* and the clade consisting of *R.* (*O.*) *amamiensis* and *R.* (*O.*) *narina* seems to have already occurred in the Pliocene, but invasion to the Yaeyama group of the southern Ryukyus by the former seems to have occurred in the Pleistocene.

As already shown by Matsui (1994), *R.* (*O.*) *utsunomiyaorum* is smaller in body size and more restricted in habitats than *R.* (*O.*) *supranarina*. This ecological asymmetry seems to have forced coexistence of the ancestors of the two species in the southern Ryukyus in the chronological order mentioned above. Divergence of *R.* (*O.*) *amamiensis* and *R.* (*O.*) *narina* should have occurred at the end of the Pliocene with the separation of the Amami and Okinawa island groups within the central Ryukyus.

By contrast, southeastern dispersal of *Odorrana*, as represented by *R.* (*O.*) *hosii*, seems to have occurred much later than the northeastern dispersal in the late Miocene. Inger and Voris (2001) hypothesized that gene flow in *R.* (*O.*) *hosii* has been interrupted since pre-Pleistocene times (>1.6 MYBP) between Borneo and the Malay Peninsula or Sumatra, and according to our data, the time of separation in *R.* (*O.*) *hosii* between the Borneo and the Malay Peninsula is later (1.9–0.9 MYBP) approximates their estimation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2005.04.030.

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