Molecular Phylogeny and Biogeography of the Grass Lizards Genus *Takydromus* (Reptilia: Lacertidae) of East Asia

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Takydromus Daudin is a group of Lacertidae lizards with slender bodies and long tails. Half of the *Takydromus* spp. are endemic to islands of eastern Asia aligned along the Pacific margin of the East Asian continent. This feature offers a good opportunity to study the effects of glaciations and land connections on the speciation of East Asian fauna. We reconstructed the molecular phylogeny of *Takydromus* species via the mitochondrial 12S rRNA gene. Phylogenetic analyses using maximum-parsimony, neighbor-joining, and maximum-likelihood options do not support a two-subgenera scheme of *Takydromus* and *Platyplacopus* proposed earlier. In contrast, the phylogeny of *Takydromus* species on islands fits the sequential separation of island groups influenced by changes in sea level. The hypothesis in our prediction supports the process of vicariant speciation and multicolonization of grass lizards on eastern Asian islands. At least two obvious colonization events were followed by vicariance events. Because the molecular clock of the 12 rRNA gene was not rejected in our model test, it is possible to estimate times of speciation events. As the most isolated and basal species compared to other temperate and subtropical species of *Takydromus*, the separation period of *T. smaragdinus* in the central Ryukyus is the crucial point in estimating the evolutionary rate. Quaternary-origin or Tertiary-origin models are proposed and discussed.   © 2002 Elsevier Science (USA)

INTRODUCTION

The East Asian grass lizard genus *Takydromus* Daudin in the family Lacertidae is distributed in the Oriental and Palearctic regions with 16 or 17 species currently recognized (Arnold, 1997). *Takydromus* spp. are frequently found in grasslands, but some species prefer dense bush or forest environments (Ziegler *et al.,* 1998; Ziegler and Bischoff, 1999). Most *Takydromus* spp.

have slender bodies and extra-long tails (e.g., with a tail length usually over 300% of snout–vent length (SVL) in *T. sexlineatus,* and nearly 420% of SVL in *T. sauteri*). In some species, the ability to curl their tails helps them climb and move in dense vegetation (Arnold, 1997).

The systematic status of the genus *Takydromus* is controversial traditionally. Early taxonomists split several *Takydromus* spp. into the genera *Apeltonotus* and *Platyplacopus* (Boulenger, 1917). *Apeltonotus,* represented by *T. dorsalis,* was characterized by smaller dorsal scales. Later, the extremely rare *A. sylvaticus* with similar characters was described from Fujian Province, southeastern China (Zhao and Adler, 1993). *Platyplacopus* was characterized by the distinctive digit structure observed on *T. kuehnei,* with lamellae beneath the compressed toes. *T. intermedius* and *Apeltonotus* species were later transferred to *Platyplacopus* based on their differentiated digits (Pope, 1935). However, this character varies considerably among *Platyplacopus* and remaining *Takydromus* species (Arnold, 1997). Arnold (1989) proposed the union of these two genera, but later suggested a revision of *Takydromus* into the subgenera *Takydromus* and *Platyplacopus* based on phylogenetic analyses of morphological characters (Arnold, 1997). The subgenus *Platyplacopus* included *T. sauteri, T. smaragdinus, T. toyamai,* and the former *Platyplacopus* species. The remaining species were placed in the subgenus *Takydromus*.

Despite the controversial phylogeny, one of the most spectacular features of grass lizards is the high percentage of endemism. Half of *Takydromus* spp. are endemic to islands aligned along the Pacific coast of the East Asian continent. These islands include several groups: mainland Japan, the Ryukyu Archipelago, and Taiwan (Fig. 1). *T. tachydromoides* is found on mainland Japan, with *T. smaragdinus* in the Amami and Okinawa group, *T. toyamai* in the Miyako group, and *T. dorsalis* in the Yaeyama group, and four species are endemic to Taiwan. *T. hsuehshanensis* is found only in the Central Mountain Range of Taiwan at over 2000 m. *T. sauteri* and *T. stejnegeri* are distributed on the east-

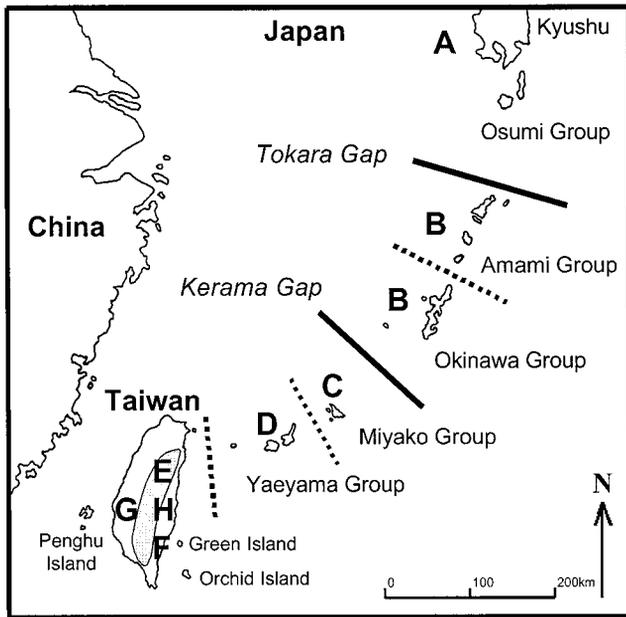1 To whom correspondence should be addressed. E-mail: biofv025@scc.ntnu.edu.tw.

FIG. 1. The distribution of the eight island-endemic *Takydromus* species. (A) *T. tachydromoides* from mainland Japan including Kyushu and the Osumi Group; (B) *T. smaragdinus* from the Amami Group and the Okinawa Group; (C) *T. toyamai* only from the Miyako Group; (D) *T. dorsalis* only from the Yaeyama Group; (E) *T. hsueh-shanensis* from the Central Mountain Range (the shaded area) at elevations higher than 2000 m in Taiwan; (F) *T. sauteri* only in eastern Taiwan including Orchid Island; (G) *T. stejnegeri* only in western Taiwan including the Penghu Archipelago; and (H) *T. formosanus* distributed throughout Taiwan including Green Island, but with greater abundance on the eastern side.

ern and western sides of the mountain, respectively, while *T. formosanus* is distributed throughout the island (Ota, 1998, 2000; Lue *et al.,* 1999).

Multiple geological events of land connections and oceanic isolation in eastern Asia have likely contributed to the diversification and distribution of *Takydromus* spp. on these islands. Located at the margin of the Eurasian, Pacific, and Philippine Sea plates, these islands have experienced tremendous geological alterations since the late Tertiary, e.g., a vertical height elevation in Taiwan caused by the severe interaction between plates (Ho, 1982). Glaciations repetitively altered sea levels and allowed interchange of connection patterns among islands and continental Asia (Kimura, 2000). Connections offered the opportunities for biological dispersal, while submergence of land bridges brought about distributive isolation among related taxa. If this was a major element influencing the formation of the highly endemic fauna of eastern Asian islands, then the phylogenetic relationships of terrestrial taxa including grass lizards should reflect these paleogeographic events. This phenomenon has been observed by UPGMA clustering of herpetofauna among these island groups (Ota, 2000).

In this study, 13 *Takydromus* spp. including all is-

land-endemic species were collected, and the complete mitochondrial 12S rRNA gene was sequenced. We used mtDNA sequence data to (i) construct a phylogenetic relationship of *Takydromus* and test the hypothesis of Arnold's morphological phylogeny and the validity of two subgenera, (ii) evaluate alternative models of the biogeographic history of *Takydromus* on eastern Asian islands, and (iii) discuss general implications for the historical biogeography of Eastern Asia.

MATERIALS AND METHODS

Sample Collection

During 1997–1999, we obtained 13 *Takydromus* species (Table 1). *T. dorsalis* was provided by Professor Hidetoshi Ota (Ryukyu University), and tissues of *T. amurensis* were provided by Dr. Szu-lung Chen (Kyoto University). We collected the remaining 11 species and these are now preserved in the Department of Biology, National Taiwan Normal University. Except for *T. toyamai* and *T. intermedius,* at least two individuals were sequenced in each species. Specimens of *Eremius argus* in our collection were used as the outgroup.

Genomic DNA was extracted from muscle tissue by a standard phenol/chloroform protocol. A complete mitochondrial 12S ribosomal RNA (rRNA) gene and flanking valine tRNA and 16S rRNA genes were amplified as a single fragment with "vertebrate-universal" primers, PL: 5′-AGTCTGCTCAAAAAGATTAATGTTAA-3′ and PH: 5′-TCTTGGTCTGAAACCTCAGTTACCTA-3′, designed by Wang *et al.* (2000). Double-stranded PCRs were performed in 50-μl reaction volumes with the following thermal cycles: 1 cycle at 94°C (3 min); 35 cycles at 94°C (30 s), 55°C (40 s), and 72°C (70 s); and 1 cycle at 72°C (10 min). PCR products were run on 2% low-melt (65°C) NuSieve GTG agarose (BMA BioProducts) in 1 × TAE running buffer. The target fragment was gel-isolated and purified following the protocol for the Viogene PCR fragment purification system (Viogene). The purified PCR products were subsequently used as the template in the direct DNA sequencing reaction. Both strands were sequenced with the Big-Dye Terminator Sequencing Kit (PE Applied Biosystems) with the same primers used for PCR amplification. In addition, two internal primers, PL2: 5′-ACAAACTAGGATTAGATACCC-3′ and PH2: 5′-TATCGATTATAGGACAGGCTCC-3′, were designed to facilitate complete sequencing. Sequencing products were run on an ABI 377 automated sequence analyzer (PE Corp.). The sequences obtained in this study have been submitted to GenBank under accession numbers listed in Table 1.

Sequence Alignment, Secondary Structure, and Phylogenetic Analyses

Sequences were initially assembled with the Pileup option in the GCG package (Wisconsin Package Ver-

TABLE 1

Sample Localities, Sample Sizes, Haplotype Nos., 12S rRNA Lengths, and GenBank Accession Nos. of Sequence Data in Our Analysis

Species	Origin	Sample	Haplotype	12S length	Accession No.
T. amurensis	Seoul City, South Korea	3	3	951–954	AF217044–46
T. dorsalis	Ishigaki Island, Yaeyama Group, Ryukyu Archipelago, Japan	2	2	954	AF217047 AY032596
T. formosanus	Her-ping, Hualien County, Taiwan	1	1	952	AF217048
	Nan-ao, Yilan County, Taiwan	1	1	953	AF217049
	Tian-shyang, Hua-lien County, Taiwan	1	1	951	AF217050
T. hsuehshanensis	Shiueh Mountain, Taiwan	1	1	958	AF217051
	Her-huan Mountain, Taiwan	2	2	958	AF217052–53
	Nan-hwu Mountain, Taiwan	1	1	958	AF217054
T. kuehnei	Muh-jah, Taipei, Taiwan	2	2	955	AF217055–56
	Shin-dian, Taipei, Taiwan	2	2*	955	AF217057–58
T. sauteri	Li-rong Mountain, Taiwan	1	1	949	AF217059
	Man-chou, Pingtung County, Taiwan	1	1*	949	AF217060
	Orchid Island, Taiwan	1	1*	949	AF217061
T. septentrionalis	Matsu Archipelago, Fujian Province, China	4	1	953	AF217062–65
T. sexlineatus	Hongkong, China	3	3	954	AF217066–68
T. smaragdinus	Okinawa Island, Ryukyu Archipelago, Japan	3	3	951–952	AF217069–71
T. stejnegeri	Penghu Island, Penghu, Taiwan	1	1	952	AF217072
	Jyi-jyi, Nantou County, Taiwan	1	1	952	AF217073
	Wang-an Island, Penghu, Taiwan	1	1	952	AF217074
	Shin-dian, Taipei, Taiwan	1	1	952	AF217075
	Nei-pu, Pingtung County, Taiwan	1	1	952	AF217076
T. tachydromoides	Yakushima Island, Japan	3	2	955	AF217077–79
	Kyoto City, Japan	1	1	954	AF217080
T. toyamai	Miyako Island, Miyako Group, Ryukyu Archipelago, Japan	1	1	955	AF217081
T. intermedius	Chengdu, China	1	1	950	AY032597
T. sexlineatus	Sapa, Vietnam Fu, 2000		1	887	AF206589
Eremias argus	Songhua River, Harbin City, China	1	1	949	AF217082
	Jyilin Province, China	1	1	950	AF217083
Gallotia galloli	Fu, 2000		1	899	AF206587
Psammodromus algirus	Fu, 2000		1	884	AF206588
Lacerta media	Fu, 2000		1	875	AF206590
Lacerta (Archaeolacerta) bedriagae	Fu, 2000		1	882	AF206592
Lacerta (Archaeolacerta) monticola	Fu, 2000		1	883	AF206593
Lacerta (Zootoca) vivipara	Fu, 2000		1	884	AF206594
Lacerta (Timon) pater	Fu, 2000		1	886	AF206596
Lacerta (Darevskia) valentini	Fu, 2000		1	886	AF206597
Algyroides fitzingeri	Fu, 2000		1	882	AF206598
Podarcis sicula	Fu, 2000		1	888	AF206599
Lacerta (Teira) perspicillata	Fu, 2000		1	890	AF206602

Note. Sequence data of outgroups are mostly obtained from Fu (2000). The length of outgroup sequence is about 70 bp shorter than that of *Takydromus* because information at the anterior part of 12S rRNA was not completely obtained.

* A repetitive occurrence of a shared haplotype, which is identical to another individual collected from a different sample locality.

sion 10.1, Genetics Computer Group, Madison, WI) and then checked and adjusted to the extent possible to confirm with secondary structure models (Neefs *et al.,* 1993; De Peer *et al.,* 1994; Springer *et al.,* 1995; Hickson *et al.,* 1996; Richards and Moore, 1996). The secondary structure of *Takydromus* 12S rRNA was constructed manually, and loops and stems were determined in all species. To investigate whether transitions and transversions in the *Takydromus* 12S rRNA gene may be saturated, the pairwise substitution rate, defined as numbers of transitions or transversions divided by the length of comparison, was plot-

ted against pairwise Tamura–Nei distances (Tamura and Nei, 1993).

Phylogenetic analyses were performed with PAUP* 4.0b6 (Swofford, 2001). The 12S rRNA sequences of *T. sexlineatus* collected from Vietnam and another nine Eurasian lacertid species (Fu, 2000) were retrieved from GenBank for analyses (Table 1). The loop sequence at the 49th stem (44 bp in length) was excluded from further analyses due to its ambiguous alignment. Maximum-parsimony (MP) analyses were performed with heuristic searches with 100 random additions of sequences to search for the most-parsimonious trees.

TABLE 2

Pairwise Comparison of *Takydromus* Species by Tamura–Nei's Distance

OTUs	SAU	INT	DOR	TAC	AMU	SEP	STE	TOY	HSU	FOR	KUE	SEXHK	SEXVN
SMA	0.0810	0.0929	0.0898	0.0971	0.1099	0.1021	0.1071	0.1100	0.1042	0.1060	0.1262	0.0998	0.1376
SAU		0.0722	0.0726	0.0925	0.0933	0.1080	0.1066	0.1035	0.1011	0.0997	0.1231	0.1017	0.1407
DOR			0.0543	0.1074	0.1007	0.1018	0.0992	0.1069	0.0937	0.0980	0.1345	0.1138	0.1344
TAC				0.1004	0.0904	0.1089	0.0995	0.1013	0.0919	0.0983	0.1201	0.1051	0.1440
AMU					0.1055	0.0969	0.0973	0.0986	0.0901	0.0873	0.1336	0.1244	0.1533
SEP						0.0827	0.0871	0.0964	0.0861	0.0775	0.1446	0.1169	0.1556
STE							0.0236	0.0441	0.0629	0.0601	0.1547	0.1175	0.1571
TOY								0.0385	0.0562	0.0531	0.1446	0.1158	0.1590
HSU									0.0662	0.0750	0.1489	0.1190	0.1617
FOR										0.0389	0.1465	0.1194	0.1556
KUE											0.1311	0.1177	0.1520
SEXHK												0.1059	0.0819
SEXVN													0.1236

Note. SMA, *T. smaragdinus*; SAU, *T. sauteri*; INT, *T. intermedius*; DOR, *T. dorsalis*; TAC, *T. tachydromoides*; AMU, *T. amurensis*; SEP, *T. septentrionalis*; STE, *T. stejnegeri*; TOY, *T. toyamai*; HSU, *T. hsuehshanensis*; FOR, *T. formosanus*; KUE, *T. kuehnei*; SEXHK, *T. sexlineatus* from Hong Kong; SEXVN, *T. sexlineatus* from Vietnam (Fu, 2000).

Bootstrapping with 10,000 pseudo replicates and a heuristic search were used to examine the robustness of clades in the resulting trees. Neighbor-joining (NJ) analysis based on Tamura–Nei distances (Tamura and Nei, 1993) was performed and corrections were made for unequal base frequencies and different transition and transversion rates. The robustness of NJ phylogenies was assessed by the 10,000-bootstrap option. For maximum-likelihood (ML) analysis, the best-fit model of DNA substitution and the parameter estimates used for tree construction were chosen by performing hierarchical likelihood ratio tests (Huelesenbeck and Crandall, 1997; Harris and Crandall, 2000) with PAUP 4.0b6 (Swofford, 2001) and Modeltest 3.0 (Posada and Crandall, 1998). Likelihood-ratio tests indicated that the Tamura–Nei model with the general time-reversible option was the most appropriate for subsequent ML analyses (Table 3). Heuristic ML searches were performed with the fast stepwise-addition, and confidential values were estimated from 10,000 puzzling steps in the ML analysis. In addition, a molecular clock likelihood ratio test (LRT), $2\Delta = \log L_{\mathrm{no\ clock}} - \log L_{\mathrm{clock}}$, which is distributed as χ^2 with $(n-2)$ degrees of freedom where n is the number of sequences (Muse and Weir, 1992), was also performed by TREE-PUZZLE 5.0 (Schmidt *et al.,* 2000) to determine whether there was a statistical difference in evolutionary rates among clades. For testing the alternative hypotheses of vicariant or dispersal speciation models, Kishino–Hasegawa test (Kishino and Hasegawa, 1989) and Templeton test (Templeton, 1983) were preformed in PAUP 4.0b6.

RESULTS

Sequence Characteristics

We obtained 32 haplotypes from the 40 *Takydromus* individuals and 2 haplotypes from *E. argus*. All haplotypes were submitted to GenBank (Table 1). The lengths of *Takydromus* 12S rRNA ranged from 949 (*T. sauteri*) to 958 bp (*T. hsuehshanensis*). Intraspecific length polymorphisms were observed in *T. amurensis, T. tachydromoides, T. smaragdinus,* and *T. formosanus,* with insertions or deletions of 1–3 bp. The 12S rRNA of *E. argus* was 949 or 950 bp in length, with a 1-bp insertion/deletion between the two haplotypes. Intraspecific sequence variation ranged from 0 to 0.57% (*T. stejnegeri*), and interspecific divergence ranged from 2.36% (*T. stejnegeri* vs *T. septentrionalis*) to 15.90% (*T. stejnegeri* vs *T. sexlineatus* from Vietnam) (Table 2). Comparisons between outgroups and ingroups ranged from 11.62% (*Lacerta monticola* vs *T. dorsalis*) to 21.23% (*Psammodromus algirus* vs *T. sexlineatus* from Vietnam).

Sequences of 990 bp for each species were considered for analyses of substitution patterns after the alignment was manually adjusted to the models of 12S rRNA secondary structure in vertebrates (Neefs *et al.,* 1993; De Peer *et al.,* 1994; Springer *et al.,* 1995; Hickson *et al.,* 1996). Among the 990 nucleotides, 475 characters were defined as "stem" regions, and 515 characters were defined as "loops." In stem regions, both transition and transversion rates revealed a nearly linear relationship with genetic distance between species pairs (Fig. 2A). In loop regions, the transversion rate remained linear, but the transition rate became curved when the genetic distance exceeded 0.15 (Fig. 2B), indicating that substitutions in loop regions were saturated at the intergeneric level. The overall sequence (stem + loop) presented linear relationships in transversion but slightly curved in transition when the genetic distance exceed 0.18 (Fig. 2C), indicating that this gene was suitable for intrageneric analysis, but not across genera.
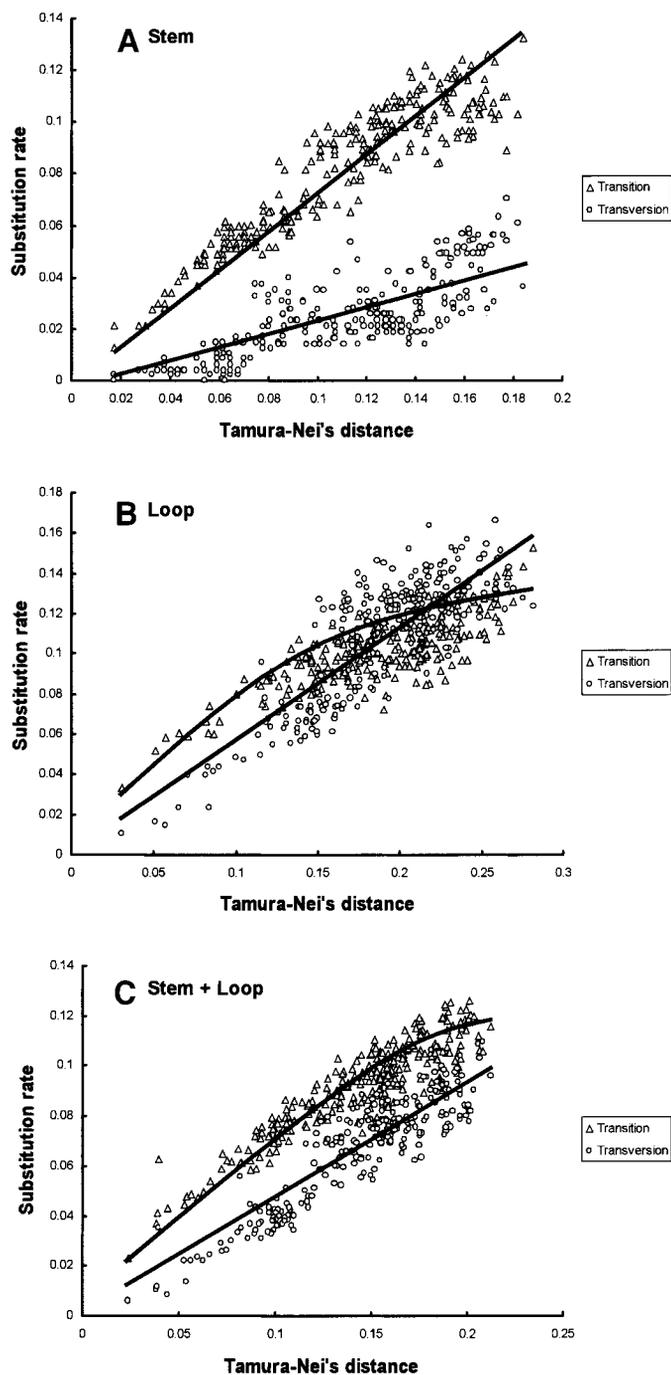
FIG. 2. Relationships between genetic distance and ratio of transitions/transversions. (A) Stem region; (B) loop region; (C) combined data set. Transversions remain linear against the genetic distances for all datasets, but transitions become curved in the loop and the combined dataset at the intergeneric level (genetic distance over 0.15).

Phylogenetic Analyses

Because intraspecific polymorphisms were much lower than interspecific divergences in *Takydromus,* we applied the consensus sequence of the 12S rRNA

gene of each species for phylogenetic analyses. We obtained 14 ingroup OTUs (13 species in our collections plus *T. sexlineatus* from Fu (2000)) and 12 outgroup OTUs (*E. argus* plus 11 species retrieved from Gen-Bank). Ambiguous regions with poor alignment consisting of 44 characters were eliminated from phylogenetic analyses. In the remaining 946 characters, 425 (44.93%) were variable, and 300 (31.72%) of the variable characters were parsimony informative. Parsimony analysis revealed a single MP tree (Fig. 3) with a tree length of 1496, a consistency index (CI) of 0.4519, a homoplasy index (HI) of 0.5481, a retention index (RI) of 0.5036, and a rescaled consistency index of 0.2276. The tree topology strongly supports *Takydromus* as a monophyletic group with 100% bootstrap support. Five internal nodes revealed high topology confidence with bootstrap values greater than 90%; (*T. kuehnei, T. sexlineatus* VN), (*T. intermedius, T. dorsalis*), (*T. hsuehshanensis, T. formosanus*), (*T. toyamai, T. septentrionalis, T. stejnegeri*), and the combination of the last two clades. Three nodes revealed marginal significance (70% < BPs < 90%), including the grouping of (*T. sauteri, (T. dorsalis, T. intermedius*)), and two other internal branches indicating the assemblage of lower groups. Most nodes among outgroup genera revealed bootstrap values lower than 50% (not shown), which might be the major reason for the low CI and RI values. This phenomenon reinforced our previous prediction on the poor resolution ability of the 12S rRNA gene for intergeneric analyses in lacertids.

Although the arrangement of outgroup species differed, neighbor-joining analysis produced an ingroup topology identical to that of the MP tree as shown in Fig. 3. Four nodes retained high bootstrap probabilities (>90%), and five nodes revealed marginal significance (70–90%). The monophyly of *Takydromus* remained 100% supported. ML analysis was based on the results of the Modeltest likelihood ratio test (Table 3), suggesting the application of Tamura–Nei's Model (Tamura and Nei, 1993) and the general time-reversible option. It revealed an ingroup topology nearly identical to those of the MP and NJ trees (-ln likelihood = 7699.27). Most nodes remained fairly well supported with quartet-puzzling steps between 65 and 90%. The only difference was the poor resolution among *T. tachydromoides, T. amurensis,* and the neighboring five-species monophyletic group. This node was later solved in another quartet-puzzling test with 1000 replicates in TREE-PUZZLE 5.0. *T. amurensis* was clustered with the monophyletic five-species group in this test (74%), showing the relationship of (*T. tachydromoides,* (*T. amurensis,* five species)). This result was identical to the previous MP and NJ tree topologies on this node.

According to the consistency of different tree criteria, we used this gene tree to stand for the possible speciation tree. Thirteen nodes (from a to m) and three major species groups were defined for further discussion (Fig.
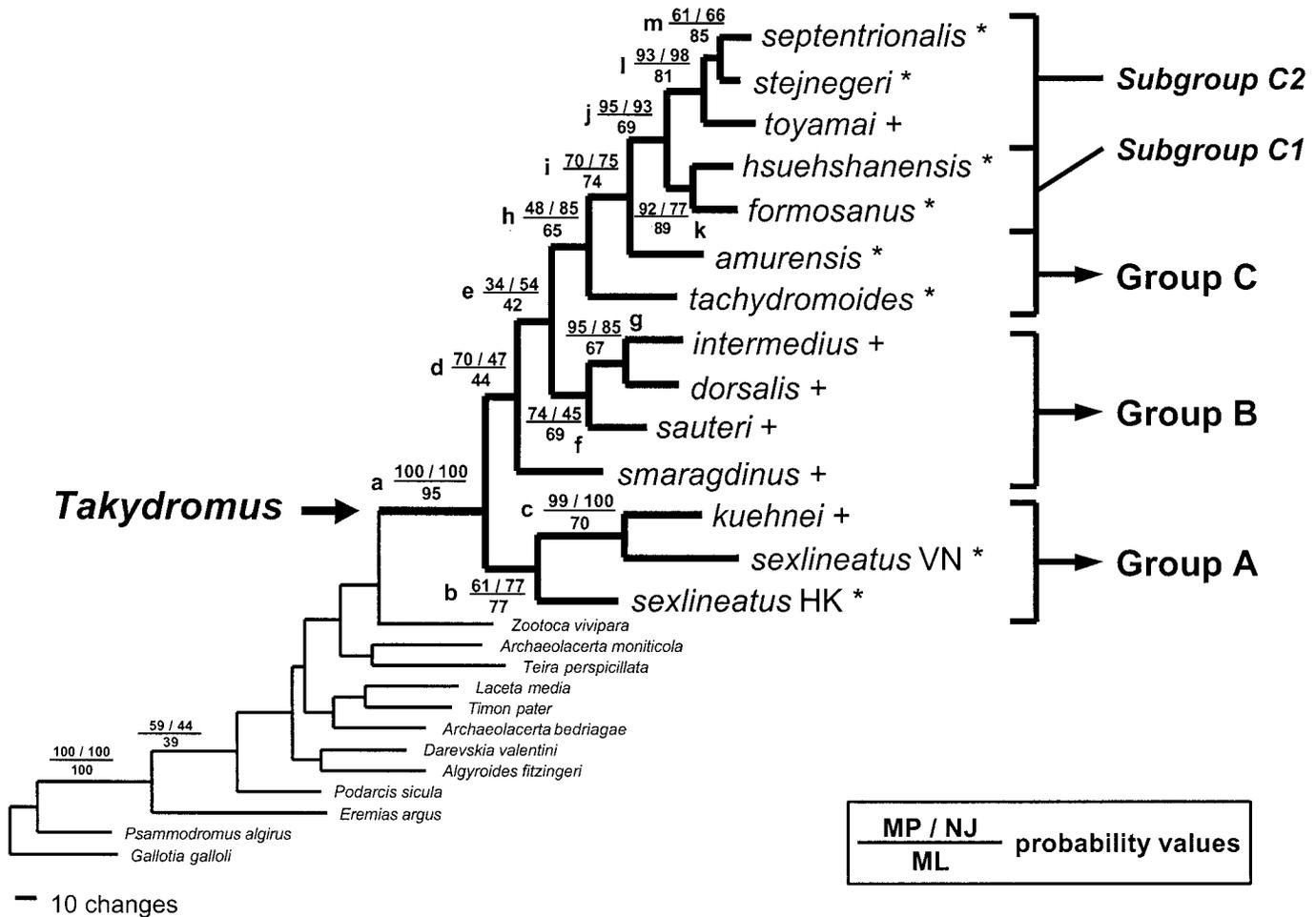
FIG. 3. Phylogenetic analyses of the mitochondrial 12S rRNA gene of *Takydromous*. Shown is the tree inferred by the maximum-parsimony (MP) method. Identical ingroup topologies were obtained from the neighbor-joining (NJ) and maximum-likelihood (ML) methods. "+" and "*" denote the division of the subgenera of *Platyplacopus* and *Takydromus* by Arnold (1997), respectively. We defined three major groups and 13 nodes (from a to m) to facilitate discussion. Probability percentages of bootstrap replicates (MP and NJ trees) and quartet puzzling analysis replicates (ML tree) supporting each branching pattern are given beside the corresponding nodes.

3). Group A contained *T. kuehnei* and *T. sexlineatus*, Group B contained *T. smaragdinus, T. sauteri, T. dorsalis,* and *T. intermedius,* and Group C contained the remaining species with two subgroups further defined. Subgroup C1 contained *T. hsuehshanensis* and *T. formosanus,* and Subgroup C2 contained *T. septentrionalis, T. stejnegeri,* and *T. toyamai.* It was controversial whether we should include *T. smaragdinus* in Group B since Groups A and C were monophyletic but Group B was not. However, we preferred this grouping based on the followed reasons. First, *T. smaragdinus* is the most basal and ancient species compared to other Group B and C species. According to our deduction (to be described below), Group B is obviously an earlier-evolved clade compared to Group C. Under such a situation, we think it is reasonable to connect *T. smaragdinus* to the serial events of Group B speciation. Second, although the tree topology indicated a (*T. smaragdinus,* (Group B, Group C)) relationship, the bootstrap values on re-

lated nodes ("d" and "e") are less supported. These bootstrap values are lower because of the existence of another possible relationship, a ((*T. smaragdinus,* Group B), Group C) relationship. The probability of clustering *T. smaragdinus* to Group C species is comparatively much lower. Such relationships, while not shown in the decisive tree topology, indicate a much closer relationship of *T. smaragdinus* to Group B species than to Group C species.

Molecular Clock Test

To apply the 12S tree topology to an assessment of speciation events of *Takydromus* on islands of eastern Asia, a maximum-likelihood ratio test was performed to examine the molecular clock hypothesis for Groups B and C. We excluded Group A species in this analysis because (1) *T. sexlineatus* from Vietnam is a long-branch species and (2), being distributed mainly in southern Asia, they are not directly related to specia-

TABLE 3

Test of the Hypotheses Relating to the Models of Evolution Appropriate for Phylogeny Reconstruction (Huelsenbeck and Crandall, 1997)

Null hypothesis	Null Model	Alternative Model	$-\ln L_0$	$-\ln L_1$	df	P
Equal base frequencies	H_0: JC	H_1: F81[a]	8951.49	8898.10	3	<0.000001
Transition = transversion	H_0: F81	H_1: HKY[b]	8898.10	8610.31	1	<0.000001
Equal transition rates	H_0: HKY	H_1: TrN[c]	8610.31	8585.10	1	<0.000001
Equal transversion rates	H_0: TrN	H_1: TIM[d]	8585.10	8582.94	1	0.037488
Equal rates among sites	H_0: TrN	H_1: TrNG[e]	8585.10	7695.25	1	<0.000001
No invariable sites	H_0: TrNG	H_1: <u>TrNIG</u>[f]	7695.25	7687.40	1	0.000075
*Molecular clock likelihood ratio test	H_0: <u>with clock</u>	H_1: without clock	3556.12	3561.78	10	0.3331

Note. The underlined model indicate the final suggestion.

* Likelihood ratio test (Muse and Weir, 1992) for examining whether a molecular clock exists among ingroup species. Only *Takydromus* species (but excluding *T. kuehnei* and *T. sexlineatus* from Vietnam) joined the latter analysis.

[a] Unequal base frequencies: G = 0.1232, A = 0.3895, T = 0.2604, C = 0.2269.

[b] Unequal ratio between transition and transversion.

[c] Unequal transition rates: R[A-G] = 4.7564, R[C-T] = 6.5449.

[d] Equal transversion rates: R[A-C] = [A-T] = [C-G] = [G-T] = 1.0000.

[e] Gamma distribution shape parameter: G = 0.6150.

[f] Proportion of invariable sites: I = 0.3757.

tion events on eastern Asian islands. A critical significance level of 33.31% was obtained in this test (Table 3). Because the simpler (clocklike) tree cannot be rejected at a significance level of 5%, we do not reject the application of a molecular clock to Groups B and C.

DISCUSSION

Molecular Phylogeny of Takydromus

Our data do not support the division of *Takydromus* into the subgenera *Platyplacopus* and *Takydromus* (Arnold, 1997). The bootstrap values clustering this genus (node a in Fig. 3) are 100% in both the MP and the NJ trees and 95% in the ML quartet-puzzling test. Species of the subgenus *Platyplacopus* and the subgenus *Takydromus* (denoted as "+" and "*" in Fig. 3, respectively) were mixed in the phylogenetic tree. The highly probable clustering of (*T. toyamai, T. stejnegeri, T. septentrionalis*) revealed an assemblage of the subgenera *Takydromus* and *Platyplacopus* species while the clustering of the (*T. kuehnei, T. sexlineatus*) group revealed another. Because neither of these subgenera was monophyletic based on molecular phylogeny, we are not convinced of the subgeneric definitions.

The phylogenetic status of *T. sexlineatus* is worthy of note. The DNA sequence of a single *T. sexlineatus* specimen collected from Vietnam (Fu, 2000) was clustered with *T. kuehnei* (Taiwan) with very high bootstrap values (100% in NJ, 99% in the MP tree) and the same species collected from Hong Kong revealed an unexpected high genetic distance (0.1236) to the Vietnam specimen (Table 2). This value is much higher than most of the other interspecific pairings in this genus. Even for the two tightly clustered species of (*T. kuehnei* and *T. sexlineatus* VN), the genetic distance is

still quite high (0.0819). Apparently these organisms belong to distinctly different species, and one of them either is misidentified or belongs to a new species. *T. sexlineatus* is the most problematic *Takydromus* species with the widest distribution throughout southern China, Vietnam, Laos, Cambodia, Thailand, Burma, Assam, the northern Malay peninsula, Borneo, Java, and Sumatra (Arnold, 1997). Two subspecies are recognized: *T. s. sexlineatus* Daudin 1802 and *T. s. ocellatus* Guerin-Meneville 1829. Specimens from Vietnam and southern China are usually regarded as *T. s. ocellatus,* and this subspecific name was still applied recently both in Hong Kong and in Vietnam faunal lists (Karsen *et al.,* 1986; Bogadek and Lau, 1997; Ziegler *et al.,* 1998, 1999). However, the type locality of this subspecies (described as *T. ocellatus*) was lost and controversial and considered to be either from southern China or from Vietnam (Zhao and Adler, 1993). Such a situation causes confusion in distinguishing *Takydromus* species in both regions.

Tropical *Takydromus* species require further investigations. *T. wolteri* has been reported from Vietnam, but is usually regarded a Palearctic species; the occurrence of this species in Vietnam is doubtful (Ziegler *et al.,* 1998, 1999). Instead, a new subspecies, *T. kuehnei vietnamensis* (Ziegler and Bischoff, 1999), and a new species, *T. hani* (Chou *et al.,* 2001), were recently described in Vietnam. Fu's specimen is probably a misidentification of one of these two species, or perhaps one of our specimens belongs to another cryptic species of *T. sexlineatus*. These findings suggest that the biodiversity of *Takydromus* fauna is probably more diverse than indicated by our present observations. We suspect that more cryptic *Takydromus* species in tropical regions will be discovered as additional field studies or
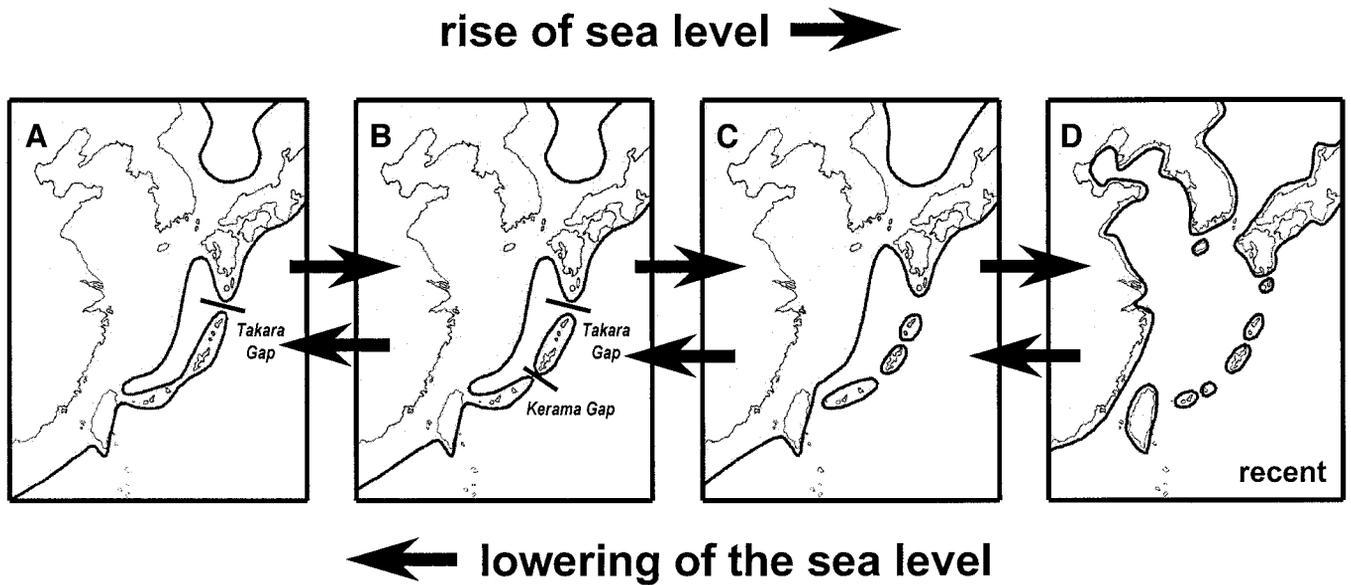
rise of sea level ➡

⬅ lowering of the sea level

FIG. 4. Simplified paleogeographic maps of the East China Sea and islands of eastern Asia, modified from Toda *et al.* (1999) and Kimura (2000). The connection patterns of these island groups are influenced by the sea level alterations due to glaciations. The arrows in both directions indicate the fluctuating and reversible relationships between the scenarios.

molecular studies are performed, especially for those widely distributed or morphologically variable species.

Biogeography of Takydromus on East Asian Islands

Most *Takydromus* biodiversity occurs on islands of eastern Asia. Located at the margin between the Eurasian and the Philippine Sea plates, these islands have experienced tremendous geological alterations since their formation around 10 million years ago. Sea level alteration caused by glaciations changed the pattern of connections among island groups. The process of alteration in land connections could be either deduced from the offshore topography or referred from paleogeographic models (e.g., Kimura, 2000). According to recent information, most offshore regions under the East China Sea would dry up during glaciations, causing the appearance of a larger continental land area (Fig. 4C). In some cases, more severe glaciations would provide the opportunity for a connection between the Southern Ryukyus (including the Miyako Group and the Yaeyama Group) and the continent of Asia via the land area extended from northeastern Taiwan (Fig. 4B). In the most severe glaciations, as the most rare geological cases, the Central Ryukyus would connect to the Asian continent through a peninsular-like land bridge (Fig. 4A). When sea levels rose again, this peninsula would sequentially break up. The Okinawa–Amami Group was isolated first, then the Miyako–Yaeyama Group, and finally the division between the Okinawa and the Amami Groups, between the Miyako and the Yaeyama Groups, and between Taiwan and mainland Asia (Figs. 4A–4D). Such repetitive and reversible processes must

have been repeated several times since the formation of these islands in the Miocene.

In most cases, terrestrial animal species with poor oversea dispersal abilities could only disperse by dry lands. The peninsular-like land bridge during glaciations offered an excellent but the only opportunity to disperse and colonize from mainland Asia to the Ryukyus. However, when the glaciations ended, oceanic isolation would occur, and vicariant events began. If vicariant speciation is the major mechanism for the formation of island-endemic species in eastern Asia, the biogeography of terrestrial species should be tightly related to the sequential separation of island groups. As an idealized model, the terrestrial phylogeny should reveal a relationship of ((Amami, Okinawa), ((Miyako, Yaeyama), (Taiwan, China))), reflecting the sequential vicariant process. An UPGMA clustering of the similarity index for reptile fauna on these islands showed the existence of this tendency (Ota, 2000). Taxonomic studies for certain reptile lineages also suggested the affinity between closely related island groups (Hikida and Ota, 1997; Hikida and Motokawa, 1999).

The phylogeny of *Takydromus* species on these islands indeed supports this evolutionary model: a vicariant speciation process was observed in Group B and Group C2 species. Group B species revealed the relationship of (*T. smaragdinus*, (*T. sauteri*, (*T. dorsalis, T. intermedius*))). The hypothetical model for the speciation process is proposed in Fig. 5. *T. smaragdinus,* endemic to the Okinawa–Amami Group, is the
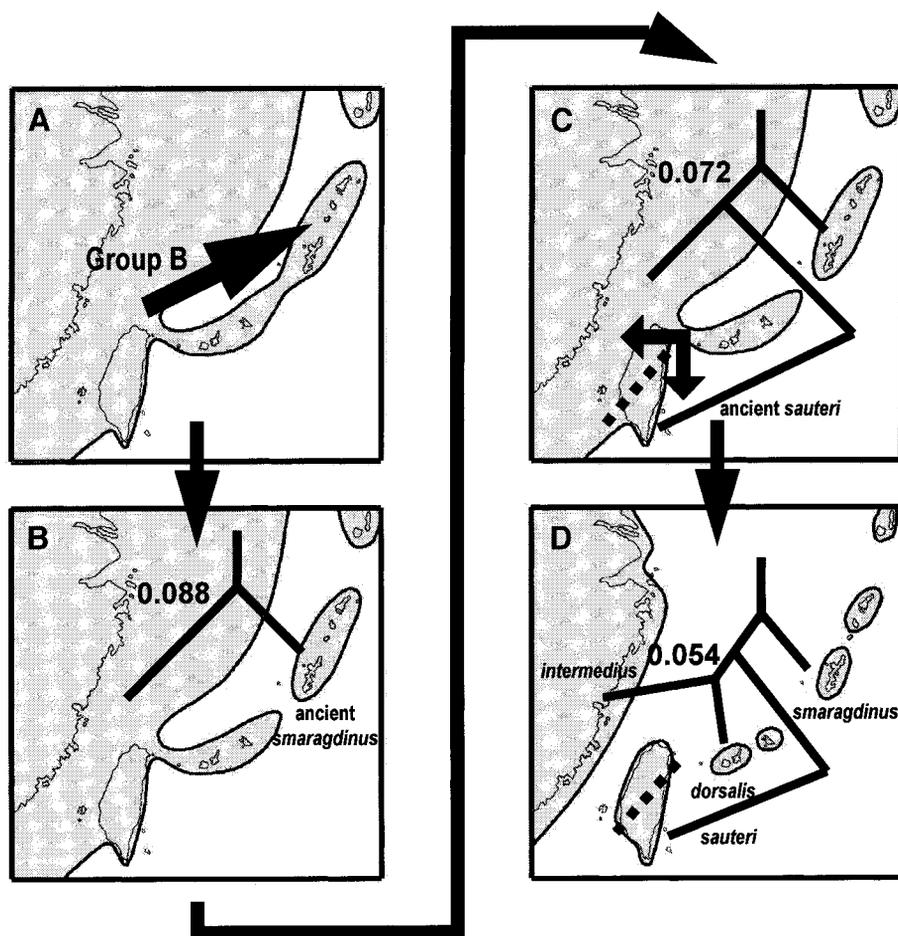
FIG. 5. Hypothesis on the speciation process of Group B species. (A) *Takydromus* dispersed through a land connection; (B) separation of the Okinawa Group isolated ancient *T. smaragdinus*; (C) either a vicariance or a dispersal model could be applied to the speciation of *T. sauteri* isolated by the Central Mountain Range (the dashed line); (D) a final separation caused the allopatric speciation between *T. dorsalis* and *T. intermedius*. The values beside the nodes reveal the interspecific genetic distances between branches, which offered the clues on timing these speciation events.

most basal and ancient species of Groups B and C, but with a closer phylogenetic relationship to Group B species. It is now distributed only on the farthest and most isolated islands. We deduced that ancient *Takydromus* species reached their modern distributions in Okinawa through a land connection (Fig. 5A). When the land bridge submerged, the Okinawa–Amami Group was isolated and caused the separation between ancient *T. smaragdinus* and other species (node d in Fig. 3; Fig. 5B). Mapping the distribution of Group B species reveals the biogeographical relationship of (Okinawa, (Taiwan, (Yaeyama, China))). In our hypotheses, we proposed the speciation of *T. sauteri* in eastern Taiwan as an independent allopatric speciation event, which caused a mismatch in the biogeographic model. The Central Mountain Range in Taiwan (the dashed line in Fig. 5) formed a major geographic barrier between this species and the others. It could be either a vicariant event (uplift of the mountains caused the isolation) or a dispersal event (lizards crossed the barrier and entered eastern Taiwan) (Fig.

5C), depending on which event (speciation versus geological alteration) occurred earlier. The remaining species would follow the relationship of (Okinawa, (Yaeyama, China)) which shows a partial match to the idealized phylogenetic relationships.

The phylogeny of Group C2 species (*T. toyamai,* (*T. stejnegeri, T. septentrionalis*)) also fits the vicariant hypothesis reflecting the biogeographic relationship of (Miyako, (Taiwan, China)) (Fig. 6). Speciation processes following the sequential breakup of land connections were also observed in this clade. Here we noticed that the interspecific genetic distance among C2 species range between 0.024 and 0.041, only half of that among Group B species (0.054–0.088). If species in these two groups evolved at similar rates, the speciation of C2 species must have occurred later than that of Group B species. In other words, the colonization, separations, and speciation of Group C2 species should have been caused by later geological events. Such a multicolonization hypothesis has been proposed in population genetic studies on the Indian rice frog (*Rana*
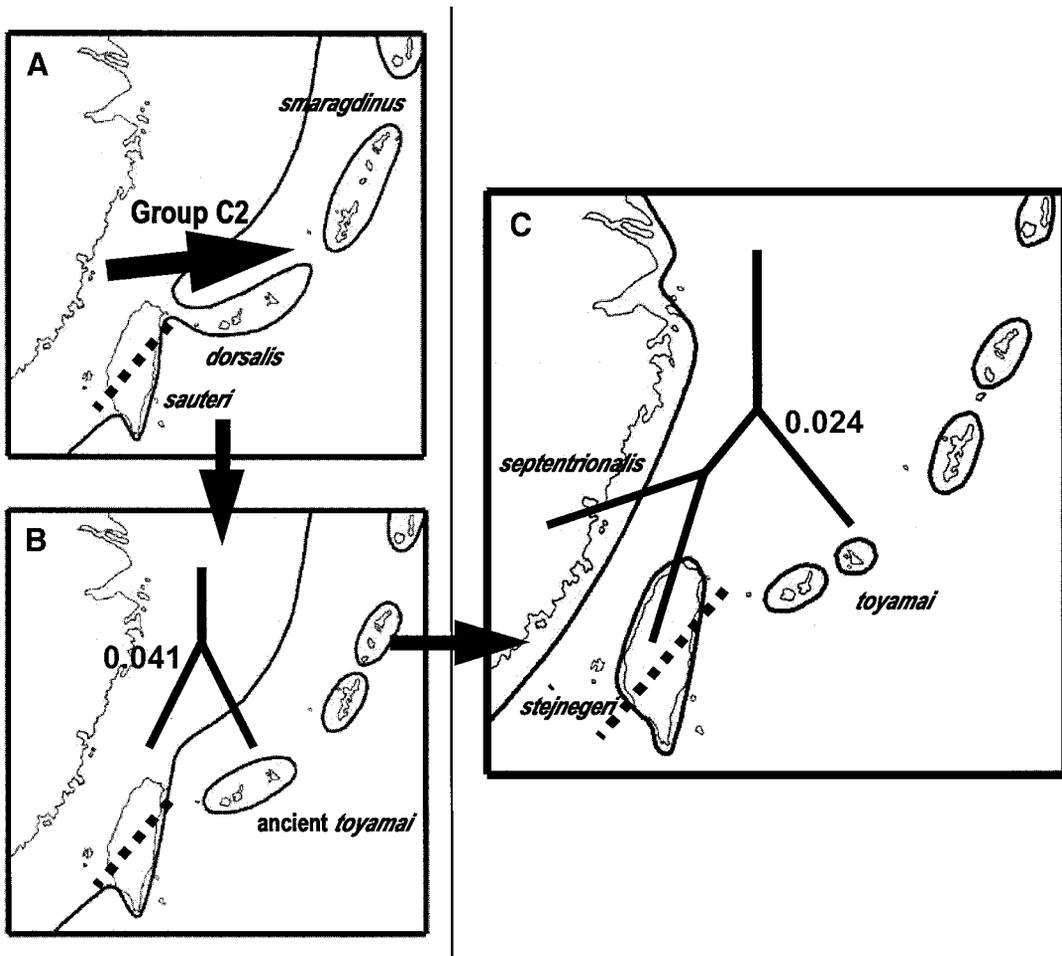
FIG. 6. Hypothesis on the speciation process of Group C2 species. (A) *Takydromus* entered the Miyako Group in a later time period when Group B species were already present; (B) separation between ancient *T. toyamai* and remaining taxa; (C) separation between *T. stejnegeri* and *T. septentrionalis*. Genetic distances between branches are shown. Under our multicolonization hypothesis, Group B species should have existed and occupied localities similar to modern distributions when Group C2 species arrived (A).

limnocharis) distributed on East Asian islands (Toda *et al.*, 1997, 1998; Toda, 1999).

Two proposals account for the absence of Group C2 in the Okinawa–Amami Group. First, the connection of continental Asia to Central Ryukyus is a very uncommon geological event; thus, Group C species have never obtained opportunities to reach this region. Second, niche competition may exist among older and younger *Takydromus* lineages. It is worthy of notice that different *Takydromus* species never coexist in the islands of Ryukyus. Principles of island biogeography (MacArthur and Wilson, 1966) could be applied to explain those "lost lineages," e.g., the lack of Group C species in the Yaeyama Group or the lack of Group B species in the Miyako Group and western Taiwan. Competition might have caused frequent extinction events and the coexistence of *Takydromus* species on these islands because of their smaller terrestrial surfaces. The effects of niche competition must be extremely severe on tiny islets like those of the Miyako or the Yaeyama Groups.

Another example of allopatric speciation in north-

eastern Asia explains the speciation of *T. tachydromoides* from other Group C species. It is reasonable to deduce that the oceanic separation between Japan and Korea led to the isolation and speciation of *T. tachydromoides*. The mean genetic distance between *T. tachydromoides* and other Group C species is 0.096, very similar to the genetic distance between *T. smaragdinus* and other species (0.100). We infer that these two species were isolated in a similar geological period. This radiation pattern offers another explanation for the comparatively low bootstrap values on nodes d, e, and h, which revealed poor resolution for solving the relationship among *T. smaragdinus*, *T. tachydromoides,* and other species.

Instead of vicariance, speciation in the Ryukyus may be caused by dispersals. For example, skinks of the genus *Eumeces* in eastern Asia, with a probably better ability of oversea dispersal, revealed the hypothetical speciation mechanisms of both vicariance and dispersals (Hikida, 1993; Hikida and Motokawa, 1999). If dispersals have occurred in the *Takydromus* lineages, the

phylogenetic relationship of Group B should be (*T. intermedius*, (*T. sauteri*, (*T. dorsalis, T. smaragdinus*))), and for Group C2 the relationship should be (*T. septentrionalis*, (*T. stejnegeri, T. toyamai*)). However, a dispersal speciation model on the islands of eastern Asia was rejected by both the Kishino–Hasegawa test ($P = 0.0038$) and the Templeton–Wilcoxon signed-rank test ($P = 0.0066$), with the tree length of the alternative phylogenetic tree being 15 steps longer than that of the original MP tree. We deduced that vicariance should be the main mechanism for the speciation of island *Takydromus*.

Although time estimation by molecular clocks is controversial, estimating the approximate time of divergence can provide a crude pattern to help us understand the formation process of the fauna on the islands of eastern Asia. Taxon-specific clocks are usually reliable in local time, local range, or specific taxonomic levels. Because the model test on temperate and subtropical *Takydromus* species did not reject the molecular clock (Table 3), we assume that they evolved at a similar rate. The crucial point in this estimation is the separation of *T. smaragdinus,* the most isolated species basal to Group B and C members. If we could obtain the timing of the separation between *T. smaragdinus* and other species, the age of each node on the phylogenetic tree could be deduced.

Paleogeography of eastern Asian islands in the Pleistocene has been proposed by Kizaki and Oshiro (1980) and Kimura (2000). According to their hypotheses, the last connection of Okinawa Island to mainland Asia was in the early Pleistocene, at around 1.0–1.3 MYA. Since the possibility of oversea dispersal is excluded in island *Takydromus,* this should be the lower limit of the time scale for *T. smaragdinus* to separate from other species. The genetic distances between *T. smaragdinus* and other temperate and subtropical species range between 0.081 and 0.110 (Table 2), with a mean of 0.100. Therefore, the evolutionary rate of *Takydromus* 12S rRNA gene is about 0.100/MY between pairs of lineages. The interspecific genetic distances among Group B species range between 0.088 (*T. smaragdinus* vs other species) and 0.054 (*T. dorsalis* vs *T. intermedius*) (Fig. 5). Under the estimation of the molecular clock, we could restrict the speciation time of Group B species to 0.88–0.54 MYA. Similarly, the genetic distances among Group C2 species range between 0.041 (*T. toyamai* vs other species) and 0.024 (*T. stejnegeri* vs *T. septentrionalis*) (Fig. 6), indicating the speciation time of Group C2 from 0.41 to 0.24 MYA. According to Kimura's hypothesis, the latter period is close to another severe sea level change that occurred in the late Pleistocene. Estimation of the speciation time scale reinforces our multicolonization hypothesis: colonization occurred at least twice, and two series of vicariant events have occurred in the evolutionary history of *Takydromus*. Under this hypothesis, it is obvious that

Group B species should have existed and occupied localities similar to modern distributions when Group C2 species arrived (Fig. 6A).

However, if the time scale employed from the above estimation is corrected, the evolutionary rate of *Takydromus* 12S rRNA gene (0.1/MY/pair) is approximately five times higher than the common evolutionary rate of vertebrate mitochondrial genomes (0.02/MY/pair) (Brown *et al.,* 1979; Avise, 2000). A possible explanation for this is the acceleration in the evolutionary rate for these lizards, since Squamata mitochondrial genes have been found to evolve at various rates in different taxonomic units according to our recent comparisons among several local organisms (S.-M. Lin, unpublished data). Nevertheless, the evolutionary rate of the Lacertidae 12S rRNA gene could be crudely estimated by referring to the researches on the biogeography of this family (Fu, 1998; Harris *et al.,* 1998). According to their inferences on the branching process of lacertid lineages, an evolutionary rate of 0.01–0.03/MY/pair could be obtained. Inference on the biogeography of varanid lizards (Reptilia: Varanidae) offered a similar but even slower evolutionary rate for this gene (Fuller *et al.,* 1998). Under such circumstance, the unexpected rate bias of the *Takydromus* 12S rRNA gene seems to be less probable.

The alternative explanation indicates that *T. smaragdinus* should have a separation time scale longer than 1.0–1.3 MY. Instead of the previous Quaternary-origin model, a Tertiary-origin model seems to be more reasonable. Based on the highly divergent fauna and the deep branch length obtained from molecular evidence from several endemic organisms in Okinawa (Takeda and Ota, 1996; Toda *et al.,* 1999), Hikida and Ota (1997) inferred a longer isolation time scale of the Okinawa–Amami Group. Their hypothesis assumed that the early Pleistocene land bridge did not connect to the Okinawa Group; thus, these islands had been completely isolated since the early Pliocene (Hikida and Ota, 1997; Hikida and Motokawa, 1999). Based on this model, the isolation of *T. smaragdinus* is about 8 MY. This separation time scale implies a 0.0125/MY/pair evolutionary rate in *Takydromus,* closer to the previous estimations on Squamata 12S rRNA genes. Under the estimation of this hypothesis, the speciation events of Group B species occurred from 7.0 to 4.3 MYA, while Group C2 species evolved during 3.3 to 1.9 MYA. The colonization events and serial vicariant speciation were still observed to have occurred twice. Our multicolonization hypothesis is still supported in this model, but their speciation age is advanced to the Tertiary. This time scale is also supported by the research on other endemic vertebrates in the Central Ryukyus, such as vipers or rodents (Toda *et al.* 1999; Suzuki *et al.,* 2000).

Another possibility that did not reject the Tertiary-origin model is that the later geological events did not have a significant influence on the already-existing

species. Island *Takydromus* species may have evolved and existed at their modern positions since the Tertiary, with later glaciations making few alterations in their current distributions. Although hypothetical intraspecific competition and extinction may have occurred during their secondary contact, it did not influence the major distribution pattern of *Takydromus* species in the eastern Asian islands.

Applications of different models provide different explanations for the speciation of *T. sauteri*. The Central Mountain Range in Taiwan is the main barrier isolating this species. The mean genetic distance between *T. sauteri* and its sister taxa is 0.072, with a 0.72-MY separation age in the Quaternary-origin model and a 5.8-MY age in the Tertiary-origin model. The sudden rise in elevation of the Central Mountain region is deduced to begin during the late Miocene to the early Pliocene (Ho, 1982), which is quite close to the second estimation. If speciation of *Takydromus* follows the Quaternary-origin model, it would be a dispersal event for ancestral *T. sauteri* because the mountains had existed long before its speciation. If the Tertiary-origin model applies, it would be a vicariant event because the timing of *T. sauteri* separated from other species fits well with the tremendous geological alterations. This phenomenon should not just be coincident.

In conclusion, the purpose of this paper is to try to resolve the phylogeny of eastern Asian *Takydromus* and propose a hypothetical speciation process of this taxon deduced from geological inferences. Vicariant speciation events were deduced according to the match between molecular phylogeny and paleogeography, which is influenced by the alterations in sea levels during glaciations. Because different *Takydromus* lineages reveal different scales for their interspecific genetic distances, a multicolonization hypothesis was proposed under the application of a molecular clock. Alternative models for timing the occurrence of speciation events were discussed and the Tertiary-origin model seems to be more probable than the Quaternary-origin model. We do not expect simple models to resolve the complete course of speciation events that occurred on these islands, but to provide a crude and reasonable scenario to explain the richness of faunal endemism in this region. The speciation process and phylogeography of organisms on eastern Asian Islands are highlighted, especially for comparisons between different taxonomic units.

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