Phylogeny, Taxonomy, and Biogeography of the Oriental Pitvipers of the Genus *Trimeresurus* (Reptilia: Viperidae: Crotalinae): A Molecular Perspective

Ming-Chung Tu¹, Hurng-Yi Wang¹, Mung-Pei Tsai², Mamoru Toda³, Wen-Jen Lee¹, Fu-Ji Zhang⁴, and Hidetoshi Ota⁵*

¹Department of Biology, National Taiwan Normal University, Taipei, 117 Taiwan
²Institute of Zoology, Academia Sinica, Taipei, 115 Taiwan
³Department of Zoology, Graduate School of Science, Kyoto University, Sakyo, Kyoto, 606-8502 Japan
⁴Chengdu Institute of Biology, The Chinese Academy of Sciences, Chengdu, 610041 People’s Republic of China
⁵Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa, 903-0213 Japan

**ABSTRACT**—Based on sequence variation in 806 bp of the mitochondrial 12S rRNA gene, phylogenetic relationships were inferred for 14 species of *Trimeresurus* (sensu lato) including all East Asian members. Samples analyzed also included representatives of all assemblages of species that are frequently treated as separate genera except for *T. mangshanensis*, a type species of the recently described monotypic genus *Ermia*. Results support some previous accounts chiefly from morphological studies, such as distinct divergence of *T. wagleri* from the remainder, and monophyly of *T. mucrosquamatus, T. flavoviridis, T. jerdonii, T. elegans* and *T. tokarensis*. On the other hand, our results negated a putative close affinity of *T. monticola* and *T. okinavensis*, and indicated the sister relationship of the latter with *T. gracilis*. Phylogenetic relationships revealed in this study suggested that the genus *Trimeresurus* dispersed into the Ryukyu region at least three times, and that *T. flavoviridis* and *T. tokarensis* from the central Ryukyus constitute a relict clade.

**INTRODUCTION**

*Trimeresurus* Lacepede, as defined by Brattstrom (1964) (henceforth referred to as *Trimeresurus* [sensu lato]), is the largest group of venomous snakes in tropical-subtropical Asia, consisting of some 40 species (David and Tong, 1997; McDiarmid et al., 1999). A few species of the genus are especially dangerous and thus are of great concern to public-health authorities and medical workers in some areas (Gopala-krishnakone and Chou, 1990). Distribution of *Trimeresurus* (sensu lato), covering not only a broad part of the Asian continent but also a number of adjacent archipelagos with complex geohistories, makes the genus a particularly suitable model to investigate biological consequences of past geological events as expressed in a phylogeographical pattern of extant species.

A number of authors have addressed phylogenetic relationships within *Trimeresurus* (sensu lato) based on morphological characters, some hypothesizing scenarios for the divergence process of the genus (Brattstrom, 1964; Zhang, 1995), others proposing its taxonomic subdivisions (Burger, 1971; Hoge and Romano-Hoge, 1981, 1983; Zhang, 1993). Nevertheless, very little consensus has yet been attained to date for almost every relevant issue (e.g., see comments by Groombridge [1986], Toriba [1989] and McDiarmid et al. [1999] on the taxonomic changes proposed by Burger [1971], Hoge and Romano-Hoge [1983] and Zhang [1993], respectively).

Recently a few preliminary molecular studies, while confirming great divergences within *Trimeresurus* (sensu lato) predicted by previous morphological studies (see above) (Kraus et al., 1996; Knight et al., 1992), elucidated prominent convergences in some of the traditionally used taxonomic characters of this and other crotalinae genera (Cullings et al., 1997; Malhotra and Thorpe, 1997; Vidal et al., 1997; Parkinson, 1999). These findings suggest the presence of severe constraints in the use of morphological characters for the recon-
struction of phylogenetic relationships in this and related genera.

For the purpose of testing some previous hypotheses regarding the phylogeny and biogeography of *Trimeresurus* (sensu lato), we sequenced a fragment of the mitochondrial 12S ribosomal RNA gene (12S rRNA) for representatives of the genus. Sequence variation in this domain is usually used to resolve higher level phylogenetic relationships in reptiles (Heise et al., 1995; Honda et al., 1999; Ota et al., 1999), but has also been shown to be effective to infer the infrageneric phylogeny of viperid snakes (e.g., Knight et al., 1993). Analyses of sequence data suggest relationships which substantially differ from those hypothesized for *Trimeresurus* (sensu lato) based on previous morphological studies. Also, our results yield new insights into the historical biogeographical analysis of terrestrial organisms in the subtropical East Asian islands.

**MATERIALS AND METHODS**

Eighteen specimens of 14 species and one subspecies of *Trimeresurus* (sensu lato) were used in this study (Table 1: Appendix 1). Of these taxa, four species and one subspecies (*T. monticola*, *T. monticola* makazayazaya, *T. jerdonii*, *T. gracilis*, and *T. puniceus*) were examined by the molecular technique for the first time. The taxa examined include all eight species of the genus occurring in Taiwan and the Ryukyu Archipelago, as well as representatives of all species assembles of *Trimeresurus* (sensu lato) but one (*Erinia Zhang*) that are frequently treated as separate genera (i.e., *Ovophis Burger*, *Protobothrops Hoge et Romano-Hoge*, *Tropidolaemus Wagler*, and *Trimeresurus sensu stricto*) (Hoge and Romano-Hoge, 1983; Toriba, 1993; Zhang, 1993; Wüster et al., 1993; McDiarmid et al., 1999). Specimens of *Deinagkistrodon acutus* and *Vipera russelli* that, respectively, represent another crotaline genus and a different viperid subfamily, Viperinae, were also included in the analyses as outgroups. Each specimen was designated as constituting an independent operational taxonomic unit (OTU) by itself for the phylogenetic analyses.

Crude DNA was extracted from fresh or 95% ethanol-preserved muscle samples following Kocher et al. (1989), but with incubation extended for approximately ten hours. After proteinase K digestion, DNA was purified by two times of phenol extraction, once or twice of phenol/chloroform/isoamyl alcohol (25:24:1) extraction, and once of chloroform/isoamyl alcohol (24:1) extraction, each followed by precipitation in ethanol with 1/10 volume NaOAC.

A portion of the 12S rRNA gene of the mitochondrial genome was amplified by the polymerase chain reaction (PCR) using two primers—SN1: 5’-ATGCTGCAGAAAAAGATTAAGTTA-3’; and SN2: 5’-TCTTTGGTCTGAAACCTCAGTACCTA-3’ (Wang and Tu, 1997). PCR reactions were performed in 50 ul volumes consisting of 10 mM Tris-HCl (pH 9.0), 50mM KCl, 15mM MgCl2, 0.1% [w/v] gelatin, 1% Triton X-100, 0.4 pM primer, 0.2 mM dNTP, 50–100 ng of crude DNA, and 1 U Taq polymerase (InvITAO, Germany). The temperature regimen of 35 cycles, subsequent to the two minutes of initial denaturation at 94°C, was 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C.

PCR products were purified with the Gene Clean III elution kit (Bio 101, CA). Both DNA strands were sequenced using dye terminator cycle-sequencing reactions that were subsequently loaded on an Applied Biosystems 377A automatic sequencer. The numbering system followed Anderson et al. (1981).

DNA sequences were aligned by using the default parameters of CLUSTAL W (Thompson et al., 1994). Adjustments were made visually on the basis of maximum nucleotide similarity. Gaps and insertions (indels) were excluded from the subsequent analyses.

Based on the aligned sequences, phylogenetic relationships among OTUs were inferred using neighbor-joining (NJ: Saitou and Nei, 1987) and maximum parsimony (MP) methods. For the former, three distance models (i.e., Jukes and Cantor’s [1969] one parameter model, Kimura’s [1980] two parameter model, and Tamura and Nei’s [1993] model) were used to correct pairwise distances for multiple hits. Resultant distance matrices were subjected to NJ analyses using PHYLIP 3.5c (Felsenstein, 1993). Degrees of supports for internal branches of the resultant tree were assessed by 1,000 bootstrap replications (Felsenstein, 1985) for each weighting scheme used. By using PAUP version 4.0 (Swoford, 1998), we further examined the content of phylogenetic information in our data set by checking the skewness of the tree distribution and the g1 values (Hillis and Huelsenbeck, 1992) for 10,000 random trees in each weighting scheme.

MP analysis was performed using PAUP*, in which each nucleotide base was regarded as an unordered character, and the four kinds of salts as different character states. A total of 1,000 bootstrap pseudoreplications (Felsenstein, 1985) were conducted using the

| Table 1. | Species and subspecies of *Trimeresurus* (sensu lato), and two outgroup species examined in this study. Information regarding their distributions was taken from Toriba (1993) and McDiarmid et al. (1999). See Appendix 1 for locality data for materials used in our analyses. |
|---|---|---|
| Species/subspecies | Sample size | Distribution |
| *Trimeresurus albolabris* | 1 | Northern India through Southeast Asia to southern China |
| *T. elegans* | 1 | The Yaeyama Group of the southern Ryukyus, Japan |
| *T. flavovirdis* | 1 | The Okinawa and Amami Groups of the central Ryukyus, Japan |
| *T. gracilis* | 1 | Taiwan |
| *T. jerdonii* | 1 | Northern India, northern Indochina, and southern China |
| *T. monticola monticola* | 1 | Nepal, northern India, Myanmar, and southern China |
| *T. monticola makazayazaya* | 2 | Eastern China including Taiwan |
| *T. murosquamatus* | 2 | Northern India through southern China to Taiwan |
| *T. okinavensis* | 1 | The Okinawa and Amami Groups of the central Ryukyus, Japan |
| *T. popeiorum* | 1 | Northern India and Southeast Asia |
| *T. puniceus* | 1 | Southern Thailand, Malay Peninsula, and adjacent islands |
| *T. stejnegeri* | 2 | Northern India through Indochina and southern China to Taiwan |
| *T. tokarensis* | 1 | Southern Tokara islands of the central Ryukyus, Japan |
| *T. trigoncephalus* | 1 | Sri Lanka |
| *T. wagleri* | 1 | Southeast Asia including the Philippines |
| *Deinagkistrodon acutus* | 1 | Northern Indochina through southern China to Taiwan |
| *Vipera russelli* | 1 | South Asia through Southeast Asia and southern China to Taiwan |
heuristic algorithm of PAUP*. The result of this analysis was expressed as a 50% majority-rule consensus bootstrap tree. Skewness of the tree distribution and the g1 values for 10,000 random trees were also calculated.

RESULTS

Aligned sequence of 12S rRNA gene consisted of 806 bp including indels (Appendix 2). The first 230 and 200 sites of T. wagleri and D. acutus, respectively, could not be sequenced despite our several attempts. These portions were treated as gaps, and were excluded from the analyses.

Comparisons of aligned sequence revealed 362 variable sites, of which 200 were phylogenetically informative. The base composition was slightly biased, with the average nucleotide

![Diagram of phylogenetic tree](image)

**Fig. 1.** Neighbor-joining (NJ) tree derived from Kimura's (1980) two parameter distance matrix for 12S rRNA sequence data. Numerals above nodes indicate proportion of support in 1000 bootstrap replications (BP). Branches without BP values were not supported in ≥ 50% of the replicates. Numerals below nodes are reference numbers used in the text.
frequencies of A, T, C and G being 37.6%, 19.8%, 25.1% and 17.5%, respectively. The estimated g1 values for trees resulting from our analyses were lower than critical values (p=0.01: Hillis and Huelsenbeck, 1992), indicating that the region sequenced contained phylogenetic information.

Distance values resulting from the three different models were very similar to each other. Likewise, resultant NJ trees showed no distinct differences in branching topology or relative branch length among the three models, either. Therefore, we provide results of NJ analysis using Kimura's distance matrix only (Fig. 1) (distance matrices and results of NJ using the remaining models are available from H.-Y. Wang upon request).

In the NJ analysis, monophyly of the genus Trimeresurus sensu lato was supported only by a low bootstrap proportion (BP:<50%), whereas the monophyly of conspecific and consubspecific OTUs invariably received a complete bootstrap support. Five major nodes (nodes 1–5) were recognized in NJ tree, of which two consisted of single species, T. monticola (node 1) and T. wagleri (node 5), whereas another one with a complete bootstrap support (node 3) consisted of T. gracilis and T. okinavensis. Of the remaining two major nodes, one with a rather poor BP support (node 4) consisted of exclusively arboreal species, with T. popeiorum possibly initially diverging from the remainder, followed by T. trigonocephalus and T. puniceus in order. The remaining major node with a complete bootstrap support (node 2) was further divided into two nodes, one consisting of T. mucrosquamatus and T. elegans (node 6), and the other of T. jerdonii, T. flavoviridis and T. tokarensis (node 7). The last two species received a 100% bootstrap support for their sister-group relationship, whereas the sister-group relationship of these species and T. jerdonii was supported with a lower, but still substantial bootstrap proportion (67%).

The 50% majority-rule consensus tree resulting from the MP analysis did not contradict with NJ tree in branching topol-

Fig. 2. Majority-rule consensus cladogram derived from maximum parsimony analysis (MP) of 12S rRNA sequence data. Numerals above nodes indicate proportion of support in 1000 bootstrap pseudoreplications. Numerals below nodes are reference numbers used in the text. Consistency index excluding uninformative sites (Kluge and Farris, 1969) =0.457; retention index (Farris, 1989) =0.530.
ology (Fig. 2), but differs from the latter in the degree of supports for a few nodes. For example, monophyly of the genus *Trimeresurus* sensu lato was supported with a much higher BP value (85%), whereas the value in support for the node 7 (54%) was lower than the corresponding value in NJ tree (see above).

**DISCUSSION**

**Phylogeny**

Our results corroborate the monophyly of *T. murosquamatus*, *T. flavoviridis*, *T. jerdonii*, *T. elegans*, and *T. tokarensis*, as was explicitly or implicitly predicted by a few previous authors (Hoge and Romano-Hoge, 1981; Kraus et al., 1996; David and Ineich, 1999; Parkinson, 1999). Moreover, initial divergence of *T. wagleri* from the remaining members of *Trimeresurus* (sensu lato) in our analyses (Figs. 1 and 2) agrees well with the relationships inferred by Brattstrom (1964) on the basis of morphological analysis. However, such relationships of *T. wagleri* and other species contradict with the hypothesis resulting from molecular analyses by Parkinson (1999), in which *T. wagleri* is considered to be closer to *T. albolarbis* and *T. stejnegeri* than are some other species common to our study (i.e., *T. elegans*, *T. flavoviridis*, *T. tokarensis*, and *T. okinavensis*). Moreover, *T. wagleri* is shown to be closest to *D. acutus* in Parkinson’s (1999) trees. Because either his trees or ours failed to receive sufficiently high bootstrap supports (> 90%; Shaffer et al., 1997) for these relationships, it is obvious that unambiguous solutions of these inconsistencies on the molecular scale characters between *T. gracilis* and *T. okinavensis*. It is thus possible that external relationships illustrated by more comprehensive allozyme analyses substantially contradict with the relationships indicated by the present analyses.

**Taxonomic implications**

The relationships depicted by our analyses (Figs. 1 and 2) support the validity of *Tropidolaemus*, which had usually been regarded as synonymous with *Trimeresurus* (e.g., see a synonym list of *wagleri* in David and Vogel [1996]), and was resurrected to accommodate *wagleri* by Hoge and Romano-Hoge (1981) on the basis of Burger’s (1971) morphological data. For further confirmation for the current taxonomic arrangement of *Tropidolaemus*, additional analyses are desired by incorporating sequence data for *luttoni* from India, a putative second species of the genus (David and Vogel, 1998).

Our results also support the validity of *Protobothrops* (node 2), which was originally described for *T. murosquamatus* (type species), *T. murosquamatus* and *T. jerdonii*, and subsequently also thought to include *T. elegans* and *T. tokarensis*, as well as *T. kaubalbacki*, *T. strigatus* and *T. xiangchengensis* (Hoge and Romano-Hoge, 1983; Kraus et al., 1996; David and Ineich, 1999; Parkinson, 1999). In contrast, relationships revealed here negate the validity of *Ovophis* as arranged by Hoge and Romano-Hoge (1981). Use of the generic name, *Ovophis*, thus should be avoided until more plausible delimitation and morphological redefinitions are made on the basis of comprehensive phylogenetic analyses for *Ovophis* sensu Hoge and Romano-Hoge (1981). Considering the possible close relationships of *monticola* and the node 2 species as suggested in our analyses (Figs. 1 and 2), use of the generic name, *Protobothrops*, though most likely being monophyletic by itself (see above), should be also avoided until the relationships of other species assigned to *Ovophis* by Hoge and Romano-Hoge (1981) are clarified.

It is obvious that the remaining portion of *Trimeresurus* (sensu lato) is yet highly heterogeneous, because it includes at least two distinct lineages, one represented by two strictly terrestrial species (*T. okinavensis* and *T. gracilis*) and the other by several exclusively arboreal species (*T. popeiorum*, *T. albolarbis*, *T. stejnegeri*, *T. puniceus* and *T. trigonocephalus*). More comprehensive analyses incorporating data for the re-
remaining species of *Trimeresurus* (sensu lato), including *T. gramineus* (type species of the genus: McDiarmid et al., 1999) and *T. mangshanensis* (type species of *Ermia*: Zhang, 1993), are strongly desired to elucidate detailed process of divergence of those terrestrial and arboreal species, and to revise their classification at the generic level.

There is a remarkable sequence divergence between *T. monticola monticola* from southern continental China and the two samples of *T. m. makazayazaya*, one from eastern continental China and the other from Taiwan (Fig. 1). Because values of Kimura’s (1980) distance between these supposedly conspecific subspecies (0.055–0.058) are as great as or greater than those between some different species (e.g., 0.043 between *T. mucrosquamatus* and *T. elegans*, 0.035 between *T. tokarensis* and *T. flavoviridis*, and 0.055 between *T. gracilis* and *T. okinavensis*), it is probable that *monticola* and *makazayazaya* actually represent two different species. This suggests the necessity for detailed analyses of geographic variation in the highly polytypic *T. monticola* (McDiarmid et al., 1999; Zhao et al., 1998).

**Biogeography**

It is assumed that in some vertebrate examples, taxonomic diversity in Taiwan has increased through a series of *in situ* divergences facilitated by the large size and diverse geomorphology of this island (e.g., Yu, 1995; Ota, 1997). With respect to *Trimeresurus* (sensu lato), however, the diversity within Taiwan seems to be attributable to the multiple colonizations rather than to the *in situ* divergences, because the four Taiwanese representatives, while being distant from each other genetically, have close relatives outside Taiwan. Toda et al. (1999) analyzed allozyme variation in *Trimeresurus* (sensu lato), exclusive of *T. gracilis* and *T. stejnegeri* from the East Asian islands. Based on the resultant phylogeny, ((*mucrosquamatus*, *elegans*) (*flavoviridis*, *tokarensis*)), they assumed a landbridge dispersal of the genus into the Ryukyu Archipelago and attributed the divergence between the *mucrosquamatus*-*elegans* clade and the *flavoviridis-tokarensis* clade to a vicariance event involved by the initial insularization of the central Ryukyus in the late Pliocene. They also referred to the relatively large genetic distance between those clades (Nei’s [1978] D=0.198–0.289) as evidence for long isolation of the central Ryukyus from the southern Ryukyus and Taiwan.

However, present analyses with the inclusion of *T. gracilis* and *T. jerdonii* besides others that were not included in Toda et al.’s (1999) analyses yielded an essentially different picture for the historical biogeography of the East Asian *Trimeresurus* (sensu lato). First of all, present results suggest that *T. okinavensis* of the central Ryukyus was derived from a lineage distinct from *T. monticola*. The closest affinity of *T. okinavensis* with the geographically disjunct *T. gracilis*, a species endemic to the high altitude area of Taiwan (Ota, 1991), suggests an extremely relictual nature of the *okinavensis-gracilis* clade (Darlington, 1957).

More surprising is the possible sister relationship of the *flavoviridis-tokarensis* clade with *T. jerdonii* from the inland area of continental China, not with the *mucrosquamatus-
elegans clade from the immediate south (Fig. 3) as was postulated by Toda et al. (1999). Such a relationship suggests that T. flavoviridis and T. tokarensis were derived from an ancestral form that dispersed to the Ryukyus independently from the dispersal of the T. mucrosquamatus-elegans clade to this archipelago. This further suggests that the sister lineage of the T. flavoviridis-tokarensis clade, once occurring in the southern Ryukyus, Taiwan and eastern continent, has subsequently disappeared from these broad areas.

There remain four species of Trimeresurus in continental China that have not yet been examined phylogenetically (T. mangshanensis, T. medoensis, T. tibetanus, and T. xiangchagensis: Zhao and Adler, 1993; Zhao et al., 1998). Thus, one may argue that one or more of them may be even closer to the flavoviridis-tokarensis clade than T. jerdonii is. However, even if this is actually the case, it will not lead to any substantial changes in the above-mentioned view on the historical biogeography of T. flavoviridis and T. tokarensis, because geographic ranges of those four species were also confined to far inland of the continent like that of T. jerdonii (Zhao and Adler, 1993; Zhao et al., 1998). It is thus likely that the flavoviridis-tokarensis clade of the central Ryukyus is in a relict state like several other reptiles occurring in this region (Hikida and Ota, 1997; Ota, 1998).

As such, it is likely that the current Trimeresurus assembly of the Ryukyus was derived from three independent dispersals from Taiwan and the continent—one by the common ancestor of T. gracilis and T. okinavensis, another by the common ancestor of T. jerdonii and the T. flavoviridis-tokarensis clade, and the other by the common ancestor of T. mucrosquamatus and T. elegans. Present results also emphasize the importance of examining relevant inland representatives of the continent, if any, for appropriate biogeographical assessment of possibly relict organisms in the central Ryukyus.

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Appendix 1. Specimens examined in this study. Catalogue numbers of voucher specimens are given in parentheses.

*Trimeresurus mucrosquamatus*: Hongya, Sichuan, continental China (uncatalogued specimen); Chishang, Kaohsiung, Taiwan (National Taiwan Normal University [NTNU] B201574).

*T. elegans*: Yaeyama Group, Okinawa, Japan (NTNU B201580).

*T. tokarensis*: Kodakarajima Island, Tokara Group, Kagoshima, Japan (Kyoto University Zoological Collection [KUZ] 21104).

*T. flavoviridis*: Kumejima Island, Okinawa Group, Okinawa, Japan (KUZ 45840).

*T. jerdonii*: Hongya, Sichuan, continental China (uncatalogued specimen).

*T. monticola makazayazaya*: Yangmingshang, Taipei, Taiwan (NTNU B200800; Yizhang, Hunan, continental China (uncatalogued specimen).

*T. m. monticola*: Kunming, Yunnan, continental China (NTNU B201401).

*T. stejnegeri*: Fushan, Ilan, Taiwan (NTNU B201588); Kunming, Yunnan, continental China (uncatalogued specimen).

*T. albolabris*: Foochow, Fujian, continental China (NTNU B201408).

*T. puniceus*: Sumatra, Indonesia (private collection of G. Vogel).

*T. gracilis*: Alishan, Chiayi, Taiwan (uncatalogued specimen).

*T. okinavensis*: Tokunoshima Island, Amami Group, Kagoshima, Japan (KUZ 45871).

*T. popeiorum*: Sakaerat, Thailand (NTNU B200511).


*Tropidolaemus wagleri*: Kuching, Sarawak, Malaysia (NTNU B200512).

*Deinagkistrodon acutus*: Hoping, Taichung, Taiwan (uncatalogued specimen).

*Vipera russelli*: Fanshan, Pintung, Taiwan (NTNU B201587).

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