Insights into the phylogenetic position and phylogeography of the monospecific skink-parasite genus *Neoentomelas* (Nematoda : Rhabditida : Rhabdiasidae), with special reference to the effects of the reproductive mode on the genetic diversity

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RT: Phylogeny and genetic diversity of *Neoentomelas*

Short summary (≤ 60 words, jargon free)

Neoentomelas asatoi is a parasitic nematode that infests only a scincid lizard species in Ryukyu Archipelago, Japan. We clarified the phylogenetic distinctiveness of *Neoentomelas* in Rhabdiasidae, and revealed at least three dispersal events of the major clades within *N. asatoi*. Our results provide new insight into the evolutionary history of Rhabdiasidae and the diversification factors of parasites.

Abstract. *Neoentomelas asatoi* Hasegawa, 1989 is a parasitic nematode that infests only the scincid lizard *Ateuchosaurus pellopleurus* (Hallowell, 1861), which inhabits the forest floor in Northern and Central Ryukyu Archipelago, Japan. As a member of Rhabdiasidae, the reproductive mode of *N. asatoi* is characterized by the alternation of the protandrous hermaphroditic mode and gonochoristic mode throughout its life cycle. The intrafamily phylogenetic position and intraspecific diversity of this nematode species were inferred by molecular phylogenetic analyses. The results revealed the phylogenetic distinctiveness of *Neoentomelas* Hasegawa, 1989 in Rhabdiasidae, which supports the unique generic status of *Neoentomelas* within the family. The intraspecific phylogenetic analyses of *N. asatoi* revealed a minor concordant phylogenetic pattern with the host and mosaic geographic arrangement of the major clades, which was discordant with the host. The analyses and distribution pattern of subclades suggested that this geographic arrangement can be explained by at least three dispersal events and subsequent switching to indigenous host populations. Colonization events might be promoted by the high establishment rate of new populations stemming from the parthenogenesis-like reproduction mode of *N. asatoi*. The present study demonstrated that reproductive modes can affect the intraspecific genetic diversity of parasites.

Additional keywords: *Ateuchosaurus pellopleurus*, endoparasite, Japan, Ryukyu Archipelago

Introduction

Vicariance events of host species and host-switching events of parasites are essential for the formation of diversification and distribution patterns of parasites (Koehler *et al.* 2009; Badets *et al.* 2011; Sands *et al.* 2017). The dispersal ability of organisms influences their intraspecific genetic diversity and speciation rate (Peterson and Denno 1998; Kisel and Barraclough 2010; Ikeda *et al.* 2012): low dispersal ability leads to a low rate of gene flow and promotes interpopulation genetic divergence, and high dispersal ability leads to a high rate of gene flow and suppresses interpopulation genetic divergence. Therefore, the mobility of hosts and host richness (the number of possible host taxa) are the major determinants of the dispersal ability of parasites (e.g., Blasco-Costa and Poulin 2013; Falk and Perkins 2013), and the vicariance of the host population would serve as barriers for the interpopulation gene flow of parasites that have sedentary hosts or narrow host ranges. In this case, cophylogenetic patterns would be expected between hosts and parasites. However, many previous studies have reported discrepant phylogenetic and distribution patterns between hosts and parasites, which have sedentary hosts or narrow host ranges at various scales; such discrepancies are speculated to be the result of asymmetric geographic range expansion and/or host switching events (e.g., Wickström *et al.* 2003; Haukisalmi *et al.* 2016). Thus, it is important to determine the degree of diversification pattern of parasites that can be explained by host divergence or host switching to improve our knowledge of the diversification factors of parasites. Comparative phylogeographic studies on certain hosts and parasites are useful to determine which phylogeographic events have occurred.

The Ryukyu Archipelago, which consists of three parts—the Northern, Central and Southern Ryukyus, is located between the Japanese main islands and Taiwan (Fig. 1). Previous systematic and phylogeographic studies have revealed great specific and genetic diversity of reptiles in this archipelago (Ota 1998; Okamoto 2017). These studies also pointed out the high endemism of reptilian fauna in Central Ryukyus (Ota 1998; Okamoto 2017), and thus, these putative host species can be expected to harbor diverse and unique parasites. In contrast to our knowledge about the endemism and phylogeographic features of reptiles inhabiting the Ryukyu Archipelago, little is known about the diversity and diversification process of parasites in the reptiles distributed in this area (but also see Hasegawa and Iwatsuki 1984; Hasegawa 1985).

The rhabdiasid *Neoentomelas* Hasegawa, 1989 is a monospecific nematode genus known from Ryukyu Archipelago, which contains only the species *Neoentomelas asatoi*

Hasegawa, 1989. Recent studies have clarified that rhabdiasid genera exhibit certain host specificity (Kuzmin and Tkach 2011; Kuzmin 2013; Tkach *et al.* 2014); *N. asatoi* also exhibits strict host specificity and only infects the lung of the scincid lizard *Ateuchosaurus pellopleurus* (Hallowell, 1861), which inhabits the forest floor throughout Central Ryukyus and part of Northern Ryukyus (Hasegawa 1989, 1990, 1992; Sata 2015). Morphological and phylogeographic studies have suggested the endemism of *A. pellopleurus* in Central Ryukyus and its recent range expansion from Central Ryukyus to Northern Ryukyus (Ota *et al.* 1999; Makino *et al.* 2020). A molecular phylogenetic study also revealed the deep genetic divergence between the Okinawa Islands population and the Amami Islands to the northern Ryukyus population (Makino *et al.* 2020).

The known geographic range of *N. asatoi* covers most of the geographic range of *A. pellopleurus*: Amamioshima Island, Okinawajima Island, and Kumejima Island (Hasegawa 1989, 1990, 1992). Although the life cycle of this nematode has not been elucidated, all investigated rhabdiasid species require no intermediate host in their life cycle. The life cycle of most rhabdiasid species is characterized by the alternation of parasitic and free-living generations; the former is reproduced by a protandrous hermaphroditic mode, and the latter is reproduced by a gonochoristic mode (Anderson 2000; Kuzmin 2013). Protandrous hermaphroditism requires only a single individual for reproduction and can allow single individuals to establish new populations, such as in parthenogenesis. Hence, it is estimated that *N. asatoi* has a high success rate of new-population establishment, and thus its phylogeographic pattern is affected by its reproductive mode. Accordingly, the comparison of phylogeographic patterns of *N. asatoi* with *A. pellopleurus* is essential for improving our understanding of the diversification process of parasites reproduced by the parthenogenesis-like mode.

Rhabdiasidae currently comprises eight genera, and most of the species dwell in the lungs of reptiles or amphibians (Kuzmin 2013; Tkach *et al.* 2014). Several authors predicted that *Neoentomelas* and *Kurilonema* Shcherbak & Sharpilo, 1969 share the most recent common ancestor because they have highly similar morphological and ecological features, i.e., both genera possess ‘a large buccal capsule with a chitinized wall’, and their host ranges are mostly limited to scincid lizards (Shcherbak and Sharpilo 1969; Hasegawa 1989, 1990, 1992; Telford 1997; Bursey *et al.* 2005; Sata 2015). Additionally, *Entomelas* Travassos, 1930 and *Kurilonema* also share morphological features, including a large buccal capsule with a chitinized wall, and are distinguished from each other only by the characteristics of teeth in the bottom of the buccal capsule;

Entomelas possesses teeth, while *Kurilonema* lacks teeth (Baker 1980; Kuzmin and Sharpilo 2002). The morphological similarities among these genera lead to arguments about the validity of their taxonomic status as a distinct genus (Baker 1980; Kuzmin and Sharpilo 2002). Therefore, systematic accounts of *Neoentomelas*, *Kurilonema* and *Entomelas* should be elucidated to elucidate the evolutionary history of these rhabdiasid genera.

In the present study, we aim to clarify the systematic accounts of *Neoentomelas*, *Kurilonema*, and *Entomelas* as distinct genera by inferring the intrafamily phylogenetic relationship of Rhabdiasidae with molecular phylogenetic analyses based on nuclear DNA sequences. We also aimed to elucidate whether the interspecific genetic diversification pattern of *N. asatoi* deviates from the phylogeographic pattern of *A. pellopleurus* by comparing the intraspecific phylogenetic pattern inferred from mitochondrial and nuclear DNA sequences with the molecular phylogenetic tree of *A. pellopleurus* estimated by Makino *et al.* (2020). Accordingly, we reinforce the taxonomic stability of rhabdiasid nematodes and provide new insight into diversification factors of parasites.

Materials and methods

Sampling

From 2014 to 2018, a total of 123 individuals of *A. pellopleurus* were collected from 21 localities on 15 islands of Central and Northern Ryukyus (Fig. 1). Hosts were euthanized by oral injection with sodium pentobarbital and then dissected. The euthanized hosts were stored at −80 °C until dissection. Their lungs were examined under a stereoscopic microscope (model Eclipse Ni-U, Nikon, Tokyo, Japan); nematodes were collected when they were present in the lungs. The nematodes obtained were fixed in hot 70% glycerin ethanol, cleared in 100% glycerin, and morphologically identified to species. The identified specimens were preserved in 70% ethanol at −20 °C until DNA extraction. The locality names where *N. asatoi* was collected are listed in Table 1. We also collected *Kurilonema markovi* Shcherbak & Sharpilo, 1969 (Rhabdiasidae) from *Plestiodon* Duméril & Bibron, 1839 lizards as additional operational taxonomic units for intrafamily phylogenetic inference and as outgroups for intraspecific phylogenetic inference. The details of the parasitic record will be described in future papers. All hosts were collected and handled in accordance with the Regulations of Animal Experimentations at Kyoto University (approval numbers: H24014, H2711).

Several representative specimens of *N. asatoi* (KUZ Z2731–Z2750; Table S1) and all

lizard specimens examined in this study were deposited in the Zoological Collection of Kyoto University (KUZ) as the voucher specimens of this study.

DNA sequencing

We used 74 specimens of *N. asatoi* for molecular phylogenetic analyses. Genomic DNA was extracted from each specimen following the method described by Sata (2018). Two fragments of mitochondrial DNA, cytochrome *c* oxidase subunit I (*COI*) and 12S ribosomal DNA (*12S*), and nuclear DNA fragments, including the 3′ end of 18S rDNA, internal transcribed spacer (*ITS*) 1, 5.8S rDNA, ITS 2, and the 5′ end of 28S rDNA (*18S–28S*), were amplified by polymerase chain reaction (PCR) using a TaKaRa Ex Taq kit (Takara Bio Inc., Kusatsu, Japan) and GeneAmp PCR Systems 2700 or 9700 (Thermo Fisher Scientific, Waltham, MA, USA). The primer sets and PCR conditions for amplifications followed Prosser *et al.* (2013) for *COI*, Casiraghi *et al.* (2004) for *12S*, and Tkach *et al.* (2014) for *18S–28S*. We also used a newly designed primer (nkITSf: 5′-ACCGGGTAAAAGTCGTAACAAG-3′) instead of a 'rift', which is a forward primer used in Tkach *et al*. (2014), for the forward primer to amplify *18S–28S* under modified PCR conditions.

The PCR products were purified with ExoSAP-IT reagent (Thermo Fisher Scientific). Sequencing reactions were performed using a BigDye Terminator Cycle Sequencing Kit (ver. 3.1, Thermo Fisher Scientific) with the primers described in Messing (1983) for *COI* (M13F and M13R); the primers corresponded to those used for PCR for *12S*; and the primers described by Tkach *et al.* (2014) and newly designed primers, nkITSf, nkf01 (5′-GCATATCAGTAAGCGGAGGA-3′), nkf02 (5′-AAACACGGACCAAGGAGTCTAG-3′), and nkr01 (5′-TCCTCCGCTTACTGATATGC-3′) for *18S–28S*. The products were cleaned by ethanol precipitation and sequenced using an Applied Biosystems 3130*xl* Genetic Analyzer (Thermo Fisher Scientific). All determined sequences were deposited with the DNA Data Bank of Japan (COI: LC632082–LC632157; 12S: LC632158–LC632226; 18S–28S: LC631491–LC631542).

Phylogenetic inferences

Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) based on both datasets of nuclear (for the intrafamily relationship of Rhabdiasidae) and mitochondrial DNA sequences (for the intraspecific relationship of *N. asatoi*).

1. The phylogenetic position of *Neoentomelas* and other related genera was estimated

based on 18S–28S sequences. In total, 44 rhabdiasid operational taxonomic units (OTUs) were included (i.e., two *Neoentomelas*, three *Kurilonema*, six *Serpentirhabdias*, five *Entomelas*, three *Pneumonema* and 30 *Rhabdias* OTUs) (Tables S2 and S3). The sequences were edited with MEGA 5 (Tamura *et al.* 2011) and aligned with MAFFT L-INS-i (ver. 7.427, see https://mafft.cbrc.jp/alignment/software/; Katoh and Standley 2013). Regions difficult to align because of alignment gaps were removed manually; thus, the final sequences yielded 2084 bp of aligned positions for *18S–28S*. The best-fit partition scheme and models were identified based on the corrected Akaike information criterion (AICc) using PartitionFinder (ver. 2.1.1, see http://www.robertlanfear.com/partitionfinder/; Lanfear *et al.* 2017) with the 'all' algorithm: for 18S and 5.8S, GTR+G; for ITS 1, GTR+I+G; for ITS 2, GTR+I+G; and for 28S, GTR+G. The ML phylogenetic tree was calculated using IQ-TREE (ver. 2.1.3, see http://www.iqtree.org/; Minh *et al.* 2020) with nonparametric bootstrapping (BS) conducted with 1000 replicates. BI tree and Bayesian posterior probabilities (PP) were estimated using MrBayes (ver. 3.2.7a, see https://nbisweden.github.io/MrBayes/download.html; Ronquist *et al.* 2012). Two independent runs for four Markov chains were conducted for 1 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer (ver. 1.7.1, see http://tree.bio.ed.ac.uk/software/tracer/; Rambaut *et al.* 2018), and the first 2501 trees were discarded based on the results.

2. The phylogenetic relationships within the available *N. asatoi* samples were estimated based on *COI* and *12S* sequences. The mitochondrial (sequences of all loci were concatenated) dataset was collapsed to only those with unique mtDNA haplotypes (individuals possessing identical sequences in the sequenced loci were treated as those with the same haplotype, even if some of them had missing loci). In total, 37 *N. asatoi* OTUs were included as ingroup taxa (Tables 1, S2). The sequences were edited with MEGA 5 and aligned with Clustal W (Thompson *et al.* 1994) for COI and MAFFT L-INS-i for *12S*. Regions difficult to align because of alignment gaps were removed manually for *12S*; thus, the final sequences yielded 655 bp of aligned positions for *COI* and 528 bp for *12S*. The best-fit partition scheme and models were identified based on AICc using PartitionFinder with the 'all' algorithm. The selected partition scheme and models were as follows: for the *COI* 1st position, GTR+G; for the *COI* 2nd position, GTR; for the *COI* 3rd position, HKY+G; for *12S*, HKY+G. The ML phylogenetic tree was also calculated using IQ-TREE with nonparametric BS conducted with 1000 replicates. BI tree and Bayesian PP were estimated using MrBayes; two independent runs for four Markov chains were conducted for 2 million generations, and

the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer, and the first 5001 trees were discarded based on the results.

3. The haplotype relationships within the available *N. asatoi* samples were estimated based on *18S–28S* sequences. In total, 47 *N. asatoi* OTUs were included as ingroup taxa (Tables 1, S2). The sequences were edited with MEGA 5 and aligned with Clustal W, and the final sequences yielded 1441 bp of aligned positions. The haplotype relationships of *18S–28S* were inferred by statistical parsimony with the TCS (Clement *et al.* 2000) algorithm implemented in PopART (ver. 1.7, see http://popart.otago.ac.nz/index.shtml; Leigh and Bryant 2015).

Results

Sequencing

We successfully sequenced 615–655 bp of *COI* for 74 specimens of *N. asatoi*, 521–528 bp of *12S* for 68 specimens, and 1371–2123 bp of *18S–28S* for 50 specimens. Unfortunately, the sequence quality of the *18S–28S* sequence of Clade I (see below) was too poor, which can be ascribed to the slippage of Taq DNA polymerase at a poly-T region. The haplotype compositions at each island are shown in Table 1.

Intrafamily phylogenetic relationship

Both ML and BI phylogenetic inference based on the *18S–28S* DNA sequence dataset yielded almost identical topologies; thus, the BI tree with BI and ML support values is shown in Fig. 2. Phylogenetic inference reconstructed each of the six rhabdiasid genera as distinct clades. The sister relationship between *Neoentomelas* and *Kurilonema* was strongly supported by both analyses (PP = 1.0; BP = 89%). The monophyly of *Entomelas* + *Rhabdias* + *Pneumonema* was also strongly supported by both analyses (PP = 0.99; BP = 91%). The monophyly of *Neoentomelas* + *Kurilonema* and *Entomelas* was not supported by the present inference, and the sister clade of the *Entomelas* clade could not be clarified by the present datasets.

Intraspecific phylogenetic relationship

Both ML and BI phylogenetic inference based on the *COI* and *12S* DNA sequence datasets yielded mostly identical topologies; thus, the BI tree with BI and ML support values is shown in Fig. 3. Phylogenetic inference reconstructed three major clades, Clades I, II, and III. Clade I occurs only on Kumejima Island (haplotype K1), and Clade II occurs only on Tokunoshima

Island (haplotypes Tku1, 2). Clade III consisted of seven subclades: subclade Mishima (occurring on the Iou and Takeshima Islands; haplotypes M1, 2), subclade Tokara (occurring on Suwanosejima and Kodakarajima Islands; haplotypes T1–T3), subclade Okinawa (occurring on Okinawajima Island; haplotypes O1–O3), subclade Tokashiki (occurring on Tokashikijima Island; haplotypes Tka1, 2), subclade Hamahiga (occurring on Hamahigalima Island; haplotype Hm1), subclade Kume (occurring on Kumejima Island; haplotypes K2–K6); and subclade Iheya–Amami (occurring on Iheyajima and Amamioshima Islands; haplotypes I1–I4, Aa1–5, and Ay1–Ay9). The populations that occurred on the Islands of Iheyajima and Amamioshima were composed of a single clade (PP = 0.94; BP = 64%), although they were located on different groups of islands: Iheyajima Island was grouped into the Okinawa Islands, and Amamioshima Island was grouped into the Amami Islands. Furthermore, the present analyses strongly supported the sister relationship between subclade Tokara (occurring in northern Ryukyus and southern Tokara Islands) and subclade Okinawa (occurring in the Okinawa Islands) in Clade III (PP = 0.91; BP = 72%). Kumejima Island accommodated two deeply diverged lineages, one belonging to Clade I and another to Clade III.

Statistical parsimony network

The statistical parsimony network of 57 haplotypes of *18S–28S* is shown in Fig. 4. In this network, two distinct clusters separated by four steps were recognized (A and B in Fig. 4). The two haplotypes (H4 and H5) of Cluster A occurred only on Tokunoshima Island, and the other six haplotypes (H1–H3 and H6–H8) of Cluster B occurred on the remaining islands. In the Iheyajima and Amami Islands, a single identical haplotype (H6) was shared. This haplotype was also detected in Ogimi Village, Okinawajima Island. Although the haplotype that occurred on Kodakarajima Island was identified as H6, this sequence was distinguished from ‘true’ H6 by a single indel-derived polymorphism.

Discussion

Phylogenetic position of Neoentomelas *and taxonomic implications*

Our phylogenies revealed that three genera, *Neoentomelas*, *Kurilonema* and *Entomelas*, represent distinct monophyletic groups, and *Neoentomelas* form sister clades of *Kurilonema*. Several previous studies predicted the close relationship of *Neoentomelas* and *Kurilonema*, as they possess noticeable morphological and ecological similarities (Hasegawa 1989; Kuzmin and Sharpilo 2002; Kuzmin and Tkach 2011). The present results provide the first molecular

phylogenetic support for the close relationship between the two genera, and their shared morphological characteristics, a large buccal capsule with a chitinized wall, can be regarded as a synapomorphy. However, because they exhibit certain genetic and morphological (well-developed dorsoventral lips in *Neoentomelas* vs. small lips in *Kurilonema*) differences, each taxon might be treated as a distinct genus. Accordingly, the possession of a large buccal capsule with a chitinized wall should be regarded as a synapomorphy for the *Neoentomelas* and *Kurilonema* clades.

The known host range of *Neoentomelas* is restricted to *A. pellopleurus* (Hasegawa 1989, 1990, 1992), and *Kurilonema* are mainly *Plestiodon* (in Japan) (Shcherbak and Sharpilo 1969; Telford 1997; Bursey *et al.* 2005; Sata 2015) and *Pinoyscincus* Linkem, Diesmos & Brown, 2011 (in the Philippines) (formerly *Sphenomorphus* Fitzinger, 1843; see Linkem *et al.* 2011) lizards (Kuzmin and Tkach 2011). *Ateuchosaurus* and *Pinoyscincus* belong to Lygosominae in Scincidae and *Plestiodon* to Scincinae in Scincidae (Greer and Shea 2000; Pyron *et al.* 2013; Makino *et al.* 2020). Given the current host ranges of *Neoentomelas* and *Kurilonema*, the host of the most recent common ancestor of these two genera should be scincid lizards.

Our results did not support the monophyly of *Kurilonema* and *Entomelas*. These species exhibit high morphological similarity and are distinguished from each other only by the presence or absence of teeth at the bottom of the buccal cavity (Shcherbak and Sharpilo 1969; Baker 1980; Kuzmin and Sharpilo 2002). Baker (1980) regarded the presence or absence of teeth as insufficient to propose the genus and synonymized *Kurilonema* with *Entomelas*. Kuzmin and Sharpilo (2002) emphasized the validity of the morphology of the anterior region of rhabdiasid nematodes to differentiate their higher taxa and regarded their generic status as distinct. Our phylogeny supported the distinct taxonomic status of these genera, as indicated by Kuzmin and Sharpilo (2002), and the presence of teeth should be a synapomorphy of *Entomelas*. It is also noteworthy that they differ in their host usages, i.e., *Kurilonema* species mainly utilize Scincidae lizards, and *Entomelas* species mainly utilize Anguidae lizards as hosts (Kuzmin 2013). Furthermore, since our phylogeny did not support the monophyly of the *Neoentomelas* + *Kurilonema* clade and *Entomelas*, the apparent sharing of close morphological features, i.e., the large buccal capsule with a chitinized wall, between *Kurilonema* and *Entomelas* may have arisen independently.

Phylogeography of Neoentomelas asatoi

The present intraspecific phylogenetic analysis based on the mitochondrial DNA sequences revealed three major clades (Clades I–III). Clades II and III could also be recognized as

313 Clusters A and B by the *18S–28S* haplotypes. These three clads showed mosaic geographic
314 arrangement. Clades I and II occurred only on Kumejima Island and Tokunoshima Island,
315 respectively, and Clade III occurred in all studied areas except Tokunoshima Island. Given
316 that *N. asatoi* was originally described from Okinwajima Island, a monophyletic group
317 comprising Clades II and III should represent the 'true' *N. asatoi*. Future detailed
318 morphological and genetic analyses are desired to reveal the taxonomic status of Clade III.
319 All subclades occurring in the Okinawa Islands, except one subclade occurring on
320 Kumejima Island, clustered into a single clade (Clade III), and this clade deeply diverged
321 from Clade II, which occurred only on Tokunoshima Island in the Amami Islands. This
322 pattern is mostly concordant with the host phylogeographic pattern, i.e., the deep allopatric
323 genetic divergence between Okinawa Islands populations and Amai Islands–northern Ryukyu
324 Islands populations (Makino *et al.* 2020). Subclades that occurred on Amamioshima Island on
325 the Amami Islands, Tokara Islands and Mishima Islands were also clustered into Clade III.
326 This pattern is discordant with the host phylogeography (Fig. 5). To explain this discordant
327 distribution pattern between *N. asatoi* and *A. pellopleurus*, two modes of phylogeographic
328 events are possible: (1) range expansions of *N. asatoi* via land bridges without host
329 colonization followed by vicariance caused by the subsidence of the bridges; and (2) recent
330 dispersal of *N. asatoi* and subsequent colonization without host colonization.
331 The Ryukyu Archipelago evolved from a continental margin to an island arc. The
332 paleogeographic history of Central Ryukyus is recognized as follows: (1) Central Ryukyus
333 was isolated from the surrounding areas by formation of straits in the Pliocene as a land mass;
334 (2) in the middle Pleistocene, the land mass was divided into several islands due to the
335 relative increase in sea level; and (3) in the late Pleistocene, land bridges between adjacent
336 islands were formed due to the relative decrease in sea level. During this period,
337 Amamioshima Island was connected with other islands in the Amami Islands but not with
338 islands in the Okinawa Islands. (4) Then, the land bridges subsided due to the relative
339 increase in sea level, and the present sets of islands in the Amami and Okinawa Islands were
340 constructed (Osozawa *et al.* 2012; Furukawa and Fujitani 2014). Given the long isolation
341 history between Amamioshima Island and the Okinawa Islands, the genetic affinity between
342 the Amamioshima island population in the Amami Islands and the Iheyajima population in the
343 Okinawa Islands may be explained by the recent colonization of *N. asatoi* without their host
344 lizard.
345 The mitochondrial phylogenetic relationship between Iheyajima and Amamioshima was
346 reticulated, and a single haplotype of *18S–28S* was shared by both island populations. The

phylogenetic analysis also revealed a sister relationship between subclade Iheya–Amami and a clade comprising subclade Hamahiga and subclade Kume (both subclades occur on islands that are members of the Okinawa Islands). Hence, these Okinawa Islands' lineages were paraphyletic to Amamioshima Island's lineage. The most plausible explanation of these is the occurrence of the recent colonization of *N. asatoi* from Iheyajima Island to Amamioshima Island (Fig. 5).

Thus, the following scenario would be suggested: (1) Clade II was distributed across the entire Amami Islands, and (2) invasion of the lineage(s) of Clade III from Iheyajima Island to Amamioshima Island occurred, and then the Clade II populations were excluded by some exclusive interaction with the Clade III lineage(s) there.

The Tokara Islands are geologically composed of two parts i.e., northern Tokara and southern Tokara Islands (Fig. 1), and these island groups belong to Northern Ryukyus and Central Ryukyus, respectively. Northern Ryukyus has been isolated from Central Ryukyus by a deep strait since the Pliocene. The northern Tokara Islands were formed by submarine volcanic activity in the middle Pleistocene, when isolation of the island of the Central Ryukyus began. Additionally, the southern Tokara Islands were formed by submarine volcanic activity in the early Pleistocene, and they have been isolated from other islands in the Central Ryukyus (Osozawa *et al.* 2012). Because both northern and southern Tokara Islands have been isolated from Central Ryukyus for a long time since their emergence, the close genetic affinities among Suwanosejima Island (northern Tokara Islands), Kodakarajima Island (southern Tokara Islands), and Okinawajima (Okinawa Islands) Island populations could not be explained by the range expansions promoted by the emergence of the land bridges and subsequent vicariant events caused by the submergences of the land bridges, but the recent colonization of *N. asatoi* without their host lizard. However, the direction of colonization could not be revealed by the present datasets (Fig. 5). Accordingly, multiple colonization events, at least three times, can explain the mosaic geographic arrangement of Clade III (Fig. 5).

The presence of the unique lineage in the Mishima Islands conflicts with the phylogeographic history of the host populations, which were established by its recent northward dispersal (Makino *et al.* 2020). However, we are convinced that further sampling would clarify their closely related parental population.

The present study unveiled that the mosaic geographic arrangement of the intraspecific lineages of *N. asatoi* could be explained by multiple colonization events. Multiple colonization events were also reported from the phylogenetic study of *Meteterakis* Karve,

1930 (Heterakoidea) nematode species parasitizing the intestinal tract of scincid lizards (including *A. pellopreurus*) and frogs in the Ryukyu and Japanese Archipelagos (Sata 2018). Because *Meteterakis* species exhibit a wide host range and utilize *Plestiodon* lizards, which experienced multiple overseas dispersal events in the Ryukyu Archipelago, they were expected to be highly dispersible parasites (Sata 2018). However, since *N. asatoi* utilizes only a locally diverged small forest-floor dwelling lizard, it is surprising that *N. asatoi* experienced multiple colonization events, similar to the *Meteterakis* species.

Heterakoid species are homoxenous parasites, similar to rhabdiasid species (Anderson 2000). They reproduce only by a gonochoristic mode (Anderson 2000), whereas rhabdiasid species reproduce by alternation of protandrous hermaphroditic mode and gonochoristic mode (Anderson 2000; Kuzmin 2013). Protandrous hermaphrodites require only a single individual for reproduction (i.e., uniparental reproduction), and in that sense, they are equivalent to parthenogenesis. Generally, parthenogenesis can allow single individuals to establish new populations. Thus, the multiple colonization events of *N. asatoi* can be ascribed to the high success rate of new population establishment stemming from parthenogenesis-like reproduction. The present study suggested that parthenogenesis and parthenogenesis-like reproduction can promote the population establishment of parasites in new areas and construct unexpected phylogeographic patterns from the host phylogeographic pattern. Further comparative phylogeographic studies are needed to evaluate the effect of the heterogonic life cycle on the population genetic divergence of parasites.

Concluding remarks

The present study discussed the taxonomic status of three rahbdiasid genera: *Neoentomelas*, *Kurilinema*, and *Entomelas*. Each of the three genera was reconstructed as distinct clades. Thus, our phylogeny supported the distinct generic status of these three genera in Rhabdiasidae. The mosaic geographic arrangement of the major clades of *N. asatoi* was clarified, and the intraspecific phylogenetic tree of *N. asatoi* was not completely concordant with the host tree. This arrangement was explained by at least three colonization events, and one of them was explained by northward dispersal and subsequent switching to indigenous host populations. These colonization events might be promoted by the high establishment rate of new populations stemming from the parthenogenesis-like reproduction mode. The present study suggested that reproductive modes significantly affect the intraspecific genetic diversity of parasites even though they use only a single sedentary host species. Additionally, because northward colonization events were detected in the phylogenetic study of *Meteterakis* in the

Ryukyu and the Japanese Archipelago, this might characterize the phylogeographic histories of parasites of reptiles in these areas.

Declaration of Competing Interests

The authors declare that they have no conflicts of interest.

Declaration of funding

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Data Availability Statement

The data that support this study will be shared upon reasonable request to the corresponding author.

References

Anderson, R. C. (2000). 'Nematode Parasites of Vertebrates: Their Development and Transmission,' 2nd edn. (CABI Publishing: Wallingford, UK.) doi:10.1079/9780851994215.0000

Badets, M., Whittington, I., Lalubin, F., Allienne, J-F., Maspimby, J-L., Bentz, S., Du Preez, L. H., Barton, D., Hasegawa, H., Tandon, V., Imkongwapang, R., Ohler, A., Combes, C., and Verneau, O. (2011). Correlating early evolution of parasitic platyhelminths to Gondwana breakup. *Systematic Biology* **60**, 762–731. doi:10.1093/sysbio/syr078

Baker, M. R. (1980). Revision of *Entomelas* Travassos, 1930 (Nematoda: Rhabdiasidae) with a review of genera and family. *Systematic Parasitology* **1**, 83–90. doi:10.1007/BF00009853

Blasco-Costa, I., and Poulin, R. (2013). Host traits explain the genetic structure of parasites: a meta-analysis. *Parasitology* **140**, 1316–1322. doi:10.1017/S0031182013000784

449 Bursey, C. R., Goldberg, S. R., and Telford, S. R. (2005). *Plagiorchis taiwanensis* (Digenea:
450 Plagiorchiidae), *Kurilonema markovi* (Nematoda: Rhabdiasidae) and other helminthes in
451 *Eumeces latiscutatus* (Scincidae) and *Takydromus tachydromoides* (Lacertidae) from
452 Japan. *Comparative Parasitology* **72**, 234–240. doi:10.1654/4170
453 Casiraghi, M., Bain, O., Guerrero, R., Martin, C., Pocacqua, V., Gardner, S. L., Franceschi,
454 A., and Bandi, C. (2004). Mapping the presence of *Wolbachia pipientis* on the phylogeny
455 of filarial nematodes: evidence for symbiont loss during evolution. *International Journal*
456 *for Parasitology* **34**, 191–203. doi:10.1016/j.ijpara.2003.10.004
457 Clement, M., Posada, D., and Crandall, K. A. (2000). TCS: a computer program to estimate
458 gene genealogies. *Molecular Ecology* **9**, 1657–1659. doi:10.1046/j.1365-
459 294x.2000.01020.x
460 Falk, B. G., and Perkins, S. L. (2013). Host specificity shapes population structure of
461 pinworm parasites in Caribbean reptiles. *Molecular Ecology* **22**, 4576–4590.
462 doi:10.1111/mec.12410
463 Furukawa, M., and Fujitani, T. (2014). Comparative study on Pleistocene paleogeographic
464 maps of Ryukyu Arc. *Bulletin of the Faculty of Science University of the Ryukyus* **98**, 1–8.
465 Greer, A. E., and Shea, G. M. (2000). A major new head scale character in non-lygosomine
466 scincid lizards. *Journal of Herpetology* **34**, 629–634. doi:10.2307/1565286
467 Hasegawa, H. (1985). Helminth parasites of reptiles from Okinawa, Japan. *The Biological*
468 *Magazine Okinawa* **23**, 1–11.
469 Hasegawa, H. (1989). *Neoentomelas asatoi* gen. et sp. n. (Nematoda: Rhabdiasidae) and
470 *Hedruris miyakoensis* sp. n. (Nematoda: Hedruridae) from skinks of the Ryukyu
471 Archipelago, Japan. *Proceedings of the Helminthological Society of Washington* **56**, 145–
472 150.
473 Hasegawa, H. (1990). Helminths collected from amphibians and reptiles on Amami-oshima
474 Island, Japan. *Memoirs of the National Science Museum (Tokyo)* **23**, 83–92.
475 Hasegawa, H. (1992). Parasitic helminths collected from amphibians and reptiles on Kume–
476 jima Island, Okinawa, Japan. *The Biological Magazine Okinawa* **30**, 7–13.
477 Haukisalmi, V., Hardman, L. M., Fedorov, V. B., Hoberg, E. P., and Henttonen, H. (2016).
478 Molecular systematics and Holarctic phylogeography of cestodes of the genus
479 *Anoplocephaloides* Baer, 1923 s. s. (Cyclophyllidae, Anoplocephalidae) in lemmings
480 (*Lemmus*, *Synaptomys*). *Zoologica Scripta* **45**, 88–102. doi:10.1111/zsc.12136
481 Hasegawa, H., and Iwatsuki, N. (1984). Helminth fauna of tree lizard, *Japarula polygonata* in
482 Okinawa Prefecture, Japan. *Akamata* **2**, 18–26.

Ikeda, H., Nishikawa, M., and Sota, T. (2012). Loss of flight promotes beetle diversification. *Nature Communications* **3**, 648. doi:10.1038/ncomms1659

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**, 772–780. doi:10.1093/molbev/mst010

Kisel, Y., and Barraclough, T. G. (2010). Speciation has a spatial scale that depends on levels of gene flow. *The American Naturalist* **175**, 316–334. doi:10.1086/650369

Koehler, A. V. A., Hoberg, E. P., Dokuchaev, N. E., Tranbenkova, N. A., Whitman, J. A., Nagorsen D. W., and Cook, J. A. (2009). Phylogeography of a Holarctic nematode, *Soboliphyme baturini*, among mustelids: climate change, episodic colonization, and diversification in a complex host–parasite system. *Biological Journal of the Linnean Society* **96**, 651–663. doi:10.1111/j.1095-8312.2008.01145.x

Kuzmin, Y. I., and Sharpilo, V. P. (2002). Rare and locally distributed helminth species of Palearctic: *Kurilonema markovi* (Nematoda, Rhabdiasidae), the lung parasite of the Japanese five-lined skink, *Eumeces latiscutatus* (Reptilia, Sauria, Scincidae). *Vestnik Zoologii* **36**, 61–64.

Kuzmin, Y. I. (2013). Review of Rhabdiasidae (Nematoda) from the Holarctic. *Zootaxa* **3639**, 1–76. doi:10.11646/zootaxa.3639.1.1

Kuzmin, Y. I., and Tkach, V. V. (2011). Description of a new species of *Kurilonema* (Nematoda: Rhabdiasidae) from lungs of the skink *Sphenomorphus abdictus aquilonius* (Reptilia: Squamata: Scincidae) in the Philippines. *Journal of Parasitology* **97**, 506–512. doi:10.1645/GE-2578.1

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., and Calcott, B. (2017). PartitionFinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution* **34**, 772–773. doi:10.1093/molbev/msw260

Leigh, J. W., and Bryant, D. (2015). POPART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* **6**, 1110–1116. doi:10.1111/2041-210X.12410

Linkem, C. W., Diesmos, A. C., and Brown, R. M. (2011). Molecular systematics of the Philippine forest skinks (Squamata: Scincidae: *Sphenomorphus*): testing morphological hypotheses of interspecific relationships. *Zoological Journal of the Linnean Society* **163**, 1217–1243. doi:10.1111/j.1096-3642.2011.00747.x

Makino, T., Okamoto, T., Kurita, K., Nakano, T., and Hikida, T. (2020). Origin and

517 intraspecific diversification of the scincid lizard *Ateuchosaurus pellopleurus* with
518 implications for historical island biogeography of the Central Ryukyus of Japan.
519 *Zoologischer Anzeiger* **288**, 1–10. doi:10.1016/j.jcz.2020.06.008
520 Messing, J. (1983). New M13 vectors for cloning. In 'Methods in Enzymology Recombinant
521 DNA, Part C Vol. 101'. (Eds R. Wu, L. Grossman, and K. Moldave.) pp. 20–78.
522 (Academic Press: New York, USA) doi:10.1016/0076-6879(83)01005-8
523 Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., von Haeseler,
524 A., and Lanfear, R. (2020). IQ-TREE 2: New models and efficient methods for
525 phylogenetic inference in the genomic era. *Molecular Biology and Evolution* **37**, 1530–
526 1534. doi:10.1093/molbev/msaa015
527 Okamoto, T. (2017). Historical biogeography of the terrestrial reptiles of Japan: a comparative
528 analysis of geographic ranges and molecular phylogenies. In 'Species Diversity of Animals
529 in Japan'. (Eds M. Motokawa, and H. Kajihara.) pp. 135–163. (Springer Japan KK: Tokyo,
530 Japan.) doi:10.1007/978-4-431-56432-4_5
531 Osozawa, S., Shinjo, R., Armid, A., Watanabe, Y., Horiguchi, T., and Wakabayashi, J. (2012).
532 Palaeogeographic reconstruction of the 1.55 Ma synchronous isolation of the Ryukyu
533 islands, Japan, and Taiwan and inflow of the Kuroshio warm current. *International*
534 *Geology Review* **54**, 1369–1388. doi:10.1080/00206814.2011.639954
535 Ota, H. (1998). Geographic patterns of endemism and speciation in amphibians and reptiles of
536 the Ryukyu Archipelago, Japan, with special reference to their paleogeographical
537 implications. *Researches on Population Ecology* **40**, 189–204. doi:10.1007/BF02763404
538 Ota, H., Miyaguni, H., and Hikida, T. (1999). Geographic variation in the endemic skink,
539 *Ateuchosaurus pellopleurus* from the Ryukyu Archipelago. *Journal of Herpetology* **33**,
540 106–118. doi:10.2307/1565549
541 Peterson, M. A., and Denno, R. F. (1998). The influence of dispersal and diet breadth on
542 patterns of genetic isolation by distance in phytophagous insects. *The American Naturalist*
543 **152**, 428–446. doi:10.1086/286180
544 Prosser, S. W. J., Velarde-Aguilar, M. G., León-Règagnon, V., and Hebert, P. D. N. (2013).
545 Advancing nematode barcoding: a primer cocktail for the cytochrome c oxidase subunit I
546 gene from vertebrate parasitic nematodes. *Molecular Ecology Resources* **13**, 1108–1115.
547 doi:10.1111/1755-0998.12082
548 Pyron, R., Burbrink, F. T., and Wiens, J. J. (2013). A phylogeny and revised classification of
549 Squamata, including 4161 species of lizards and snakes. *BMC Ecology and Evolution* **13**,
550 93. doi:10.1186/1471-2148-13-93.

551 Rambaut, A., Drummond, A. J., Xie, D., Baele, G., and Suchard, M. A. (2018). Posterior
552 summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology* **67**, 901–
553 904. doi:10.1093/sysbio/syy032
554 Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B.,
555 Liu, L., Suchard, M. A., and Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian
556 phylogenetic inference and model choice across a large model space. *Systematic Biology*
557 **61**, 539–542. doi:10.1093/sysbio/sys029
558 Sands, A. F., Apanaskevich, D. A., Matthee, S., Horak, I. G., and Matthee, C. A. (2017). The
559 effect of host vicariance and parasite life history on the dispersal of the multi-host
560 ectoparasite, *Hyalomma truncatum*. *Journal of Biogeography* **44**, 1124–1136.
561 doi:10.1111/jbi.12948
562 Sata, N. (2015). Distribution of parasitic nematodes in Japan with host–parasite relationship
563 of lizards of *Plestiodon* (Reptilia:Squamata: Scincidae). *Comparative Parasitology* **82**, 17–
564 24. doi:10.1654/4728.1
565 Sata, N. (2018). Allopatric speciation of *Meteterakis* (Heterakoidea: Heterakidae), a highly
566 dispersible parasitic nematode, in the East Asian islands. *Parasitology International* **67**,
567 493–500. doi:10.1016/j.parint.2018.04.008
568 Shcherbak, N. N., and Sharpilo. V. P. (1969). Data on taxonomy, ecology and parasitology of
569 reptiles from the Kuril Islands. Communication I. *Vestnik Zoologii* **4**, 18–25.
570 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5:
571 molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance,
572 and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739.
573 doi:10.1093/molbev/msr121
574 Telford, S. R. (1997). 'The Ecology of a Symbiotic Community. Vol. 1'. (Krieger: Florida,
575 USA.)
576 Tkach, V. V., Kuzmin, Y. I., and Snyder, S. D. (2014). Molecular insight into systematics, host
577 associations, life cycles and geographic distribution of the nematode family Rhabdiasidae.
578 *International Journal for Parasitology* **44**, 273–284. doi:10.1016/j.ijpara.2013.12.005
579 Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the
580 sensitivity of progressive multiple sequence alignment through sequence weighting,
581 position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**,
582 4673–4680. doi:10.1093/nar/22.22.4673
583 Wickström, L. M., Haukisalmi, V., Varis, S., Hantula, J., Fedorov, V. B., and Henttone, H.
584 (2003). Phylogeography of the circumpolar *Paranoplocephala arctica* species complex

585 (Cestoda: Anoplocephalidae) parasitizing collared lemmings (*Dicrostonyx* spp.). *Molecular*
586 *Ecology* **12**, 3359–3371. doi:10.1046/j.1365-294X.2003.01985.x

587 **Table 1. Number of specimens and genetic composition of each sampling locality examined in this study**

588 The H6 sequence marked with an asterisk (*) was distinguished from the 'true' H6 by a single indel-derived polymorphism

Locality no.	Locality name	Island group	*n* (mtDNA)	Haplotype (mtDNA)	*n* (*18S–28S*)	Haplotype (*18S–28S*)
1	Takeshima Island, Kagoshima	Mishima Islands	1	M1	n/a	n/a
2	Iojima Island, Kagoshima	Mishima Islands	5	M1, M2	3	H2
3	Suwanosejima Island, Kagoshima	Tokara Islands	3	T1	3	H1
4	Kodakarajima Island, Kagoshima	Tokara Islands	4	T2, T3	1	H6*
5	Amami City, Amamioshima Island, Kagoshima	Amami Islands	11	Aa1, Aa2, Aa3, Aa4, Aa5	6	H6
6	Yamato Village, Amamioshima Island, Kagoshima	Amami Islands	13	Ay1, Ay2, Ay3, Ay4, Ay5, Ay6, Ay7, Ay8, Ay9	7	H6
7	Tokunoshima Town, Tokunoshima Island, Kagoshima	Amami Islands	6	Tku1	5	H4, H5
8	Isen Town, Tokunoshima Island,Kagoshima	Amami Islands	1	Tku2	1	H4
9	Iheyajima Island, Okinawa	Okinawa Islands	7	I1, I2, I3, I4	5	H6
10	Ogimi Village, Okinawajima Island, Okinawa	Okinawa Islands	6	O1, O2, O3	4	H3, H6
11	Hamahigajima Island, Okinawa	Okinawa Islands	5	Hm1	2	H8
12	Tokashikijima Island, Okinawa	Okinawa Islands	3	Tka1, Tka2	3	H8
13	Kumejima Island, Okinawa	Okinawa Islands	9	K1, K2, K3, K4 K5, K6	7	H7, H8

589

FIGURE LEGENDS

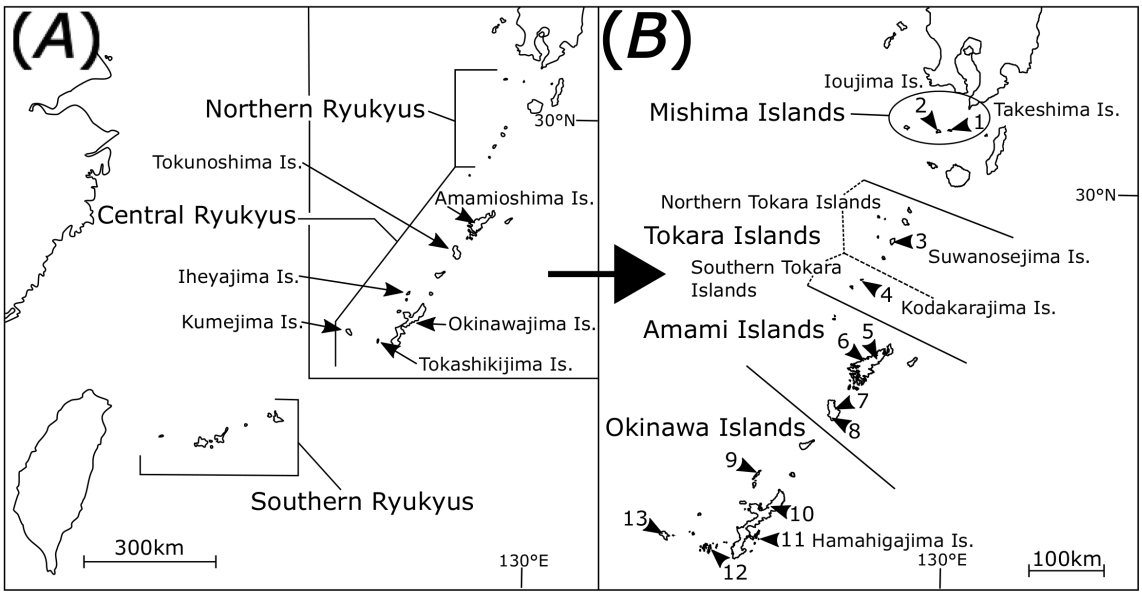
Fig. 1. Map of the Ryukyu Archipelago. (*A*) The entire studied area in the Ryukyu Archipelago; (*B*) sampling localities in the Ryukyu Archipelago. The locality numbers are consistent with those in Table 1.

Fig. 2. A Bayesian phylogenetic tree for rhabdiasid nematodes based on the nuclear *18S–28S* marker. The numbers on branches represent Bayesian posterior probabilities and bootstrap values for maximum likelihood.

Fig. 3. A Bayesian intraspecific phylogenetic tree for *Neoentomelas asatoi* based on mitochondrial markers. The numbers on branches represent Bayesian posterior probabilities and bootstrap values for maximum likelihood. The details of haplotype numbers are listed in Table 1. Vertical white bars indicate the Okinawa Islands' subclades, single gray bars indicate the Northern Ryukyus' and southern Tokara Islands' subclades, and black bars indicate the Amami Islands' subclades.

Fig. 4. Statistical parsimony network for the *18S–28S* haplotypes of *Neoentomelas asatoi*. The details of haplotype numbers are listed in Table 1.

Fig. 5. The geological distribution of the mitochondrial haplotypes of *Neoentomelas asatoi*. The ingroup phylogenetic tree is identical to that in Fig. 3. The details of haplotype numbers are listed in Table 1. Arrows indicate colonization events of *N. asatoi*. Solid circles on the phylogenetic tree and arrows indicate the colonization events from Iheyajima Island to Amamioshima Island, and solid star shapes indicate the colonization events among Okinawajima Island, Kodakarajima Island and Suwanosejima Island. Dotted lines indicate the geological distribution of two major clades of the host lizard (*Ateuchosaurus pellopleurus*), Clade Ok: a clade comprising the populations in the Okinawa Islands; Clade A + NR: a clade comprising the populations in the Amami Islands and northern Ryukyus.



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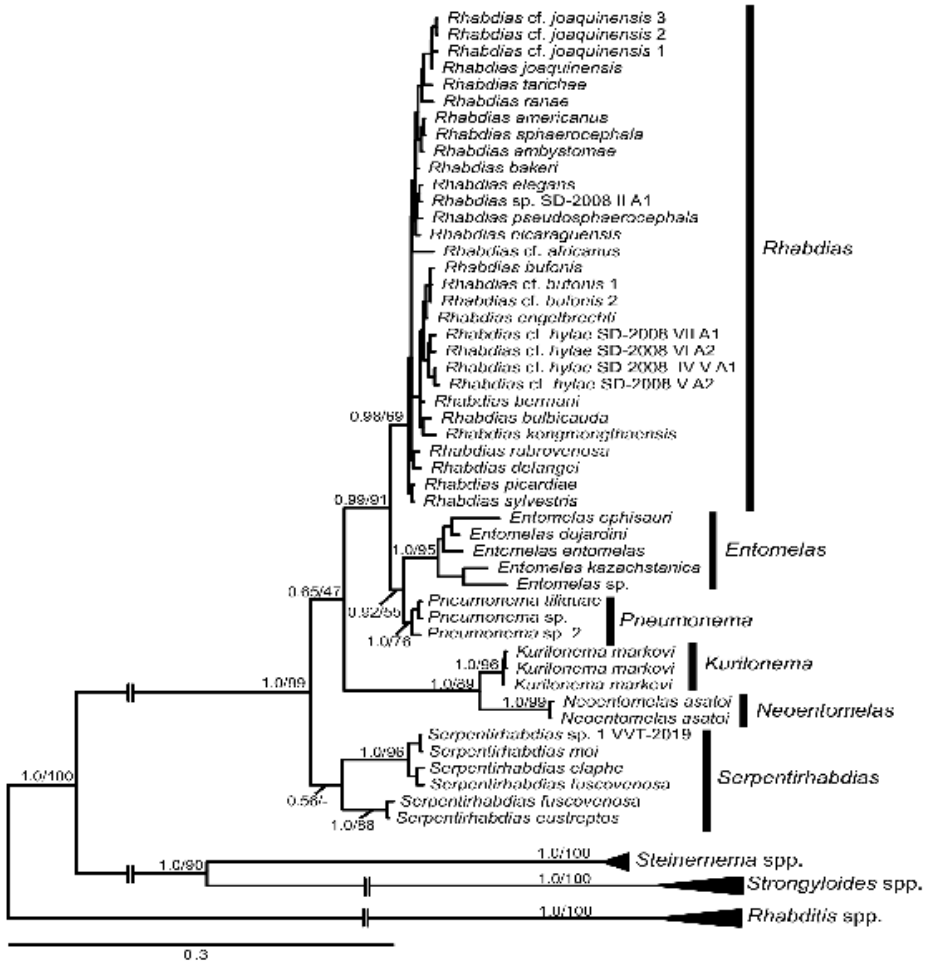

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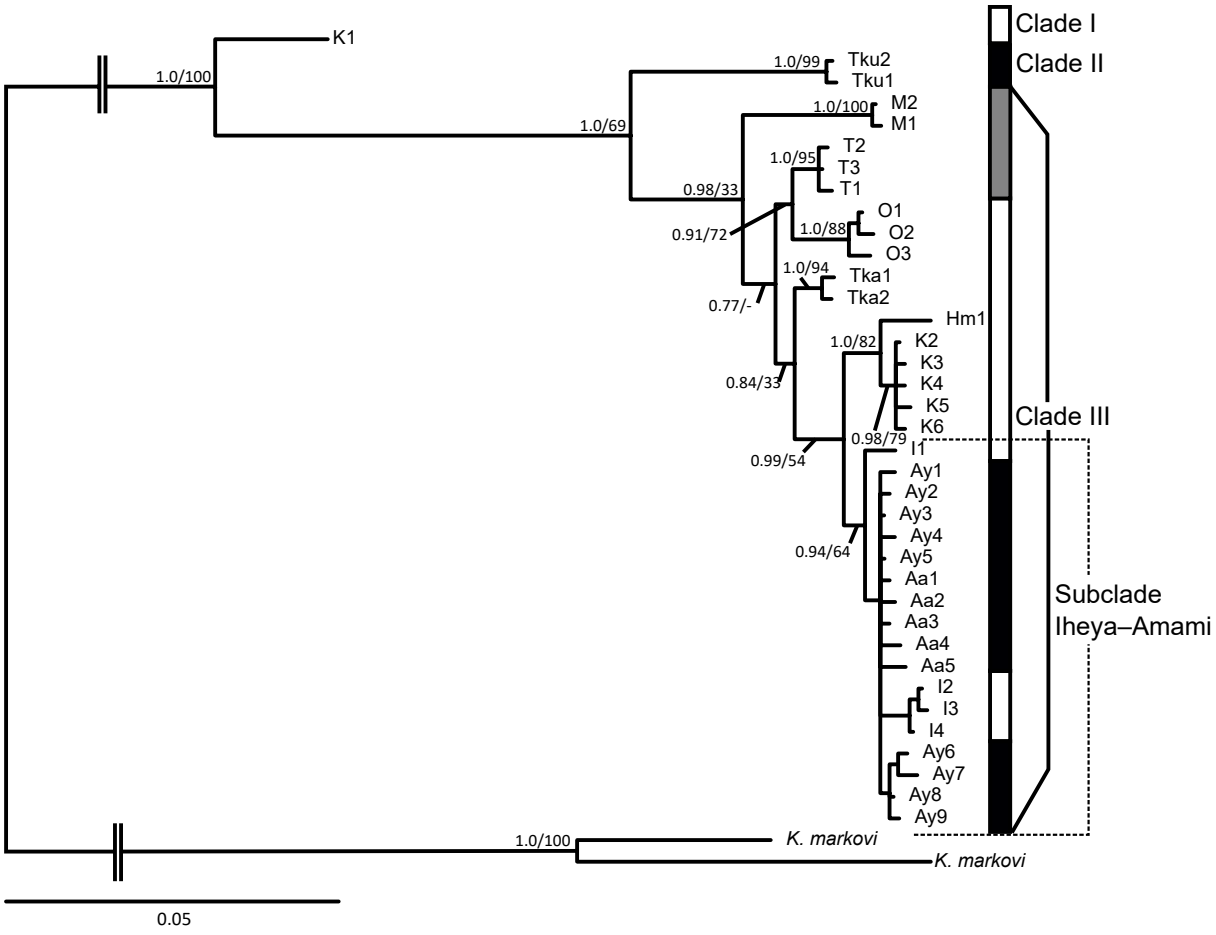
629



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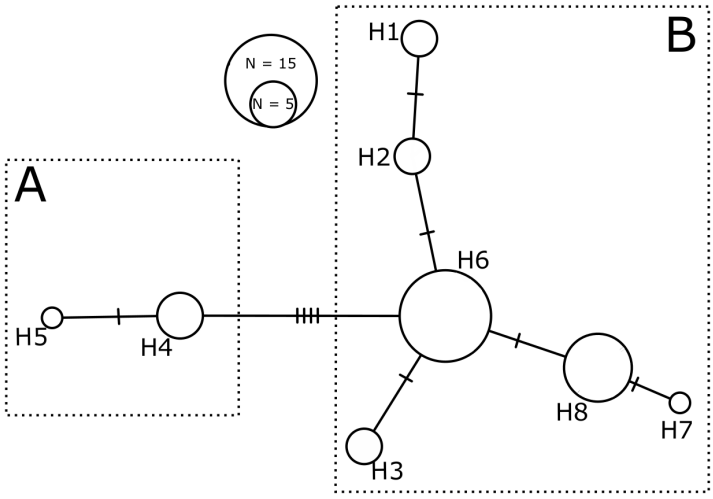
637



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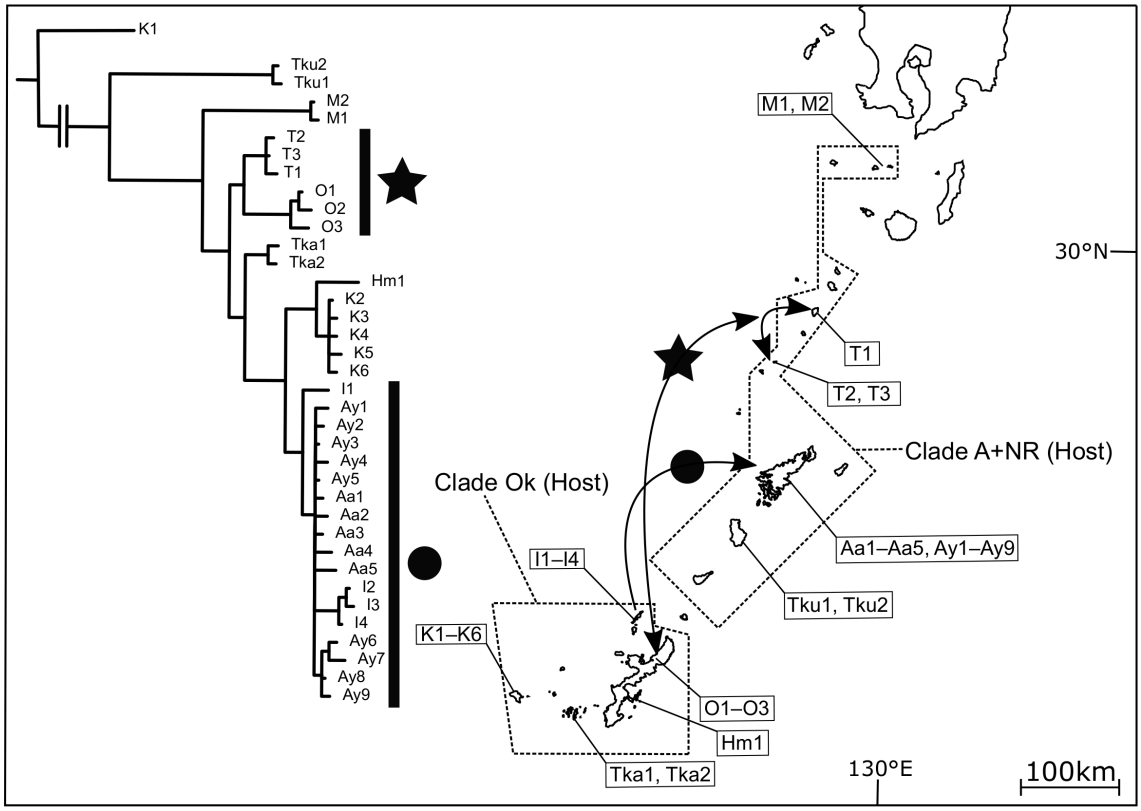

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