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ORIGINAL ARTICLE

A time-calibrated phylogenetic approach to assessing the phylogeography, colonization history and phenotypic evolution of snakes in the Japanese Izu Islands

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ABSTRACT

Aim We infer the biogeography and colonization history of a dispersal-limited terrestrial vertebrate, the Japanese four-lined ratsnake (*Elaphe quadrivirgata*), to reveal the number of times mainland populations have invaded the Izu Archipelago of Japan, the mainland sources of these colonists, and the time-scale of colonization. We compare these results with those of past studies in an attempt to uncover general biogeographical patterns. Moreover, we briefly examine the significance of colonization history when evaluating the evolution of body size and melanism of the Izu Island *E. quadrivirgata* populations.

Location The Izu Islands (Oshima, Toshima, Niijima, Shikine, Kozu, Tadanae and Mikura), a volcanic archipelago off the Pacific coast of central Japan.

Methods We obtained DNA sequences of the mitochondrial cytochrome *b* gene (1117 base pairs) from 373 individual snakes sampled from seven of the Izu Islands and 25 mainland localities. We employed partitioned Bayesian phylogenetic analyses assuming a relaxed molecular clock to estimate phylogenetic relationships among extant haplotypes and to give an explicit temporal scale to the timing of clade divergence, colonization history and tempo of body-size evolution. Moreover, we employed model-based biogeographical analysis to calculate the minimum number of times *E. quadrivirgata* colonized the Izu Islands.

Results We found evidence that three separate regions of the Izu Archipelago have been colonized independently from mainland ancestors within the past 0.58–0.20 Ma. The Izu Peninsula plus Oshima and Mikura were both colonized independently from lineages inhabiting eastern mainland Japan. The Toshima, Niijima, Shikine, Kozu and Tadanae populations all derive from a single colonization from western mainland Japan. Oshima has been subject to three or possibly four colonizations.

Main conclusions These results support the hypothesis that the extreme body-size disparity among island populations of this ratsnake evolved *in situ*. Moreover, the fact that the dwarf, melanistic population inhabiting Oshima descends from multiple mainland colonization events is evidence of an extremely strong natural selection pressure resulting in the rapid evolution of this unique morphology. These results contrast with theoretical predictions that natural selection pressures should play a decreased role on islands close to the mainland and/or subject to frequent or recent immigration.

Keywords

Bayesian phylogeny, Colubridae, *Elaphe quadrivirgata*, island biogeography, Izu Islands, LAGRANGE, multiple colonization, ratsnakes, relaxed molecular clock, reptiles.

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INTRODUCTION

Oceanic island systems have proven to be some of the most important 'natural laboratories' of ecological and evolutionary biology research (e.g. Gillespie & Roderick, 2002; Losos & Ricklefs, 2009). Because oceanic islands form with no connection to the mainland and are colonized only by limited dispersal, they serve as 'blank slates' on which we can observe the varied outcome of ecological and evolutionary processes. Moreover, geological information and the recent development of molecular dating techniques have permitted the study of these systems within a temporal framework. Much of this research has focused on the Galápagos (Grant & Grant, 2008; Parent *et al.*, 2008), Hawaiian (Shaw, 1995; Gillespie, 2004), and Canary (Emerson *et al.*, 2000; Emerson, 2002) islands. This focus on extremely remote, isolated archipelagos suggests that island-based evolutionary phenomena are most important only in the most isolated systems. However, equally important evolutionary processes under varying influences of isolation, dispersal and natural selection occur on islands in closer proximity to mainland (e.g. Camin & Ehrlich, 1958; King & Lawson, 1995, Whittaker & Fernández-Palacios, 2007).

The Izu Archipelago is a potentially important system in which to study such ecological and evolutionary processes. The Izu Archipelago is a chain of volcanic islands off the coast of eastern Japan (Fig. 1), which were probably formed within the past million years (Kaneoka *et al.*, 1970). These are oceanic islands formed *de novo* without any previous connection to the mainland. Thus, their inhabitants must have colonized via overseas dispersal from the mainland or adjacent islands. However, unlike remote oceanic islands such as the Hawaiian and Galápagos islands, the Izu Islands' close geographical proximity to mainland source populations implies relatively frequent colonization, which makes this system especially valuable for exploring ecological and evolutionary processes from a different perspective.

The flora and fauna of the Izu Islands have been the subjects of numerous ecological and evolutionary studies (birds: Higuchi, 1976; rodents: Takada *et al.*, 2006; Kageyama *et al.*, 2009; reptiles: Hasegawa & Moriguchi, 1989; Hasegawa, 1990,

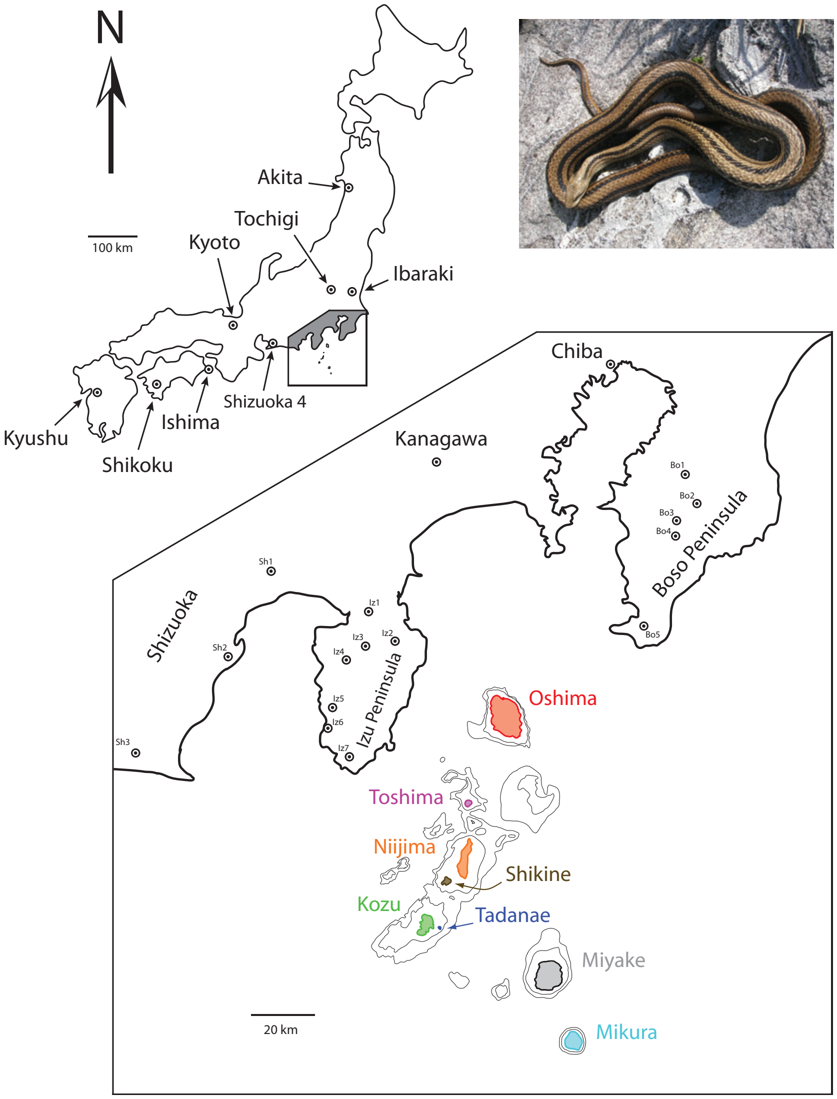

Figure 1 Sampling localities of *Elaphe quadrivirgata* populations used in this study. Bathymetric lines around the Izu Islands represent 100 and 200 m depths (only depths in the immediate vicinity of the Izu Islands are shown). Precise collecting localities are provided in Appendix S1. *Elaphe quadrivirgata* does not inhabit Miyake Island (light grey). The photograph depicts an adult of *E. quadrivirgata*.

1994, 1999, 2003; predator–prey interactions: Mori & Hasegawa, 1999; plant–animal interactions: Inoue & Amano, 1986; Abe *et al.*, 2006), but a vast majority of such studies, while informative, were conducted in the absence of a phylogeographical framework. To date, the existing phylogeographical literature on the Izu Islands is limited to plants (*Campanula punctata*: Inoue & Kawahara, 1990; *C. microdonta*: Oiki *et al.*, 2001), snails (*Euhadra*: Hayashi & Chiba, 2004), and rodents (*Apodemus speciosus*: Suzuki *et al.*, 2004; Tomozawa & Suzuki, 2008). Inoue & Kawahara (1990) and Oiki *et al.* (2001) determined that *Campanula* species colonized the entire Izu Archipelago only once, and subsequently dispersed among the islands. Hayashi & Chiba (2004) also found evidence of a single colonization of *Euhadra* snails to the Izu Islands, but remarkably, individuals on all the inhabited islands possessed an identical haplotype (i.e. fine-scale phylogeographical structure was not discernible). Suzuki *et al.* (2004), Tomozawa & Suzuki (2008) and Kageyama *et al.* (2009) presented evidence of at least two independent colonizations to the Izu Islands by two mainland clades of *Apodemus* mice.

It is apparent that our current knowledge of the general floral and faunal history of the Izu Islands remains extremely limited, and this lack of a general phylogeographical framework not only affects interpretations of the ecological and evolutionary data, but also limits our understanding of the evolutionary and geological history of the system as a whole. Thus, more empirical phylogeographical data of the Izu Island species are needed to begin elucidating general patterns of phylogeny and island colonization history.

Here, we explore the phylogeography and colonization history of the Izu Island populations of the Japanese four-lined ratsnake, *Elaphe quadrivirgata* (Boie, 1826) (Squamata: Colubridae). We chose this study organism because it is the subject of extensive previous and ongoing biological research (Hasegawa & Moriguchi, 1989; Tanaka *et al.*, 2001; Mori & Hasegawa, 2002; Hasegawa & Mori, 2008). Moreover, given that it is a dispersal-limited terrestrial vertebrate, its phylogeographical structure is less likely than many other Izu Island taxa to be affected by extensive gene flow, and it thus provides an ideal case study to further uncover the general patterns of Izu Island phylogeography.

We employ model-based biogeographical inference (Ree *et al.*, 2005; Ree & Smith, 2008), relaxed molecular clock models (Drummond *et al.*, 2006), and Bayesian phylogenetic analyses of an extensive *E. quadrivirgata* DNA data set, including a large number of individuals from all major Izu Islands that the species inhabits, to answer the following questions. How many times have the Izu Islands been colonized from the mainland? Are some islands subject to multiple colonizations? From what mainland geographical regions did these colonists originate? What is the time-scale of colonization?

Moreover, the island biogeography model of MacArthur & Wilson (1967), further refined by Whittaker *et al.* (2008), predicts that evolutionary dynamics play the dominant role in shaping the biota on large, remote islands. The Izu Islands provide an interesting test of these models' predictions because the islands differ in area (0.04–91.05 km^2) and distance from the mainland (25–260 km). There exists extreme body-size disparity (Hasegawa & Moriguchi, 1989) among island populations of *E. quadrivirgata* as well as multiple colour morphs (most notably represented by the dwarf, melanistic population inhabiting Oshima). Here we also use our time-calibrated phylogeographical analysis of *E. quadrivirgata* to evaluate body size and melanism of different island populations, as well as to provide a phylogenetic framework within which to understand the evolutionary processes that drive these differences.

MATERIALS AND METHODS

Sample collection and sequencing

We obtained non-lethal scale clip tissue samples (these populations are subjects of an ongoing long-term ecological study) from 373 individuals of *E. quadrivirgata* from seven island populations and 25 mainland localities (Fig. 1). We focused our collecting efforts on the Izu Islands and regions of mainland Japan in close geographical proximity. To capture the genetic diversity within each island of the Izu Archipelago, we sampled a large number of individuals from each island (range = 16–57, median = 35, see Appendix S1 in the Supporting Information). We included individuals from all major Izu Islands, but we were unable to obtain samples from several extremely small islands.

DNA was isolated from tissue using Qiagen DNeasy columns. The mitochondrial cytochrome *b* (cyt *b*) gene has been shown to be a useful genetic marker for elucidating population genetic structure in snakes (e.g. Burbrink *et al.*, 2000). Thus, we amplified the entire 1117-bp gene in two overlapping fragments using the following primers: H14910 (Burbrink *et al.*, 2000), 5′-GAC CTG YGA TMT GAA AAA CCA CYG TT-3′; EqintR, 5′-AAG TGG AGG GCR AAG AAT CGA GTT AAG GT-3′; EQ-mH2, 5′-AGC TTT GTC TTA CAA GGA CAA CGC-3′; EQ-mL2, 5′-CCA TGA GGA CAA ATA TCA TTC TGA G-3′ and standard polymerase chain reaction (PCR) techniques (95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s). PCR products were cleaned using ExoSap-IT (USB Corp., Cleveland, OH, USA). Purified templates were dye-labelled using BigDye (Applied Biosystems, Foster City, CA, USA) and sequenced on an ABI 3077 automated DNA sequencer (Applied Biosystems) using the same primers. Nucleotide sequences were examined and aligned by eye, and an open reading frame for this gene was verified using MacClade v. 4.08 (Maddison & Maddison, 2008). No indels were present in any individuals.

Body-size data

Body-size data were collected during field studies conducted from 1982 to 2008 on the Izu Islands of Oshima, Toshima, Niijima, Shikine, Kozu, Tadanae and Mikura, and from 2004 to 2008 for the mainland populations in eastern Japan adjacent to the Izu Islands, including the Kanto region (represented by Kanagawa, Tochigi and Ibaraki in Fig. 1), Boso Peninsula and Izu Peninsula, and from western Japan around Kyoto (Fig. 1). We measured snout–vent length (SVL) and recorded gender and colour pattern for each specimen. Individuals were also marked by unique ventral scale clipping for future identification. Data were collected only from adult specimens. We assumed the snake was an adult if the SVL of females or males was greater than the minimum SVL at which the females of that population are known to become gravid. We visually assessed body-size differences among these populations by calculating the maximum, minimum, mean, SD and SE of adult body size for the four mainland and seven Izu Island populations. We compared body-size differences statistically using ANOVA and *post hoc* tests with Bonferroni/Dunn correction (StatView; SAS Institute Inc., Cary, NC, USA).

Phylogenetic and molecular dating analyses

In order to understand the phylogenetic relationships among extant haplotypes and give an explicit temporal scale to the biogeography of *E. quadrivirgata*, we employed partitioned Bayesian phylogenetic analyses assuming a relaxed molecular clock. Bayesian phylogenetic and divergence date estimation using relaxed molecular clocks is attractive for its ability to estimate divergence dates while simultaneously incorporating rate heterogeneity among lineages and phylogenetic uncertainty (and thus estimates of dating error) in the tree estimation process (Drummond *et al.*, 2006). Estimating divergence times from molecular data requires some *a priori* estimate ages for at least one divergence. These are commonly estimated by incorporating fossil taxa as age constraints to ‘calibrate’ the relaxed molecular clock. Modern Bayesian methods allow for the incorporation of a prior distribution of ages (‘age constraints’), and thus age uncertainty, into these fossil calibrations and, ultimately, uncertainty of estimates of divergence times.

There are no known *E. quadrivirgata* fossils that can be used as calibration age constraints. Thus, we infer the divergence times of *E. quadrivirgata* populations using ‘external’ calibration age constraints (i.e. fossils of lineages outside *E. quadrivirgata*; outgroup lineages). Fortunately, a recent study (Burbrink & Lawson, 2007) inferred the phylogeny and divergence times of species representing all major lineages of ratsnakes, including *E. quadrivirgata* and its close relatives. For our time-calibrated phylogenetic analyses, we combined our newly collected data for *E. quadrivirgata* with cyt *b* data from all the other species used by Burbrink & Lawson (2007). With two exceptions (below), we used the same fossil calibrations of Burbrink & Lawson (2007), but our use of Bayesian methods of estimating phylogeny and divergence times requires that we incorporate the fossil ages slightly differently from their original study. We chose prior age distributions so that the youngest age of the distribution corresponded to the youngest age used by Burbrink & Lawson (2007) (i.e. the youngest possible age at which that lineage existed). We chose a standard deviation of the distribution so that 95% of the lognormal distribution was younger than the oldest age used by Burbrink & Lawson (2007) (i.e. 5% of the distribution sampled older ages). We used the following calibrations: C1 (earliest *Lampropeltis* fossil), C2 (earliest *Pantherophis*, see below), C4 (earliest *Coluber* and *Masticophis* fossils), and C5 (earliest *Salvadora* fossil) of Burbrink & Lawson (2007) (see that paper for further discussion of the composition and ages of these constraints). Burbrink & Lawson (2007) used the earliest known *Pantherophis* fossil to date the root of that genus (calibration C2), but this assumes the fossil is a part of the crown *Pantherophis*. However, if *Pantherophis* fossils exist, then this is evidence that the lineage leading to modern *Pantherophis* existed, and not necessarily that the crown taxa had yet diverged. Instead, we use this fossil to date the split between *Pantherophis* and its sister clade. We do not use Burbrink & Lawson’s (2007) calibration C3 because their assumption that this fossil *Zamenis* is ‘directly ancestral’ to two of the three sampled *Zamenis* is debatable.

We used BEAST v. 1.5.3 (Drummond & Rambaut, 2007) to estimate the phylogeny and divergence times of the 42 *E. quadrivirgata* haplotypes. Because different tree priors are appropriate for different phylogenetic scales (e.g. inter- and intraspecific; Drummond *et al.*, 2006), we first conducted an analysis including 56 outgroup taxa and three haplotypes that spanned the root of the *E. quadrivirgata* tree and the divergence between the α and β clades (Fig. 2). This analysis enforced the above fossil calibration age constraints (C1: lognormal mean = 0, SD = 0.843, offset = 15; C2: 0, 0.843, 16; C4: 0, 0.843, 11; C5: 0, 0.843, 20). We note that the use of an offset places a hard lower bound on the age distribution (i.e. no bound on sampling older ages). Moreover, we used specific evolutionary models for each codon position, as the use of partitioned models is known to improve phylogenetic inference by accounting for sequence evolution heterogeneity among different parts of a gene or genome (Castoe *et al.*, 2004; Nylander *et al.*, 2004; Brandley *et al.*, 2005). The most appropriate evolutionary model for each of the three partitions was determined using the Bayesian information criterion (BIC; Schwarz, 1978) after estimating parameters of 24 models of nucleotide evolution in PAUP* v. 4b10 (Swofford, 2002) using an initial neighbour-joining tree calculated from Jukes–Cantor (JC)-corrected genetic distances. The selected models include GTR+I+G for the first and third codon positions, and HKY+I+G for the second codon position. The model testing regime indicated that the GTR+I+G substitution model was the most appropriate for all three codon positions. The subsequent BEAST analyses of the complete ratsnake data set were run for 2×10^7 generations (sampled every 1000 generations) using a birth-death tree prior on rates of cladogenesis, a maximum likelihood starting tree, uncorrelated lognormal relaxed molecular clock, and the programs default prior distributions.

We then conducted an additional analysis including only the 42 *E. quadrivirgata* haplotypes, but using the date lognormal distributions of the two *E. quadrivirgata* nodes inferred by the

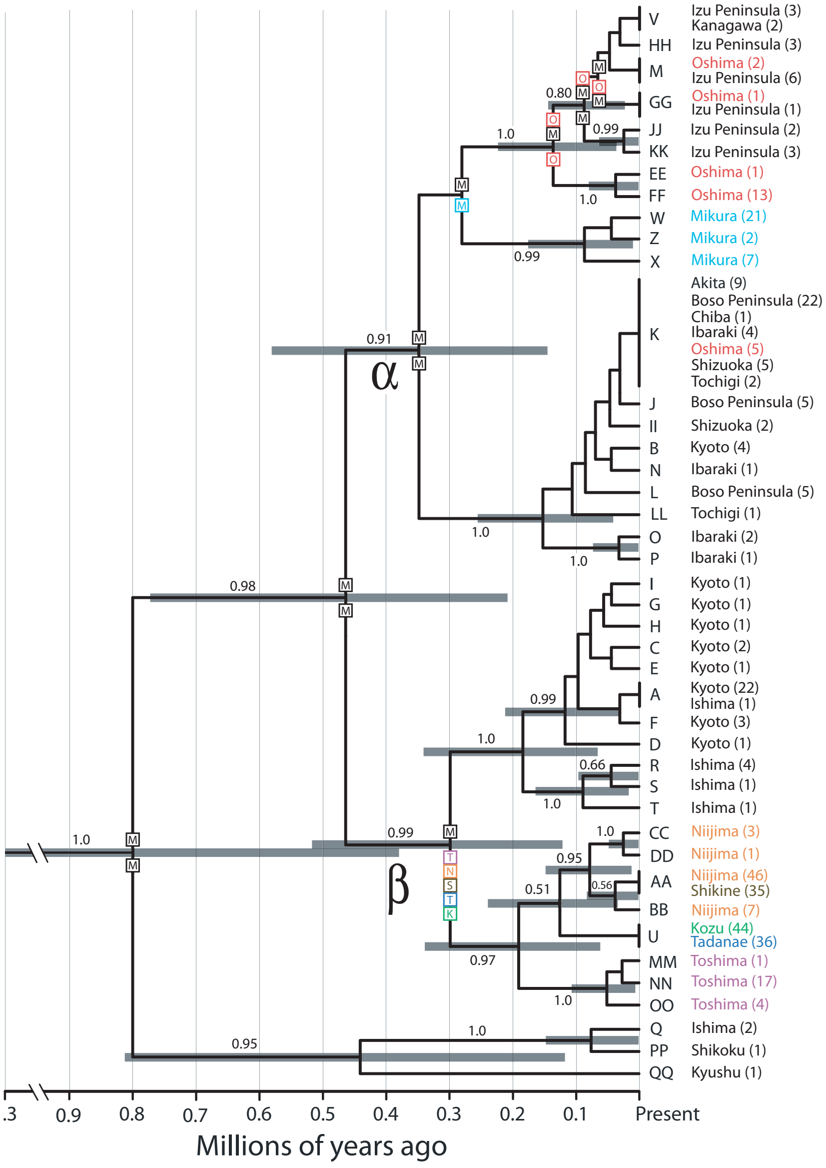

Figure 2 Results of the time-calibrated Bayesian analysis of *Elaphe quadrivirgata* haplotypes using BEAST. Letters indicate haplotype names; numbers in parentheses indicate how many sampled individuals from a specific population share that haplotype. Izu Island populations are coloured in accordance with Fig. 1. Branch lengths are in units of time. Node bars indicate the 95% credible interval of that node's age. Numbers above or below the nodes are posterior probabilities (probabilities < 0.50 not shown). Boxed letters (coloured and labelled by island) indicate results of the LAGRANGE analysis; only the optimal reconstruction is shown for simplicity, the full results are provided in Appendix S3. Species other than *E. quadrivirgata* are not shown.

first BEAST analysis as secondary calibrations [mean age (in real space) = 1.2, SD = 0.35 for the root of *E. quadrivirgata*, and mean age (in real space) = 0.48, SD = 0.50 for the divergence between the α and β clades]. This BEAST analysis used the same analytical parameters as above, but with a coalescent-constant size tree prior, substitution models re-estimated for each partition, and run for 10^7 generations. Bayes factor analyses (not shown) indicate that modelling the first and second codon positions as separate partitions is not warranted, thus we combined these into a single partition, and re-estimated models for this partition (HKY+I) and the third codon position (GTR+I). Secondary calibrations have received deserved criticism when used as errorless point calibrations (see Graur & Martin, 2004). However, we note that we have explicitly incorporated this error by using statistical distributions inferred by the primary analysis, rather than simple point calibrations.

To determine apparent stationarity, we constructed cumulative posterior probability plots for each clade using the 'cumulative' function in AWTY (Nylander *et al.*, 2008). Stationarity was assumed when the cumulative posterior probabilities of all clades stabilized. To decrease the chance of not fully sampling the posterior distribution, we repeated the BEAST analyses four times. Posterior probability estimates for each clade were then compared between the four analyses using a scatter-plot created by the 'compare' command in AWTY. If posterior probability estimates for clades were similar in all analyses, the results of both analyses were combined. Posterior probabilities (PP) ≥ 0.95 are considered strong clade support (following Huelsenbeck & Rannala, 2004).

Ho *et al.* (2005, 2007) demonstrated that using external calibrations with some data sets will result in underestimating the rate of evolution for recent divergences, and thus may overestimate times of these same divergences. This phenomenon is due to the differences in apparent rates of evolution at very different time-scales. It is unclear if a similar phenomenon would be present with our analyses, but we note that we employ partition-specific modelling (e.g. Brandley *et al.*, 2005), which is expected to capture better the parameters that influence molecular divergence times (including evolutionary rates); indeed, there is evidence that divergence dates estimated from partitioned analyses capture better the true underlying rate of evolution (Brandley *et al.*, in press). Moreover, we assume an appropriate tree prior for the intraspecific analysis (constant population-size tree prior).

Model-based biogeographical inference

We used the dispersal–extinction–cladogenesis (DEC) model in LAGRANGE v. 2 (Ree & Smith, 2008) to quantify the number of independent colonizations to (and potentially from) the Izu Islands. We constructed a geographical range matrix coding each haplotype as present or absent in one of four geographical areas: mainland (including the Izu Peninsula); Oshima; 'middle' Izu Islands (Toshima, Niijima, Shikine, Kozu and Tadanae, ordered north to south: TNSKT); and Mikura. Although alternative coding strategies are possible, we sought to strike a balance between an overly simple matrix (e.g. coding only mainland and island), and one so complex as to be computationally unfeasible, or so overparameterized that it exceeds the explanatory power of our data and phylogeny (e.g. coding each island separately). Haplotypes that inhabit more than one geographical area were coded accordingly. The LAGRANGE analysis used this geographical matrix, time calibrated Bayesian consensus tree of the haplotype relationships (with branch lengths equal to mean ages, root age = 0.80 Ma), and assumed an equal probability of dispersal among geographical states, with no range constraints other than assuming the mainland as the root state.

RESULTS

Phylogeny and divergence data

The full ratsnake phylogeny is provided in Appendix S2. Identification and distribution of the 42 unique haplotypes are provided in Table 1 and Fig. 2. Ages are presented as the mean and 95% credible interval (CI) of the posterior distribution. The partitioned Bayesian analyses achieved stationarity by 2 million generations, and posterior distributions of each parameter were calculated from the remaining post-burn-in trees from all four analyses. The phylogeny of the haplotypes, clade posterior probabilities, and 95% CI of divergence times are provided in Fig. 2 (outgroups not shown). Support for *E. quadrivirgata* monophyly is significant (PP = 1.0). The sampled haplotypes form two major clades, with one comprising populations from Ishima, Shikoku and Kyushu that is sister to a well supported (PP = 1.0) clade composed of two subclades (α and β clades; Fig. 2).

Table 1 Distribution of the 39 α and β clade *Elaphe quadrivirgata* haplotypes for the Japanese mainland, peninsula, and Izu Island sampling localities. Three haplotypes (Q, PP, QQ) that are not part of either clade are not represented in this table.

	Haplotype	
Locality	α clade	β clade
Mainland		
Akita	K	
Ibaraki	K, N, O, P	
Kanagawa	V	
Shizuoka	K, JJ	
Tochigi	K, LL	
Kyoto	B	A, C, D, E, F, G, H, I,
Ishima		A, R, S, T
Peninsula		
Boso Peninsula	K, J, L	
Izu Peninsula	M, V, GG, HH, II, KK	
Izu Islands		
Kozu		U
Mikura	X, W, Z	
Niijima		AA, BB, CC, DD
Oshima	K, M, EE, FF, GG	
Shikine		AA
Tadanae		U
Toshima		MM, NN, O

The α clade (PP = 0.91) contains haplotypes from eastern Japan (including the Izu and Boso Peninsulas; Fig. 1) and two Izu Islands, Mikura and Oshima (Fig. 2). The basal relationships of this clade are not well supported, but nonetheless we can infer that this clade began to diversify 0.34 (0.13–0.58) Ma. The population of Mikura contains three haplotypes that presumably descend from a single colonization event. The Oshima populations contain haplotypes whose closest ancestors are interspersed throughout the α clade. Of the five haplotypes found on Oshima, three (K, M and GG) are shared with populations on mainland Japan. However, most of the sampled Oshima individuals (14 of 22 individuals) have haplotypes (EE and FF) that are distinct to Oshima and form a clade that diverged from its sister lineage 0.12 (0.03–0.23) Ma.

The β clade (PP = 0.99) contains haplotypes from five Izu Islands, Niijima, Shikine, Kozu, Tadanae and Toshima (Fig. 2), and diverged from its sister clade of western Japan haplotypes 0.30 (0.11–0.52) Ma. Toshima contains three haplotypes that form a clade exclusive to that island. The Niijima population is composed of four haplotypes, the largest of which (haplotype AA: 46/57) is also shared with every sampled individual from nearby Shikine (Fig. 1). Similarly, genetic diversity is very low on Kozu and Tadanae, as every sampled individual on each island shares the same haplotype (U).

Biogeographical reconstruction

The results of the LAGRANGE analyses are shown in Fig. 2; the complete analyses, with −ln*L* values for constructions of each node, are provided in Appendix S3. The analysis unambiguously infers a single colonization of the current Izu Islands of TNSKT from a mainland β clade ancestor. Within the α clade, we estimate that Mikura was also colonized once from the mainland. The colonization history of Oshima is much more complex, with two unambiguous independent colonizations of that island from mainland ancestors (individuals with haplotypes K, M). A third colonization is represented by haplotypes EE + FF. LAGRANGE infers a potential fourth colonization from an α clade ancestor (GG). However, we cannot currently distinguish between this hypothesis and the hypothesis that the ancestor of the clade containing GG and closely related Izu Peninsula haplotypes inhabited Oshima and subsequently recolonized the mainland.

Geographical variation of body size

Mean, standard error, minimum and maximum body sizes (in mm) for the sampled populations are provided in Fig. 3. The ANOVA for the two peninsulas (Izu and Boso Peninsulas; Fig. 1) and Kanto region (Fig. 1) found no significant difference in mean SVL among populations in both adult males ($F_{2,\ 41} = 1.624$, $P > 0.20$) and females ($F_{2,\ 27} = 2.822$, $P > 0.05$). Thus, all body-size data from the three mainland regions were combined to obtain standard eastern mainland body size. Overall mean SVLs were 1003.2 mm ± 16.0 SE for adult males ($n = 44$), and 847.8 mm ± 10.6 SE for adult females ($n = 30$). Mean body size for the western mainland population from Kyoto region was 1020.6 mm ± 21.1 SE for adult males ($n = 30$), and 843.5 mm ± 9.3 SE for adult females ($n = 30$). Body sizes of the eastern and western populations of *E. quadrivirgata* were not significantly different in both males ($F_{1,\ 72} = 0.844$, $P > 0.60$) and females ($F_{1,\ 58} = 1.291$, $P > 0.40$).

The ANOVA including the combined mainland and seven island populations found significant differences in adult body size for both males ($F_{7,\ 1015} = 233.227$, $P < 0.001$) and females (SVL: $F_{7,\ 547} = 221.646$, $P < 0.0001$). *Post hoc* tests with Bonferroni/Dunn correction (Table 2) showed that adult male and female snakes from Tadanae have significantly larger body size compared with the other seven populations (Fig. 3). Adult snakes from Oshima and Toshima tended to be smaller than, or were similar to, the mainland populations. As a result, snakes from the mainland, Oshima and Toshima populations were significantly smaller than the other island populations (Table 2). Other island populations in Niijima, Shikine, Kozu

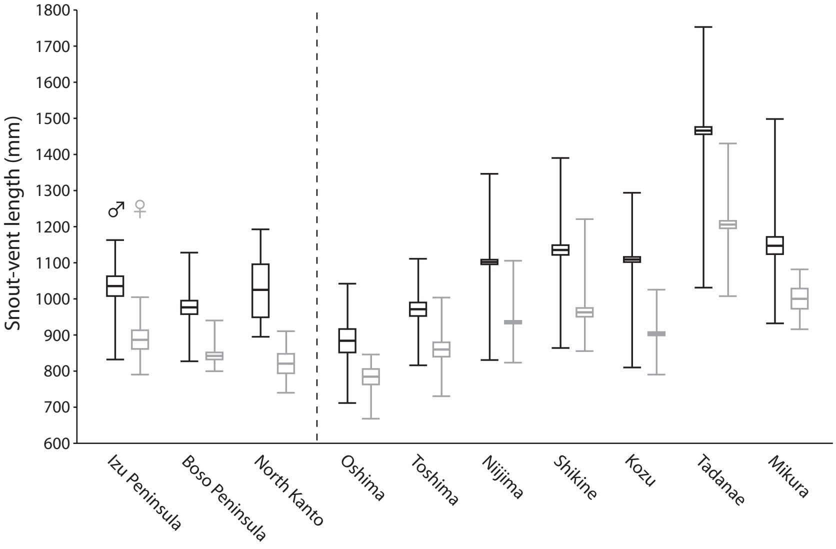

Figure 3 Body-size distributions (snout–vent length) of adult males and females of *Elaphe quadrivirgata* from mainland Japan (left side of hashed line) and Izu Island (right side of hashed line) populations. Top and bottom bars, box and centre line represent maximum, minimum, standard error and mean body size.

Table 2 Summary of the *post hoc* tests with Bonferroni/Dunn correction for body-size comparison among the Japanese mainland and seven island populations of *Elaphe quadrivirgata*. Males are above the diagonal; females below. Numerals are the absolute size difference between two populations (mm). Significant interpopulation differences ($P < 0.05$) in body size are indicated by bold numerals.

	Mainland	Oshima	Toshima	Niijima	Shikine	Kozu	Mikura	Tadanae
Mainland	–	**119.2**	32.0	**99.2**	**131.9**	**105.8**	**144.7**	**462.7**
Oshima	64.9	–	87.2	**218.4**	**251.1**	**225.0**	**263.9**	**581.9**
Toshima	10.5	75.4	–	**131.2**	**163.8**	**137.8**	**176.6**	**494.6**
Niijima	**85.8**	**151.2**	**75.8**	–	32.6	6.6	45.4	**363.4**
Shikine	**112.9**	**178.3**	**102.9**	27.1	–	26.1	12.8	**330.8**
Kozu	53.6	**119.1**	43.6	**32.1**	**59.2**	–	38.9	**356.9**
Mikura	**150.6**	**216.0**	**140.6**	64.8	37.7	**96.9**	–	**318.0**
Tadanae	**356.1**	**421.5**	**346.1**	**270.3**	**243.2**	**302.5**	**205.5**	–

and Mikura were intermediate between the dwarf Oshima and the giant Tadanae populations (Fig. 3).

DISCUSSION

Phylogeography and colonization history of Izu Island *Elaphe quadrivirgata*

The geological history of the Izu Islands no doubt played a major role in shaping the phylogeographical history of *E. quadrivirgata*. All the Izu Islands are volcanic, formed in the complex interface of the Eurasian, Pacific and Philippine plates. Unfortunately, the precise geological age of the Izu Islands arc is not known, although Kaneoka *et al.* (1970) used K–Ar geochemical dating to conclude that the Izu Islands are much younger (emergence no older than 2–3 Ma) than the Bonin Islands *c.* 800 km to the south, which formed 26–40 Ma. Furthermore, tectonic studies (Niitsuma, 1991) indicate that the Izu Peninsula was once a group of volcanic islands located far south of its present location, which collided with the main island of Honshu via the north-westward movement of the Philippine plate 1.2–0.7 Ma (Kitazato, 1997). These results imply that at least part of the Izu volcanic arc was formed in the geological aftermath of the collision that formed the Izu Peninsula. Regardless of the exact mechanism by which the islands were formed, our divergence date analyses (assuming reliable calibrations) demonstrate that none of the island populations is older than *c.* 0.9 Ma, and they may be considerably younger.

Inspection of our phylogenetic results (Fig. 2) reveals three biogeographically relevant regions with independent colonization histories: Oshima + Izu Peninsula, Mikura, and TNSKT. We discuss the colonization history of each of these regions in further detail below.

Oshima and Izu Peninsula

The island of Oshima is the largest of the Izu Islands and closest to the mainland (Fig. 1). Island biogeography theory (MacArthur & Wilson, 1967) predicts that Oshima would receive the highest number of mainland immigrants. Indeed, our biogeographical analysis reveals that Oshima was colonized by at least three (and potentially four; see below) mainland lineages that currently inhabit the Izu Peninsula and eastern mainland Japan (Figs 1 and 2). However, with these data, we cannot completely rule out that this multiple colonization scenario is an artefact of incomplete lineage sorting. Incomplete lineage sorting is a phenomenon where ancestral alleles or in our case, mitochondrial DNA haplotypes that are present before a lineage splits (i.e. when two or more populations are reproductively isolated) are retained in its descendant lineages after the divergence (Maddison, 1997). These ancestral alleles will be lost to genetic drift over time and replaced by new alleles unique to the new descendant lineages; however, during short time frames, there is a higher chance that these alleles will not be lost due to drift (e.g. Carstens & Knowles, 2007; McGuire *et al.*, 2007; Leaché, 2009).

Thus, it is possible that Oshima was colonized once from the mainland in the past, but that most of the mainland alleles have not been lost to drift. A far larger nuclear DNA data set would be required to answer this question definitively, but other evidence suggests that incomplete lineage sorting may not explain the existence of multiple mainland haplotypes on Oshima. Such a scenario would require that Oshima be colonized *c.* 0.13–0.58 Ma (the approximate age of the α clade, which is the most recent common ancestor between haplotype K and other Oshima haplotypes), and that identical haplotypes be maintained on both the mainland and Oshima since this time. Examination of our time-calibrated phylogeny shows that this time frame is sufficient to produce many new alleles (e.g. the α and β clades) and, more importantly, to eliminate mainland haplotypes (e.g. TNSTK and Mikura clades all possess unique alleles). Thus, the chance that drift would not eliminate mainland alleles in Oshima is small. Our analyses therefore strongly suggest that Oshima has been receiving multiple waves of mainland alleles since the island's emergence. One of these lineages colonized Oshima within the past *c.* 0.25 Myr and subsequently diverged into at least two endemic haplotypes (EE and FF). However, the other haplotypes inhabiting Oshima (K, M and GG) are likely to be recent immigrants from the Izu Peninsula and Kanto mainland regions (Fig. 1) with which Oshima shares these haplotypes.

Haplotypes unique to the Izu Peninsula diverged roughly coincidently with the Oshima population from an ancestral eastern Japan clade. A potential explanation for this pattern is that snakes may have colonized the Izu Peninsula proto-islands before they collided with the main island of Honshu. Okamoto *et al.* (2006) revealed a similar distribution pattern in the lizard *Plestiodon latiscutatus*, and hypothesized that the ancestors of the Izu Peninsula populations colonized the Izu Peninsula proto-islands before they collided with the main island of Honshu. Alternatively, given our current sampling of haplotypes in the clade containing haplotypes GG and EE + FF, we cannot distinguish between the hypothesis that Oshima was colonized independently by haplotypes GG and EE + FF (thus four total colonizations) and the more intriguing suggestion that it was colonized once but with subsequent recolonization of the mainland.

Mikura

The colonization history of Mikura is remarkable in terms of both the location of the putative source population and the age of the lineage (compared with the age of the extant haplotypes) that inhabits it. Mikura is the farthest of the Izu Islands from the mainland (Fig. 1) inhabited by *E. quadrivirgata*, yet the source of the single successful colonization of the island was the eastern mainland clade (α clade) rather than the western clade (β clade) that is also the source of the population that inhabits TNSKT > 50 km north-west (Figs 1 and 2). The age of the root of the α clade, which is a trichotomy including

Mikura, Oshima and primarily eastern Japan haplotypes (the closest relatives to the Mikura lineage are not well supported) is *c.* 0.44 Myr old. With the caveat of divergence dating uncertainty, and the possibility that such a pattern may also be produced through a stochastic coalescence process, these results are indicative of an old colonization event to Mikura (perhaps soon after it emerged) followed by a relatively strong bottleneck. Alternatively, this result may simply be an artefact of not sampling potential mainland stem lineages that are more closely related to the source of the Mikura immigrants.

Toshima, Niijima, Shikine, Kozu and Tadanae

The results of our phylogenetic and biogeographical analyses strongly support a single clade composed of populations that inhabit the middle Izu Islands of TNSKT (Fig. 1), and that this entire lineage diverged from western Japanese ancestors *c.* 0.30 Ma (Fig. 2). Although these data support a single colonization of the entire region, they do not indicate the sequence in which each island was inhabited. However, more information about the biogeographical history of this group may come from geological data.

Inspection of the bathymetry of the Izu Island volcanic arc (Fig. 1) reveals that the present TNSKT islands are part of a larger island block. Moreover, during times of maximum sea level recession (e.g. during Pleistocene glacial periods), these islands were potentially joined or, at least, in close proximity. Thus, given our phylogenetic results, we hypothesize that the western mainland clade ancestor (β clade, Fig. 2) of the TNSKT populations once colonized a larger landmass that subsequently became isolated into Toshima, Niijima + Shikine, and Kozu + Tadanae, or colonized one of these three smaller landmasses and quickly dispersed to the others.

Niijima contains more genetic diversity and older haplotypes (Fig. 2) than the geographically proximate Shikine (Fig. 1). This diversity, and the fact that every sampled individual from Shikine ($n = 35$) shares a haplotype identical to most of the Niijima population (haplotype HH), suggests very recent genetic mixing between the two islands. Indeed, if both islands were emergent during Pleistocene glacial periods (geological data suggest that Shikine may have formed *c.* 10,000 years ago; Yoshida, 1992), they would have been connected into a single landmass during maximum sea level recession. The most recent land connection between Niijima and Shikine may have developed after a volcanic eruption in the ninth century (Isshiki, 1987; Sugihara *et al.*, 2001; Tsukui *et al.*, 2006), but consisted of only a sand bar (Isobe & Yasuda, 1995). This connection was severed no later than the early 18th century (I. Isobe, Geological Survey of Japan, personal communication) by a series of earthquakes and associated tsunamis. Thus, it is unknown whether this cataclysm also wiped out unique Shikine haplotypes that were subsequently replaced by the widespread Niijima haplotype HH via short-distance overwater dispersal, or whether this haplotype previously inhabited Shikine and became isolated when the landmass was divided.

All sampled individuals on Kozu ($n = 44$) and Tadanae ($n = 36$) share a single haplotype. We cannot determine, with any degree of certainty, whether one island was colonized first and subsequently colonized the other, or whether this colonization occurred during a period of low sea level when these islands were connected.

Consequences of colonization history for character evolution: a test of island biogeographical model predictions

Our time-calibrated phylogeographical analysis permits examination of the patterns and processes of morphological evolution previously not possible for the Izu Island system. Most importantly, our analyses offer preliminary insight into whether the dramatic body-size differences among Izu Island populations (Hasegawa & Moriguchi, 1989) are the result of colonization from large- or small-bodied mainland founders, or whether these size morphs evolved *in situ* on each island. The results of our phylogenetic analyses clearly support the latter hypothesis, as the body-size variation observed in the Izu Island populations greatly exceeds that observed in the potential mainland source populations (Hasegawa & Moriguchi, 1989).

The predominant island biogeographical theory (MacArthur & Wilson, 1967) predicts that island biotas are influenced by rates of immigration, extinction and speciation. However, the impact of speciation was not well developed in their model and, perhaps most importantly, the model is less informative over long evolutionary and geological time-scales (see Whittaker *et al.*, 2008). Whittaker *et al.*'s (2008) general dynamic model of oceanic island biogeography refined MacArthur and Wilson's dynamic equilibrium model to explicitly incorporate geological age and especially its impact on speciation dynamics. These models predict that, given the low rates of immigration, evolutionary dynamics (e.g. natural selection and genetic drift) should play the dominant role in shaping the biota of remote islands. Conversely, evolutionary dynamics should play a decreased role on islands close to the mainland with high rates of immigration (due to gene flow from the mainland). Our time-calibrated analyses of the phylogeography of *E. quadrivirgata* provide two potential counter-examples to these predictions.

Given its large size (91.1 km^2) and proximity to mainland (*c.* 25 km), it is somewhat unsurprising (given prevailing biogeographical theory: e.g. MacArthur & Wilson, 1967; Lomolino, 1990; Whittaker *et al.*, 2008) that Oshima has been receiving the highest amounts of immigration from mainland Japan. Our time-calibrated phylogeographical analysis predicts three to four independent colonizations of Oshima within the past *c.* 0.58 Myr. However, the Oshima *E. quadrivirgata* population is unique in that all individuals are both dwarf (Table 2; Fig. 3) and melanistic (Hasegawa & Moriguchi, 1989). That this unique phenotype has potentially evolved multiple times despite frequent immigration from presumably striped, 'average' body-sized mainland ancestors (Table 2;

Fig. 3) strongly suggests that natural selection plays the dominant role in shaping the phenotype of *E. quadrivirgata* on Oshima. Although we cannot yet identify the precise causal mechanism, we note that Oshima contains far more potential vertebrate predators than the other Izu Islands, including the weasel (*Mustela itatsi*) and multiple species of predatory birds, and competitors (four species of snake; Hasegawa, 2003). If this scenario of the multiple colonization of Oshima is supported by future analyses of multiple independently evolving loci, it would suggest an extremely rapid rate of body size and pigment evolution on a par with the comparatively better known systems such as African Rift Lake cichlids (see Kocher, 2004).

The Tadanae population of *E. quadrivirgata* is remarkable in terms of having extremely large body size, and in terms of the rate at which this phenotype apparently evolved. Individuals of the Tadanae population are much larger than those of other island and mainland populations of *E. quadrivirgata* (Table 2; Fig. 3), yet share an identical haplotype with the smaller population inhabiting nearby Kozu. The genetic similarity between these two populations suggests that any morphological changes between these two populations evolved very recently and quickly. Again, this pattern is highly suggestive of strong natural selection pressures promoting large body size on Tadanae.

What natural selection forces could have been strong enough to produce this dramatic shift in body size on Tadanae? The likely selective agent is a consequence of the unique prey availability on the island. Tadanae is an extremely small island (0.15 km^2) off the coast of Kozu. Unlike Kozu (and indeed all other islands but Mikura), Tadanae is home to nesting colonies of four species of sea birds (*Synthliboramphus wumizusume*, *Calonectris leucomelas*, *Oceanodroma tristrami* and *Larus crassirostris*; Hasegawa & Moriguchi, 1989). Our previous and ongoing long-term mark–recapture studies (Hasegawa & Moriguchi, 1989; Hasegawa & Mori, 2008) have shown that adults of the Tadanae *E. quadrivirgata* population consume these relatively large (*c.* 30 g) seabird eggs and nestlings. Although we cannot reject the hypothesis that this body-size disparity results from extreme phenotypic plasticity in response to very different prey resource availability, growth trajectory analyses (Hasegawa & Mori, 2008) suggest that these differences between the Tadanae and Kozu populations reflect inherited genetic differences in size and age at maturity, and in life span.

CONCLUSIONS AND FUTURE DIRECTIONS

Our time-calibrated phylogenetic and biogeographical analyses reveal biogeographical patterns and a colonization history of the Izu Islands that are far more complex than those described by previous studies from these islands (Inoue & Kawahara, 1990; Oiki *et al.*, 2001; Hayashi & Chiba, 2004; Suzuki *et al.*, 2004). Studies focusing on *Campanula* plants (Inoue & Kawahara, 1990; Oiki *et al.*, 2001) and *Euhadra* snails (Hayashi & Chiba, 2004) found that the entire Izu Island archipelago was colonized only once, and that the islands were subsequently colonized via inter-island dispersal. In contrast, our analyses revealed at least three independent colonization events between *c.* 0.8 and *c.* 0.2 Ma, and that the island of Oshima may have been subject to repeated mainland colonization. Yet our results are not incongruent with all previous studies. Suzuki *et al.* (2004) and Tomozawa & Suzuki (2008) found that *Apodemus* mice on Niijima, Shikine, Kozu and Miyake descend from a single colonization to the Izu Islands 0.2–0.3 Ma, and that the Oshima population descends from a mainland clade distributed widely in eastern and western Japan. Similarly, Kageyama *et al.* (2009) inferred at least two separate colonizations of the Izu Islands to Oshima + Niijima + Miyake and Kozu. Thus, rather than a single colonization event to the entire Izu Islands, multiple colonizations (spanning the period from the islands' emergence to the present day) might be the predominant pattern of community assembly of the Izu Island biota. Further research based on similarly dispersal-constrained taxa will be necessary to determine this with confidence.

Finally, the Izu Islands system, given its mix of large and small oceanic islands, both close and remote to the mainland, provides a valuable opportunity to test predictions of prevailing biogeographical models. Ultimately, our results underscore the need for additional phylogeographical analysis of the other plant and animal species inhabiting the Izu Islands, with the ultimate goal of understanding both the stochastic and deterministic ecological and evolutionary processes that drive patterns of community assembly (Chase, 2003; Fukami *et al.*, 2007) in this island system.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Appendix S1 Geographical locality and haplotype of *Elaphe quadrivirgata* tissue samples used in this study.
Appendix S2 The complete time-calibrated ratsnake phylogeny used for secondary calibrations.
Appendix S3 Complete results of the LAGRANGE analysis.

BIOSKETCHES

Our research group investigates the phylogenetic history, phylogeography, morphological evolution and ecology of Japanese and East Asian snakes and lizards.

Author contributions: T.K., M.C.B, A.M., M.H. and M.H. collected the data from both the islands and mainland; A.K. collected genetic data from the mainland; T.K., M.C.B and M.H. analysed the morphological and genetic data; and M.C.B., T.K. and M.H. led the writing.

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