Divergence Process of *Sophiodela* Tiger Beetles (Coleoptera, Cicindelidae) in Japan Inferred from Molecular Phylogeny

Kaoru Tsuji [1], **Teiji Sota** [2] **and Michio Hori** [3]

[1] Center for Ecological Research, Kyoto University, 2-Hirano, Otsu, Shiga, 520–2113 Japan
E-mail: tsuji@ecology.kyoto-u.ac.jp
[2] Department of Zoology, Graduate School of Science, Kyoto University, Sakyo, Kyoto, 606–8502 Japan
[3] Kyoto University, Yoshida-Honmachi, Sakyo-ku, Kyoto, 606–8501 Japan

Abstract   Three *Sophiodela* tiger beetle species (Coleoptera, Cicindelidae) are distributed in Japan. To infer their divergence process, we examined mitochondrial cytochrome c oxidase subunit I (COI) sequences of *Sophiodela* populations in Japan and mainland East Asia. Our divergence time estimation suggests that *S. ferriei* (Fleutiaux, 1894) in the Amami Islands diverged 2.2 million years ago (mya) from a common ancestor of *S. chinensis* (DeGeer, 1774) in China and Korea, and of *S. japonica* (Thunberg, 1781) and *S. okinawana* (Nakane, 1957) in Japan. The divergence time of the latter two groups was estimated at 1.1 mya during the late Early Pleistocene. The ancestor of *S. japonica* and *S. okinawana* derived from *S. chinensis* may have expanded its range over the ancient Japanese archipelago from Honshu to Okinawajima among the Ryukyu Islands. Its immigration to Okinawajima during the Early Pleistocene may have occurred via a land bridge between the present locations of the Tokara Islands (Tokara Ridge) and Okinawajima, without passing the Amami Islands (southeast of the land bridge) and contacting with *S. ferriei*. This land bridge disappeared following the development of the Okinawa Trough in the Middle Pleistocene, resulting in complete isolation of the population (now *S. okinawana*) on Okinawajima from the population on the main islands of Japan (now *S. japonica*) around 0.9 mya.
Key words: Divergence time, Pleistocene, Tokara Ridge, East Asia, Mitochondrial cytochrome c oxidase subunit I (COI).

Introduction

Sophiodela was proposed as a subgenus of *Cicindela* by Nakane (1955), who designated *Cicindela japonica* Thunberg, 1781 as the type species, which had been treated as a subspecies of *C. chinensis* DeGeer, 1774 by Horn (1915). Rivalier (1961) proposed another subgenus, *Sericina*, comprising *C. chinensis*, *C. ferriei* Fleutiaux, 1894, and *C. cyanea* Fabricius, 1787, and treated '*japonica*' as a subspecies of *C. chinensis*. Wiesner (1992) correctly arranged *Sericina* as a junior synonym of *Sophiodela*, but included *C. cyanea* and counted '*japonica*' as a subspecies of *C. chinensis*, following Rivalier (1961). Although this classification has been widely accepted, our recent molecular phylogenetic study of *Sophiodela* and related groups (Tsuji *et al*., 2016) showed that *Sophiodela* is sister to the genus *Cosmodela*, and not directly related to the *Cicindela* genus members evaluated (i.e., subgenera *Cicindela*, *Pachydela*, and *Tribonia*). Recently, Fukuda *et al*. (2019) proposed the treatment of *Sophiodela* as a genus based on the characteristic structure of the internal sac of the male genitalia. Fukuda *et al*. (2019) recognized four species from East Asia (*S. chinensis*, *S. ferriei*, *S. japonica*, and *S. okinawana* (Nakane, 1957)) as members of *Sophiodela*, but excluded *C. cyanea* from the Indian subcontinent, and moved it to the *Cicindela* subgenus *Pancallia*. Thus, the genus *Sophiodela* is endemic to East Asia. In addition to the rearrangement of the *Sophiodela* species, new insights for *Sophiodela* subspecies are proposed (Fukuda *et al*., 2015; Tsuji *et al*., 2016; Fukuda *et al*., 2019).

Sophiodela ferriei populations in Amamioshima and Tokunoshima have been treated as subspecies *S. f. ferriei* and *S. f. indigonacea* (Wiester, 1992), but Fukuda *et al*. (2015) synonymized these based on genital characters. Similarly, the uncertain status for two subspecies of *S. chinensis* and *S. c. chinensis* from China and *S. c. flammifera* from Korea was reported (Tsuji *et al*., 2016; Fukuda *et al*., 2019).

Of the four species of *Sophiodela*, only one species, *S. chinensis*, occurs in mainland East Asia (China and Korea), whereas in Japan, three species, *S. japonica*, *S. ferriei*, and *S. okinawana* occur, and in detail, they are observed in the main islands of Japan (Kyushu, Shikoku, and Honshu), the Amami Islands, and the Okinawa Islands, respectively. The diversification of *Sophiodela* in Japan is interesting from the perspective of historical biogeography. We previously discussed their divergence process (Tsuji *et al*., 2016), but not in detail, especially for *S. japonica* and *S. okinawana*. Osozawa *et al*. (2016) performed a molecular phylogenetic analysis of *Sophiodela* species and proposed that the divergence of three species, excluding *S. ferriei*, was facilitated by the synchronous isolation of Ryukyu and other Japanese islands 1.55 million years ago (mya) (Osozawa *et al*., 2012). However, they did not consider the divergence process of *S. japonica* and *S. okinawana* in detail. In this study, we conducted a phylogeographic analysis of *Sophiodela* species in Japan based on mitochondrial cytochrome c oxidase I (COI) sequence data and inferred the colonization history of *Sophiodela* species in Japan. Although our study is similar to that by Osozawa *et al*. (2016), we used different approach to estimate divergence time with a larger sample size and provided a novel explanation for the historical process leading to the enigmatic distribution of the most ancient *Sophiodela* species, *S. ferriei*, in the Amami Islands between the ranges of recently diverged sister species, *S. japonica* and *S. okinawana* (Tsuji *et al*., 2016).

Material and Methods

In this study, we used four *Sophiodela* species, as well as *Cosmodela batesi* (Fleutiaux, 1893) as an outgroup species (Fig. 1). We performed molecular phylogenetic analysis of 262 beetles preserved in 99% ethanol, using 736-bp sequences of the mitochondrial COI gene (Table 1 & Fig. 1). DNA extraction, polymerase chain reaction (PCR) and sequencing methods were as described in Tsuji *et al*. (2016). All sequence data were deposited in the DNA Data Bank of Japan (DDBJ; see Table 1 for accession numbers). We used 89 sequences obtained in this study and 23 previously published sequences (Tsuji *et al*., 2016) in the following analysis.

We conducted a maximum likelihood (ML) analysis with COI sequences using the RAxML version 8 software (Stamatakis, 2014). Based on an optimal partitioning scheme search using the PartitionFinder software (Lanfer *et al*., 2012), the first and second codon positions and the third codon position were treated as separate partitions, and the GTR+G (general time reversible model with gamma distribution for rate heterogeneity) substitution model was applied to each partition individually. We conducted a RAPID ML analysis with 1,000 bootstrap analyses. To estimate divergence time based on COI sequences, we used the BEAST2 version 2.5.0 software (Bouckaert *et al*., 2014). We performed partitioned analysis as in the ML analysis, but applied the HKY+G substitution mode to the first/second codon position partition and GTR+G to the third codon position according to the PartitionFinder analysis results. The tree prior was the birth–death process model, and a strict clock model was used. For time calibration, we set the node age prior for the most recent common ancestor of *Sophiodela* (*S. chinensis* + *S. japonica* + *S. okinawana* + *S. ferriei*) as a normal distribution with a mean of 2.52 Ma and standard deviation (SD) of 0.5 mya, referring to the divergence time estimation provided by Tsuji *et al*. (2016). A Markov chain Monte Carlo (MCMC) run was conducted for 50 million generations, sampling every 5,000 generations. The results were verified using the TRACER version 1.7 software

Table 1. Sampling sites, cytochrome c oxidase subunit I (COI) haplotypes, and accession numbers.

Species 　Locality	COI haplotype ID [DDBJ accession number] (number of individuals)
S. chinensis	
Guizhou, China	C15 [AB822059] (1)
Henan, China	C11 [AB822043] (2), C13 [AB822044] (1), C14 [AB822053] (1)
Mt.Tianzhu, Anhui, China	C5 [LC421754] (1), C6 [LC421755] (1), C8 [AB822030] (1), C11 [AB822043*] (1)
Sichuan, China	C16 [AB822071] (1)
Gyongsangnam, Korea	C7 [AB822034*] (1), C10 [AB822031] (1)
North Chungcheong, Koera	C9 [LC421756*] (1)
Ulsan, Korea	C7 [AB822034] (9), C9 [LC421756] (1), C12 [AB822035] (1)
S. ferriei	
Amamioshima	F22 [LC421760] (1), F26 [LC421763] (1), F27 [LC421764] (2), F30 [LC421767] (1), F31 [LC421768] (1), F32 [AB822050] (3), F36 [LC421771] (1), F37 [LC421772] (1), F38 [AB822051] (1), F39 [LC421773] (1), F42 [LC421776] (1), F43 [LC421777] (1)
Tokunoshima	F23 [LC421761] (1), F24 [AB822039] (5), F25 [LC421762] (10), F28 [LC421765] (1), F29 [LC421766] (2), F31 [LC421768*] (1), F32 [AB822040] (1), F33 [LC421769] (1), F34 [AB822041] (1), F35 [LC421770] (1), F40 [LC421774] (1), F41 [LC421775] (1), F44 [LC421778] (1)
S. japonica	
Aomori	J57 [LC421789] (1), J58 [AB822047*] (4), J73 [LC421799] (2), J74 [AB822037] (3)
Fukuoka	J48-2 [LC421782] (1)
Hiroshima	J55 [AB822045*] (1), J79 [LC421806*] (1)
Ishikawa	J54 [LC421787] (1), J58 [AB822047*] (1), J95 [LC421820] (1), J96 [AB822052] (1), J97 [LC421821] (1)
Kagoshima	J51 [LC421784*] (1), J53 [LC421786*] (1), J55 [AB822045] (1), J79 [LC421806] (1), J80-2 [LC421807] (1), J81 [LC421808] (1), J94 [LC421819] (1)
Kanagawa	J45 [LC421779] (5), J50-1 [LC421805, LC426080] (8), J50-2 [LC421804] (1), J59-1 [AB822048] (9), J59-2 [LC426081] (1), J60 [LC421790] (1), J61 [AB822029] (1)
Kochi	J71 [AB822049*] (2), J107 [LC421831] (2), J108 [LC421832] (1), J106 [LC421830] (1)
Kuchinoerabujima	J80-1 [LC426078] (4)
Kumamoto	J52 [LC421785] (1), J53 [LC421786] (1)
Kyoto	J46 [LC421780] (1), J47 [LC421781] (2), J58 [AB822047*] (1), J71[AB822049*] (1), J72 [LC421798] (1), J77 [LC421802] (4), J78 [LC421803] (2), J76 [LC421801] (1), J93 [LC421818*] (1)
Mie	J58 [AB822047*] (4), J90 [LC421815] (1), J91 [LC421816] (1), J92 [LC421817] (1), J93 [LC421818] (1)
Miyazaki	J48-2 [LC421782*] (2), J51 [LC421784] (2), J53 [LC421786*] (1), J56 [LC421788] (1), J69 [LC421797] (1), J75 [LC421800] (1)
Nagano	J59-1 [AB822048*] (1)
Nagasaki	J48-1 [LC426079] (1), J48-2 [LC421782*] (2)
Niigata	J58 [AB822047*] (3), J87 [LC421812] (1), J88 [LC421813] (1), J89 [LC421814] (1)
Shimane	J48-2 [LC421782*] (1), J55 [AB822045*] (2), J68 [LC421796] (1), J71 [AB822049], (5), J79 [LC421806*] (2)
Tsushima	J49 [LC421783] (8), J62 [LC421791] (4), J63 [LC421792] (2), J64 [LC421793] (1), J65 [LC421794] (1), J66 [LC421795] (1), J67 [AB822038] (2), J105 [LC421829] (2)
Wakayama	J58 [AB822047] (2), J77 [LC421802*] (2), J84 [LC421809] (1), J85 [LC421810] (1), J86 [LC421811] (1)
Yakushima	J80-2 [LC421807*] (10), J80-3 [LC426077] (1), J83 [AB822046] (1), J98 [LC421822] (2), J99 [LC421823] (2), J100-1 {LC426076] (1), J100-2 [LC421824] (1), J101 [LC421825] (1), J102 [LC421826] (1), J103 [LC421827] (1), J104 [LC421828] (1)
S. okinawana	
Okinawajima	O17 [LC421757] (1), O18 [AB822033] (10), O19 [AB822032] (1), O21 [LC421759] (1)
Ishigakijima	O19 [AB822032*] (7), O20 [LC421758] (1)
C. batesi	
Taipei, Taiwan	B1 [LC421750] (1), B2 [LC421751] (1)
Nantou, Taiwan	B3 [LC421752] (1)
Iriomotejima	B4 [LC421753] (7)

Haplotypes J50-2 and J80-1 include undetermined nucleotides (N). Asterisks (*) indicate accession numbers of identical haplotypes previously registered from other localities.

(Rambaut *et al*., 2018). An age-calibrated tree was obtained after removing the initial 1,000 trees as burn-in. We constructed statistical parsimony networks (TCS networks; Templeton *et al*., 1992) for the three Japanese *Sophiodela* species using the PopART program (Population Analysis with Reticulate Trees; available at http://popart.otago.ac.nz).

Results and Discussion

Haplotypes of four *Sophiodela* species were grouped separately from one another (Figs. 1 & 2). *Sophiodela ferriei* populations in Amamioshima and Tokunoshima that have previously been treated as subspecies (Wiester, 1992, but see Fukuda *et al*., 2015) were not distinguished by the COI sequences (Figs. 1 & 2), which is consistent with Fukuda *et al*. (2015). Similarly, the sequences of the two subspecies of *S. chinensis* from China and Korea, were not discriminated (Figs. 1 & 2) as was found by Tsuji *et al*. (2016) (see also Fukuda *et al*., 2019). The time-calibrated COI gene tree (Fig. 3) suggests that *S. ferriei* diverged 2.2 mya (95% highest posterior density interval [HPDI], 1.2–3.3 mya) from the common ancestor of *S. chinensis*, *S. japonica*, and *S. okinawana*, followed by the divergence of *S. chinensis* from a common ancestor of *S. japonica* and *S. okinawana* 1.1 mya (95% HPDI, 0.5–1.7 mya). Finally, *S. japonica* and *S. okinawana* diverged 0.9 mya (95% HPDI, 0.4–1.4 mya). The divergence time between *S. chinensis* and a common ancestor of *S. japonica* and *S. okinawana*, 1.1 mya, was more recent than the time proposed by Osozawa *et al*. (2016), who assumed that these taxa diverged 1.55 mya after the opening of straits around the Ryukyu and other Japanese Islands based on their hypothesis (Osozawa *et al*., 2012). Note that we calibrated divergence time based on the COI molecular clock (Papadopaulou *et al*., 2010; see Tsuji *et al*., 2016 for details) and not geological evidence. However, our results do not contradict the proposition by Osozawa *et al*. (2016) that the divergence of the three *Sophiodela* species occurred after 1.55 mya.

Haplotype diversity in *S. okinawana* was small (5 haplotypes; nucleotide diversity, π = 0.0016; Fig. 2), likely due to its small population size. The most recent common ancestor (tMRCA) of *S. okinawana* was estimated at 0.2 mya (95% HPDI, 0.05–0.3 mya; Fig. 3). We found as many as 68 haplotypes in *S. japonica*, which occurred in many islands over a much wider range (Fig. 2), with tMRCA of 0.5 mya (95% HPDI, 0.2–0.8 mya; Fig. 3), and higher nucleotide diversity (π = 0.011) than those of *S. okinawana* and *S. ferriei* (π = 0.007). *Sophiodela japonica* haplotypes were highly divergent among regions. Haplotypes from Yakushima (including those from Kuchinoerabujima) and Tsushima diverged 0.3 mya (95% HPDI, 0.1–0.4 mya; Fig. 3) from those of Kyushu and western Honshu (Fig. 3), and were unique to those islands, except for one haplotype shared by Yakushima and Kyushu populations (Figs. 1 & 2). In contrast, the Kyushu population shared haplotypes with Honshu and Shikoku populations in addition to unique (private) haplotypes (Figs. 1 & 2). These findings suggest that populations in Yakushima–Kuchinoerabujima and Tsushima have long been isolated, whereas those in Kyushu and western Honshu diverged recently, and gene flow may have occurred. In Honshu and Shikoku, haplotypes were largely divergent among regions (western Honshu and Shikoku, Kinki, Kanto, and northern Honshu); however, some haplotypes were shared among regions (Figs. 1 & 2), suggesting recent divergence with gene flow among regions.

The geographic distribution of the three Japanese *Sophiodela* species is enigmatic, as the ranges of sister species *S. japonica* and *S. okinawana* are intervened by the range of *S. ferriei*, which colonized Japan much earlier than the other two species. The common ancestor of *S. japonica* and *S. okinawana*, derived from *S. chinensis*, likely immigrated to Japan from South Korea over a land bridge during the Middle Pleistocene, with one population colonizing the main islands of Japan (Kyushu, Shikoku, and Honshu). Another population, the ancestral *S. okinawana*, immigrated to Okinawajima
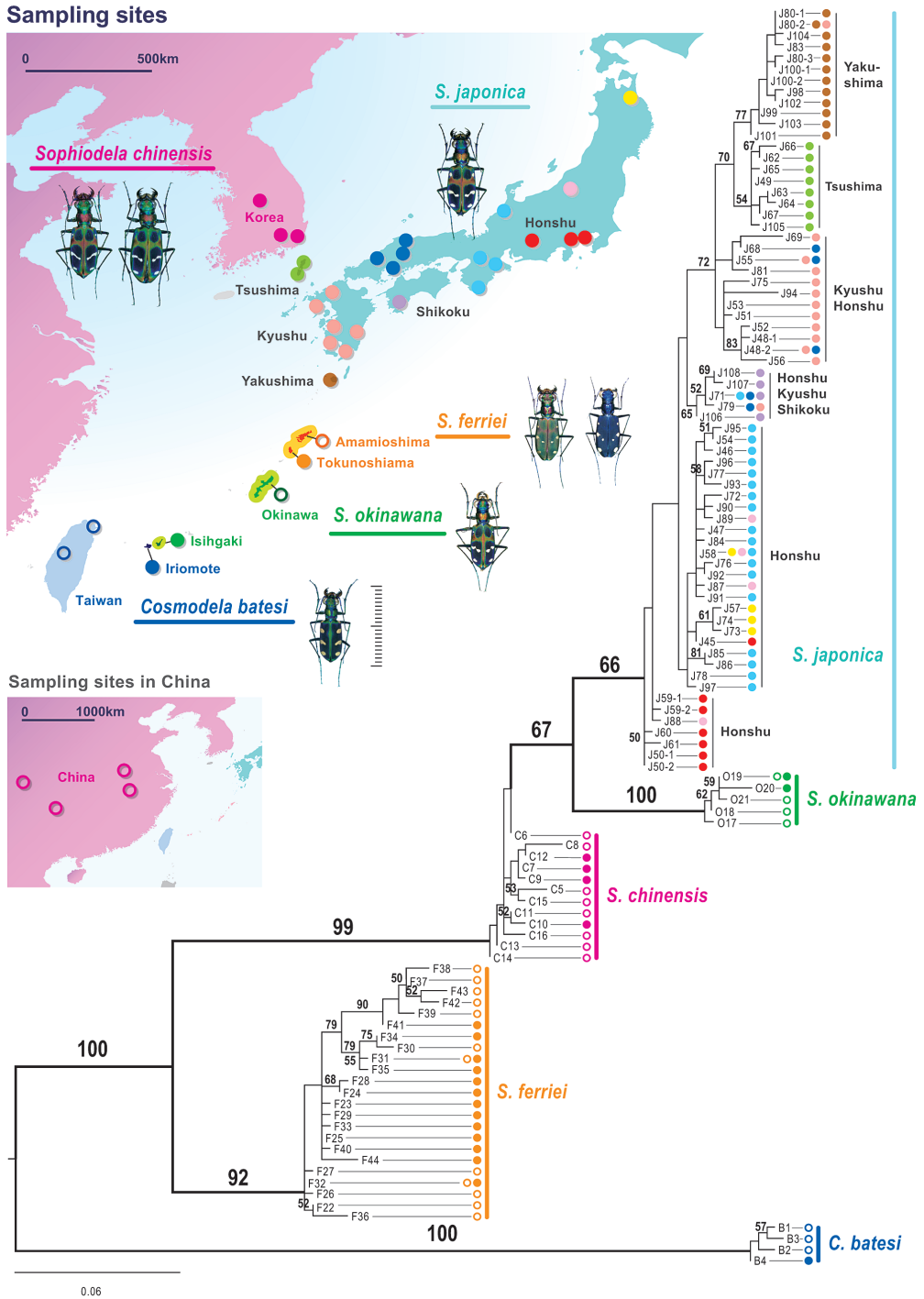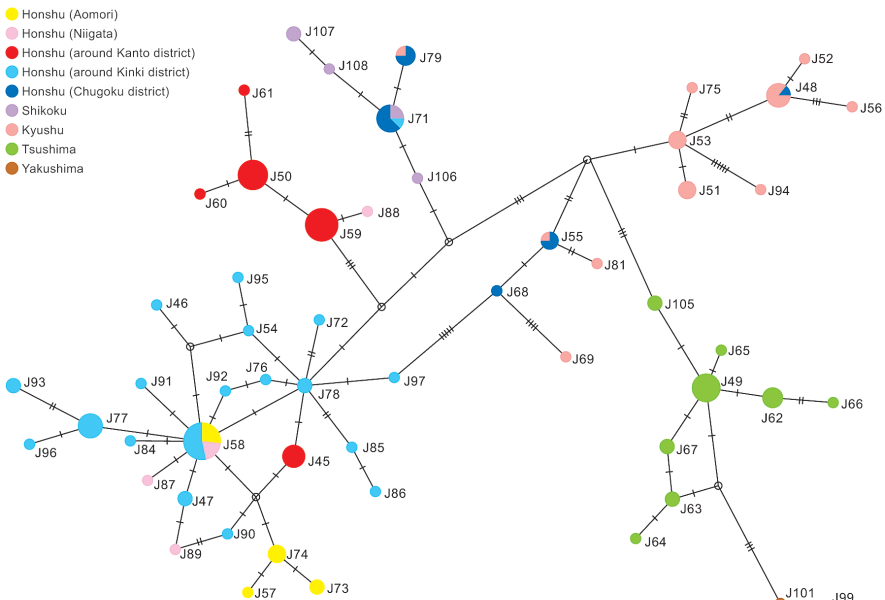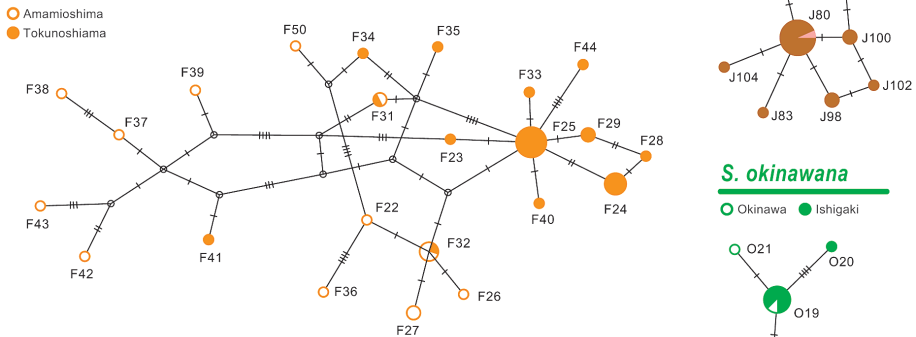
Fig. 1. Locations of sampling sites and maximum-likelihood tree based on 736-bp sequences of the COI gene. Numbers above branches are bootstrap percentages (shown when >50%). Circles with different color patterns indicate different species and geographic regions.
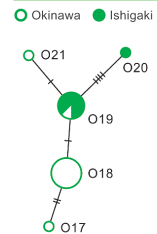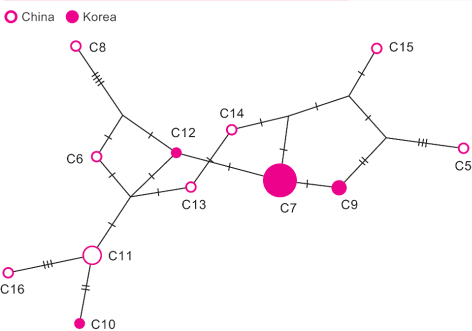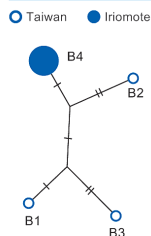
Fig. 2. Statistical parsimony networks of COI haplotypes (736 bp) for each species. Each circle represents one haplotype; its size is proportional to the number of individuals with that haplotype. Small gray open circles indicate missing haplotypes, and short lines indicate the number of base differences between haplotypes. Circles with different color patterns indicate geographic regions as in Fig. 1.
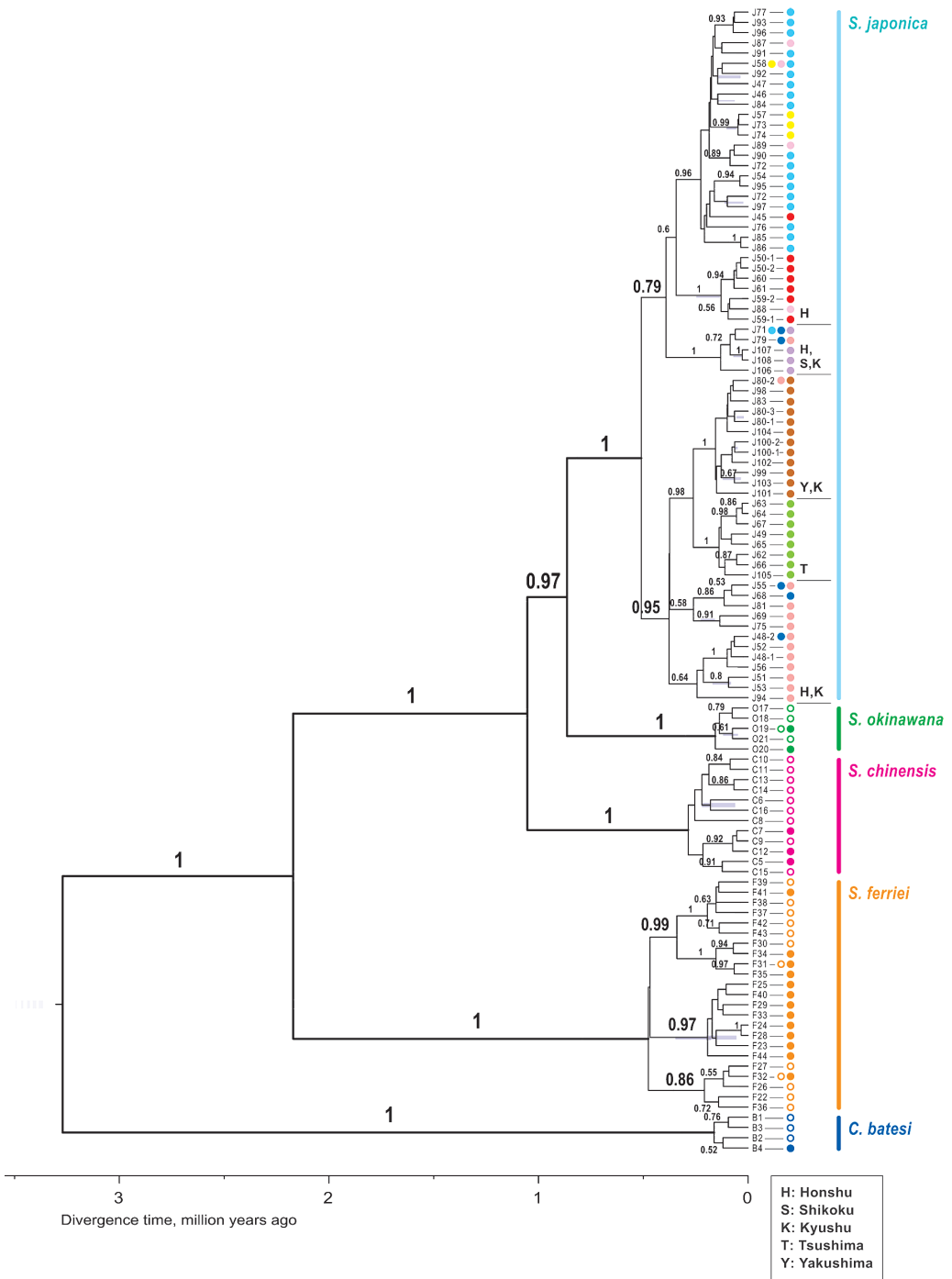
Fig. 3. Time-calibrated phylogenetic tree of *Sophiodela* species resulting from BEAST analysis of COI gene sequences. Horizontal gray bars show 95% highest posterior density intervals (HPDI) among divergence time estimates. Numbers on branches represent posterior probabilities (shown when >0.50). Circles with different color patterns indicate geographic regions as in Fig. 1.

by crossing the long distance between southern Kyushu and Okinawajima. In our previous paper, we questioned how the ancestral *S. okinawana* could have passed through the Amami Islands, where *S. ferriei* must have existed, and suggested two possibilities (Tsuji *et al*., 2016). The first is that *S. okinawana* might not have reached the Amami Islands due to the lack of available land bridges, and the second is that *S. okinawana* might have reached the Amami Islands but did not colonize successfully due to the presence of *S. ferriei* via ecological processes, such as reproductive interference and resource competition (Tsuji *et al*., 2016). Because *Sophiodela* beetles primarily inhabit inland areas, we assume that they are unable to undertake colonization by long distance flights across the sea. In the present study, we propose that the ancestral *S. okinawana* could have colonized Okinawajima without passing through the Amami Islands, based on the paleogeographical hypothesis of Kimura (1996, 2002), who proposed the existence of a land bridge connecting southern Kyushu and Okinawajima, via the Tokara Ridge northwest of the Amami Islands during the Early Pleistocene. During this period, the ancestral *S. okinawana* may have occurred widely on lands north of the Amami Islands, separated from *S. ferriei*. The land bridge disappeared in the Middle Pleistocene, following the development of the Okinawa Trough (Kimura, 1996, 2002), which may have resulted in extinction of the ancestral *S. okinawana* in the Tokara Ridge area, where islands were small. *Sophiodela* species occur on Yakushima and Kuchinoerabujima, but have not been found on smaller islands, perhaps indicating that *Sophiodela* populations cannot survive on small islands.

Recent human activity may have disturbed the natural distribution of *Sophiodela* species. *Sophiodela okinawana* is considered to have recently colonized Ishigakijima from Okinawajima (Fukuda, 2015; Satô & Takagi, 2006; Fig. 1). The existence of a common haplotype of *S. okinawana* from both islands supports this hypothesis (Figs. 1–3; Osozawa *et al*., 2016). In addition, *Cosmodela batesi* has recently been found on Iriomotejima (Hori, 2002; Satô & Takagi, 2006; Fig. 1), although this species was considered endemic to Taiwan. We found only one haplotype from the Iriomote population, which is closely related to haplotypes from Taiwan (Figs. 1 & 2; Osozawa *et al*., 2016). Low haplotype diversity in Iriomotejima suggests their recent colonization from somewhere in Taiwan. Satô & Takagi (2006) suggested that transportation development around these regions may have facilitated their immigration.

Acknowledgements

要　　約

辻かおる・曽田貞滋・堀　道雄：日本に生息するナミハンミョウ属（鞘翅目ハンミョウ科）の分岐過程に関する分子系統に基づく考察．――――日本のナミハンミョウ属 *Sophiodela* 3 種について，分岐過程を考察するため，日本と東アジア大陸（中国と韓国）に生息するナミハンミョウ属のミトコンドリア COI 遺伝子を解析した．分岐年代推定から，奄美群島に生息するアマミハンミョウはおよそ 220 万年前に大陸に広く生息

するチュウゴクナミハンミョウと日本のナミハンミョウ・オキナワハンミョウとの共通祖先から分岐したことが示唆された．その後，前期更新世末の 110 万年前ごろにチュウゴクナミハンミョウと，ナミハンミョウおよびオキナワハンミョウの共通祖先が分岐した．チュウゴクナミハンミョウから分岐したナミハンミョウとオキナワハンミョウの共通祖先は，本州から沖縄島まで分布を拡大したと考えられる．沖縄島への移入は，前期更新世に，現在のトカラ諸島が位置する場所と沖縄島の間に存在していた陸橋を伝って行われ，アマミハンミョウが生息する奄美群島は通過しなかったのではないだろうか．この陸橋は中期更新世にオキナワトラフの発達により消失した．これによって 90 万年前ごろまでにオキナワハンミョウは沖縄島に隔離され，日本本土のナミハンミョウ個体群から分岐したと考えられる．

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