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We conducted phylogenetic analyses using mitochondrial *COI* gene sequences of *Tylos granuliferus*, a semiterrestrial coastal isopod in East Asia, to clarify possible phylogeographic patterns and to assess relationships between present and past marine environments and genetic population structures. Our results strongly suggest the presence of four clades of *T. granuliferus*, one of which consists of three subclades. The distribution pattern of clades and subclades seems to have been affected by ocean current activities. Our results also suggest that historical changes in oceanic environments and the subsequence bifurcation of current streamlines affected the first and second divergences of *T. granuliferus* during the late Miocene and near the beginning of the Pliocene, respectively.

Key words: Crustacea, Malacostraca, Peracarida, Isopoda, *Tylos,* phylogeny, biogeography, mitochondrial DNA

INTRODUCTION

The processes underlying marine biodiversity constitute a topic of considerable scientific focus, as both phyletic and genetic diversity are higher among marine animals than among terrestrial animals (Gray, 1997). In coastal areas, species diversify along coastlines, showing unique distribution patterns (e.g., Burton and Lee, 1994). Because the distributions of their populations can be treated as one-dimensional (Pielou, 1979) and each population is expected to disperse through restricted routes, we can analyze the relationships between these distributions and phylogenetic, ecological, and geographic factors with comparative ease. Thus, phylogeographic research into coastal animals provides fruitful information not only about the individual biogeographic studies themselves, but also about the development of the marine biota.

The distributions of populations are affected by both environmental and historical factors (Avise, 2000). The effects of environmental factors, such as ocean currents, on coastal animals must be strong, especially because of their strong transportation capacities, and some coastal species show current-related distribution patterns. Furthermore, organisms that have relatively low migration capacities or frequencies, such as those with short planktonic periods or that inhabit areas far from offshore environments, show complex genetic structures within a single species, which correspond to past oceanic conditions (Dawson, 2001).

In contrast, some coastal species that inhabit semiterrestrial regions do not show current-related distribution patterns. Populations of these coastal species seem to show geohistorical structures rather than structures affected by currents and other oceanic conditions (e.g., *Thoropa* spp.: Fitzpatrick et al., 2009). Phylogenetic analyses of mitochondrial DNA data have revealed that some semiterrestrial coastal isopods display noncurrent-related and complex distribution patterns in several areas (*Ligia* spp.: Itani, 2000; Hurtado et al., 2010).

The genus *Tylos*, a semiterrestrial coastal isopod, is an ideal model organism with which to assess the relationships between present/past marine environments and genetic population structures. It inhabits sandy beaches in tropical and temperate regions (Kensley, 1974), not far from the high-water mark. It can only withstand seawater for several hours (Brown and Odendaal, 1994). Lacking a planktonic stage, *Tylos* also has low migratory ability, and individuals hide under the sand for most of the day (Brown and Odendaal, 1994). Thus, members of the genus are expected to show both current-related and noncurrent-related distributional patterns.

The Japanese Archipelago is an important area in which to study the ecological and evolutionary processes of coastal species because it has a long, branched shape and two warm ocean currents, the Tsushima and Kuroshio Currents, flow around it into the surrounding Sea of Japan and Pacific Ocean, respectively. Two cold currents also exist in the area: the Oyashio Current, located along the northeastern side of the archipelago; and the Liman Current, flowing along the northeastern coast of continental Asia (Fig. 1). The presence and collision of these currents influence the oceanic environments and contribute to the formation of

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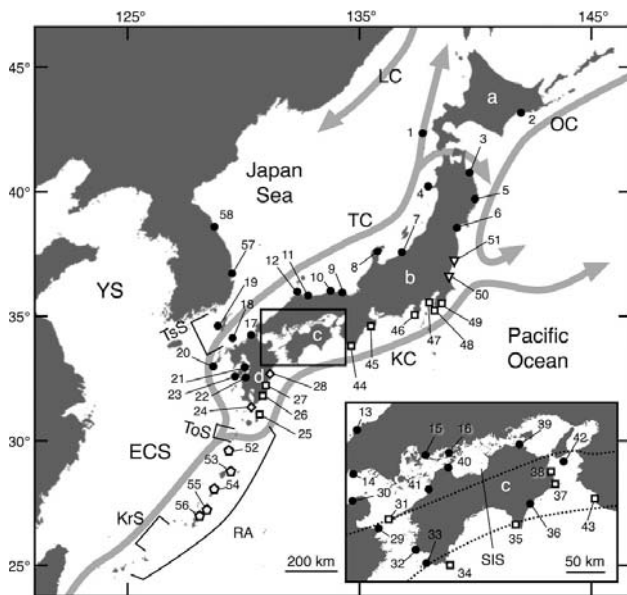　　　　　　　　　　　　E-mail: yahata@biol.tsukuba.ac.jp

Fig. 1. Sampling localities for *Tylos granuliferus*, with population numbers. The numbers of the localities are as in Table 1. Closed circles indicate populations of specimens of C-I, open pentagons indicate C-II, open squares indicate C-III, and open triangles indicate C-IV. Open lozenges indicate localities at which individuals of C-I and C-III coexist. The Japanese islands (a: Hokkaido Island; b: Honshu Island; c: Shikoku Island; d: Kyushu Island; RA, Ryukyu Archipelago) are shown with the water currents and seas around the islands (TC: Tsushima Current; KC: Kuroshio Current; OC: Oyashio Current; LC: Liman Current; YS: Yellow Sea; ECS: East China Sea; SIS: Seto Inland Sea; TsS: Tsushima Strait; ToS: Tokara Strait; and KrS: Kerama Strait).

the marine climatic zones of Japan (Nishimura, 1981; Fujikura et al., 2010).

In this study, we examined the East Asian species *T. granuliferus*, which occurs throughout Japan and adjacent areas, including Korea and Russia (Nunomura, 1990; Schmalfuss, 2003). Our aims were to analyze the genetic diversity and genetic structure of *T. granuliferus* around the Japanese Archipelago, and to discuss the paleogeographic and paleoenvironmental implications of the phylogeny established.

MATERIALS AND METHODS

Sampling

In total, 222 specimens of *T. granuliferus*, including both adults and juveniles, were collected by hand or sift-sorting from either sandy or gravel beach at 56 sites throughout Japan and at two sites on the Korean Peninsula (Table 1 and Fig. 1). Although we failed to select sampling sites in a random manner due to the rarity of the species, designation of 58 sites as representative of all populations from the Japanese Archipelago and adjacent areas should minimize any substantial error in a gross estimation of phylogenetic relationships.

All the specimens were stored in a freezer at −30°C or in 100% ethanol at room temperature before analysis. As outgroups, we included a *Tylos* sp. which obviously belongs to an undescribed species from Hachijojima Island, Japan, in the analyses, together with previously published data for *T. neozelanicus* from New Zealand (EU364624), *Ligia oceanica* (DQ442914), and *Armadillidium vulgare* (GU130251).

DNA extraction, PCR, and DNA sequencing

Total DNA was extracted from leg samples from each specimen with the DNeasy Blood and Tissue Kit (Qiagen, CA). A fragment of the mitochondrial gene encoding cytochrome oxidase subunit I (*COI*) was amplified by PCR using the primers LCO1490 and HCO2198 (Folmer et al., 1994). The cycling program used consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min, before a final extension at 72°C for 5 min.

The amplified products were purified with exonuclease and alkaline phosphatase (ExoProStar; Amersham Biosciences, NJ) and directly sequenced with the primers mentioned above using an ABI BigDye Terminator v3.1 Cycle Sequencing Kit and Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, CA). The 115 new *COI* haplotypes of *T. granuliferus* and one haplotype of *Tylos* sp. were deposited in GenBank (accession numbers: AB763432–AB763451, AB763453–AB763460, AB763462–AB763464, AB763466–AB763530, AB763532–AB763534, AB763536–AB763537, AB763539–AB763553).

Phylogenetic analyses

The partial *COI* sequences were aligned using MAFFT v.6 (Katoh and Toh, 2008). Before their analysis, the p-distances of each position of codons were plotted against those of the total sequences in order to confirm no evidence of mutation saturations by eye.

The schemes used to partition the data were a single-partition strategy and a three-partition strategy (1st, 2nd vs. 3rd). Each partitioning scheme was assessed using a Bayesian analysis implemented in BEAST 1.7.5 (Drummond et al., 2011) with an uncorrelated lognormal relaxed clock and a Yule tree model prior with 10 million generations, sampling every 1000 iterations. The three-partition strategy of the coding gene by codon position was selected as the optimal strategy for the present data using Bayes factors (Kass and Raftery, 1995) calculated in TRACER 1.5 (Rambaut and Drummond, 2007).

The phylogenetic relationships of *T. granuliferus* were reconstructed using the maximum likelihood method (ML) with RAxML (Stamatakis, 2006), with the three-partition strategy and the GTR + gamma model for each codon, which was determined to be the best model with the Akaike information criterion using Kakusan4 (Tanabe, 2011). The confidence of branches on the ML tree was assessed with 1,000 bootstrap pseudoreplicates. Tree topologies with 70% or greater bootstrap proportions (BPs) were regarded as having sufficiently resolved nodes, whereas those with BPs between 50% and 70% were considered to be weakly supported (Huelsenbeck and Hillis, 1993; Shaffer et al., 1997).

Bayesian inference (BI) using the Markov chain Monte Carlo (MCMC) technique was also performed with MrBayes 3.2 (Ronquist et al., 2012), using the partitioning schemes and substitution models described above. We initiated four independent analyses with a random starting tree that ran for 10 million generations. We used the program Tracer 1.5 (Rambaut and Drummond, 2007) to determine when the log likelihood of the sampled trees reached a stationary distribution. Because the apparent stationarity of the MCMC runs was reached at no later than one million generations, we conservatively discarded the first 25% generations from each run as "burn-in" and sampled one of every 100 generations to calculate the posterior probability for each branch on the Bayesian tree. Bayesian posterior probabilities (BPPs) of 0.95 or greater were considered significant support for a given branch (Larget and Simon, 1999; Huelsenbeck et al., 2001).

Parsimony-based haplotype networks of the *COI* region for each assemblage were constructed using TCS 1.2 (Clement et al., 2000), with the connection limit set at 95%.

Table 1. Sampling sites, number of specimens (N), haplotype information, number of polymorphic site (Np), haplotype diversity (h), nucleotide diversity (π), Tajima's D, and Fu's Fs of *Tylos granuliferus*. Populations with N < 2 were omitted. Asterisk (*) indicates significant difference between samples (*P* < 0.05). See Figs. 1 and 2 for sampling localities and haplotypes, respectively.

No.	Abbreviation	Locality	N	Haplotypes	Np	h	π	D	Fs
1	HkOk	Okushiri town, Hokkaido (Okushiri island)	1	H001(1)					
2	HkKs	Kushiro city, Hokkaido	7	H009(1), H010(1), H011(1), H012(2), H013(1), H014(1)	12	0.952	0.007	−0.522	−1.376
3	AoTa	Hachinohe city, Aomori	4	H001(2), H003(1), H004(1)	3	0.833	0.002	−0.754	−0.288
4	AkOg	Oga city, Akita	8	H001(7), H002(1)	1	0.250	0.000	−1.055	−0.182
5	IwKr	Otsuchi town, Iwate	1	H001(1)					
6	MyAz	Sichigahama town, Myagi	2	H023(1), H024(1)	2	1.000	0.003	0.000	0.693
7	NgTa	Joetsu city, Niigata	4	H001(1), H025(1), H026(1), H027(1)	5	1.000	0.004	−0.797	−1.514
8	IkHn	Noto town, Ishikawa	5	H001(2), H018(1), H019(1), H020(1)	8	0.900	0.007	1.211	0.322
9	KyHr	Kyotango city, Kyoto	1	H017(1)					
10	HyTa	Toyooka city, Hyogo	9	H001(4), H007(1), H015(2), H016(1), H017(1)	5	0.806	0.002	−0.910	−1.593
11	TtYn	Yonago city, Tottori	6	H017(2), H039(1), H040(1), H041(1), H042(1)	6	0.933	0.003	−1.367	−2.112*
12	SmMt	Matsue city, Simane	9	H001(7), H037(1), H038(1)	3	0.417	0.001	−1.513	−0.380
13	YgAb	Abu town, Yamaguchi	6	H001(3), H017(2), H043(1)	6	0.733	0.004	−0.932	1.199
14	YgOh	Houfu city, Yamaguchi	3	H044(2), H057(1)	1	0.667	0.001	0.000	0.201
15	HsMa	Takehara city, Hiroshima	1	H044(1)					
16	HsMk	Onomichi city, Hiroshima	1	H051(1)					
17	FoAs	Ashiya town, Fukuoka	10	H001(4), H005(1), H006(1), H007(1), H08(1), H049(1), H050(1)	21	0.867	0.012	−0.057	0.158
18	NsIk	Iki city, Nagasaki (Iki island)	3	H031(1), H032(1), H033(1)	11	1.000	0.012	0.000	0.807
19	NsTs	Tsushima city, Nagasaki (Tsushima island)	2	H035(1), H036(1)	9	1.000	0.014	0.000	2.197
20	NsFk	Goto city, Nagasaki (Fukue island)	5	H017(3), H028(1), H029(1)	4	0.700	0.003	−0.410	0.469
21	KmNk	Kamiamakusa city, Kumamoto	2	H062(1), H063(1)	18	1.000	0.029	0.000	2.890
22	KmMn	Minamata city, Kumamoto	1	H061(1)					
23	KmAk	Amakusa city, Kumamoto	3	H058(1), H059(1), H060(1)	19	1.000	0.020	0.000	1.389
24	KsSt	Minamiohsumi town, Kagoshima	2	H064(1), H103(1)	84	1.000	1.000	0.000	4.431
25	KsTn	Nishinoomote city, Kagoshima (Tanegashima island)	2	H091(2)	0	0.000	0.000	0.000	−
26	KsSb	Shibushi city, Kagoshima	3	H085(2), H101(1)	1	0.667	0.001	0.000	0.201
27	MzNb	Nobeoka city, Miyazaki	1	H106(1)					
28	MzKd	Kadogawa town, Miyazaki	5	H065(1), H091(2), H104(1), H105(1)	94	0.900	0.061	−1.182*	3.921
29	OiSg	Oita city, Oita	4	H044(2), H055(1), H056(1)	2	0.833	0.002	−0.710	−0.887
30	OiKs	Bungotakada city, Oita	5	H044(4), H054(1)	1	0.400	0.001	−0.817	0.090
31	EhSt	Ikata town, Ehime	4	H091(2), H092(1), H093(1)	3	0.833	0.002	−0.754	−0.288
32	EhUw	Uwajima city, Ehime	5	H044(2), H047(1), H048(2)	3	0.800	0.003	0.699	0.276
33	EhAi	Ainan town, Ehime	1	H044(1)					
34	KcTs	Tosa-shimizu city, Kochi	3	H091(2), H097(1)	2	0.667	0.002	0.000	1.061
35	KcMr	Muroto city, Kochi	3	H094(1), H095(1), H096(1)	12	1.000	0.013	0.000	0.901
36	TkKy	Kaiyo town, Tokushima	2	H066(1), H112(1)	97	1.000	0.155	0.000	4.575
37	TkAn	Anan city, Tokushima	5	H097(2), H109(1), H110(1), H111(1)	5	0.900	0.003	−1.124	−1.012
38	TkOm	Tokushima city, Tokushima	2	H097(1), H111(1)	2	1.000	0.003	0.000	0.693
39	KwHg	Higashi-kagawa city, Kagawa	4	H044(1), H052(2), H053(1)	8	0.833	0.006	−0.824	1.163
40	EhIm	Imabari city, Ehime	4	H044(4)	0	0.000	0.000	0.000	−
41	EhIy	Iyo city, Ehime	5	H044(3), H045(1), H046(1)	2	0.700	0.001	−0.973	−0.829
42	WyOh	Wakayama city, Wakayama (Okinoshima island)	1	H067(1)					
43	WySr	Shirahama town, Wakayama	5	H097(2), H103(1), H116(1), H117(1)	6	0.900	0.004	−0.191	−0.445
44	WyKs	Kushimoto town, Wakayama	5	H085(1), H097(1), H096(1), H114(1), H115(1)	7	1.000	0.005	−0.747	−2.238*
45	MeSm	Shima city, Mie	1	H085(1)					
46	SoTo	Shimoda city, Sizuoka	1	H108(1)					
47	KgJo	Miura city, Kanagawa (Jogashima island)	7	H085(5), H098(1), H099(1)	2	0.524	0.001	−1.237	−0.922
48	CbUb	Katsuura city, Chiba	6	H085(3), H088(1), H089(1), H090(1)	17	0.800	0.009	−1.482*	1.419
49	CbSr	Minami-boso city, Chiba	4	H085(2), H086(1), H087(1)	7	0.833	0.006	−0.817	0.961
50	IbHi	Hitachinaka city, Ibaraki	5	H118(3), H120(1), H121(1)	2	0.700	0.001	−0.973	−0.829
51	FsOn	Iwaki city, Fukushima	7	H118(5), H119(2)	3	0.476	0.002	0.755	2.508
52	TkTk	Toshima village, Kagoshima (Takara island)	3	H082(1), H083(1), H084(1)	41	1.000	0.044	0.000	2.185
53	KsAm	Amami city, Kagoshima (Amami-Oshima island)	6	H068(1), H069(1), H070(1), H071(1), H072(2)	25	0.933	0.017	−0.277	0.893
54	KsTk	Tokunoshima town, Kagoshima (Tokunoshima island)	3	H073(1), H074(2)	2	0.667	0.002	0.000	1.061
55	OkZt	Kunigami village, Okinawa	4	H078(1), H079(1), H080(1), H081(1)	4	1.000	0.003	−0.065	−1.741
56	OkMi	Ogimi villege, Okinawa	3	H075(1), H076(1), H077(1)	11	1.000	0.012	0.000	0.807
57	KrG	Uljin, Gyeongsangbuk-do, Korea	3	H001(2), H022(1)	1	0.667	0.001	0.000	0.201
58	KrK	Yangyang, Gangwon-do, Korea	4	H001(4)	0	0.000	0.000	0.000	−

Divergence times, genetic structure, and historical demography

Estimates of the divergence times of *T. granuliferus* were calculated with BEAST 1.7.5 (Drummond et al., 2011). It has been reported that the evolutionary rates of the *COI* genes in the Crustacea vary from 1.66%/Ma to 3.0%/Ma (Ketmaier et al., 2003), although a rate of approximately 2.0%/Ma is broadly used for the molecular clock of vertebrate mitochondrial cytochrome b (e.g., Rocha et al., 2005; Fernandes et al., 2012; Rapson et al., 2012). Because there is no reliable temporal calibration point directly applicable to our data, we used a rate of 2.5%/Ma as a representative value, which was reported to be the *COI* divergence rate in a stenasellid isopod (Ketmaier et al., 2003). Rough estimates of divergence times based on molecular clocks may give some idea of the absolute date of colonization, although their accuracy remains disputable (e.g., Avise, 2000; Heads, 2005).

The analysis was run for 60 million generations, with the first six million generations discarded as "burn-in" and the parameter values were sampled every 1000 generations, imposing a strict clock on the substitution rates (Drummond and Rambaut, 2007) and coalescent constant population size because divergences among closely related lineages follow coalescent process (e.g., Ho et al., 2005; Sota et al., 2005). The parameter estimates and convergences were checked using Tracer 1.5 (Rambaut and Drummond, 2007).

We calculated both haplotype diversity (h) and nucleotide diversity values (π) in each of the samples. Population pairwise fixation indices (F_{ST}) were calculated and their significances were tested with a non-parametric permutations approach with 1,000 permutations of haplotypes among sampling localities. To examine whether populations are at equilibrium, we calculated Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997). We chose these tests because of the increased statistical power in detecting significant changes in population size when using small sample sizes (Ramos-Onsins and Rozas, 2002). In addition, demographic parameters were estimated based on mismatch distribution analysis. These analyses were performed using Arlequin 3.5 (Excoffier et al., 2005). In these analyses, we tentatively treated clades and subclades (see below) as populations in the gross estimation of diversity of each lineage.

RESULTS

Sequence divergence

We determined 627 bp of the partial *COI* sequences with-

out any insertions or deletions for 222 individuals, and detected a total of 115 haplotypes within the species *T. granuliferus*, of which 232 positions of sequences displayed variability. Interspecific nucleotide replacements of *Tylos* ranged from 119 bp (*T. granuliferus* vs. *Tylos* sp.) to 135 bp (*Tylos* sp. vs. *T. neozelanicus*).

The genetic distances (p-distances) among the clades were relatively high (7.7%–16.6%), whereas the intra-clade genetic distances were low (Clade I [C-I]: 2.6%; C-II: 2.6%; C-III: 0.9%; and C-IV: 0.4%). The observed haplotype (h) and nucleotide diversities (π) of C-Ic were higher than those
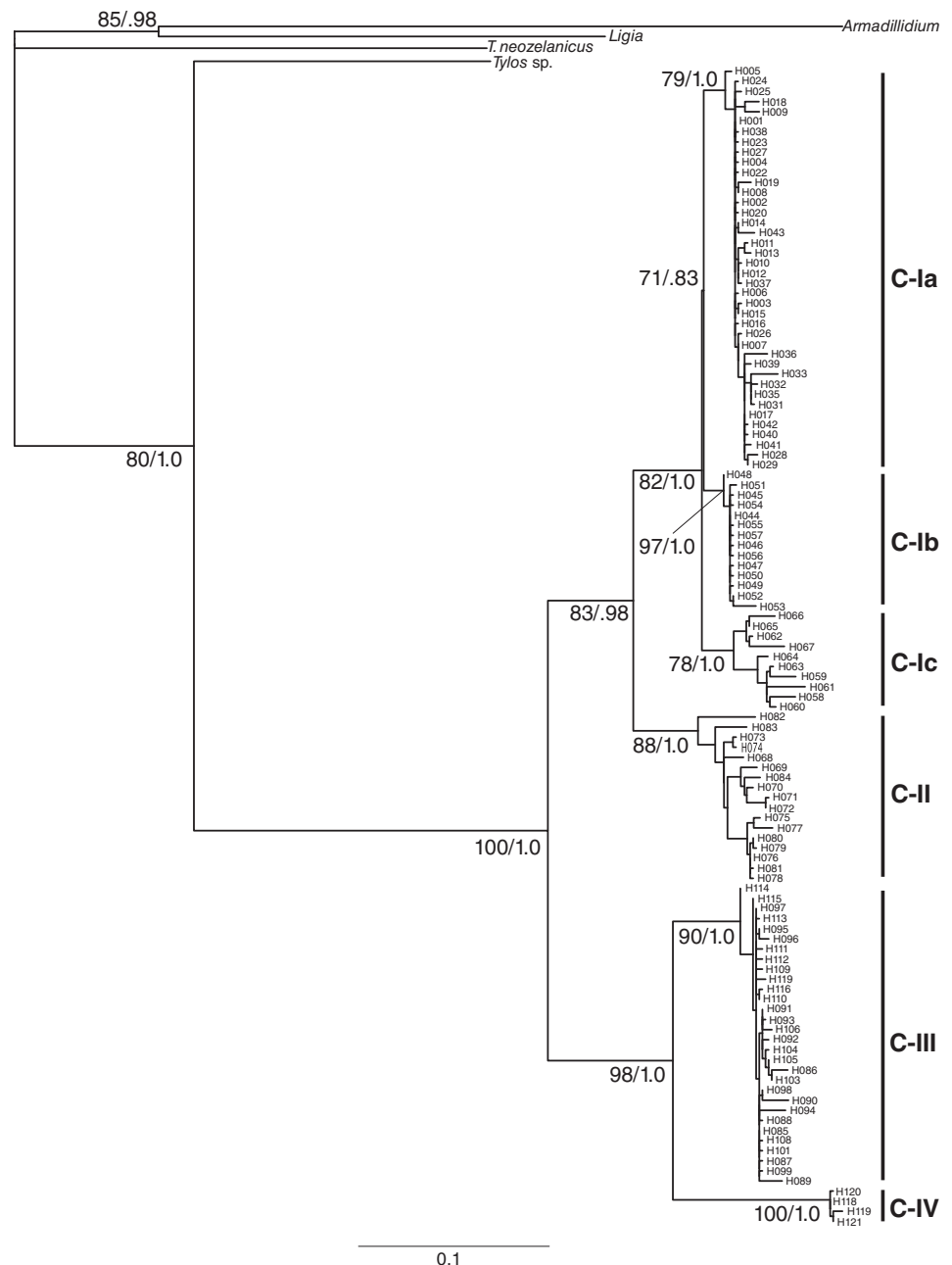

Fig. 2. Maximum likelihood (ML) tree derived from mitochondrial *COI* gene sequences of *Tylos granuliferus* (lnL = −5584). Node support is given as bootstrap proportions (BPs) for the ML analysis, followed by the Bayesian posterior probabilities (BPPs) for BI method. BP and BPP values smaller than 50% and 0.70, respectively, as well as those within clades and subclades, are omitted.

of C-Ia and C-Ib (Table 3).

Phylogenetic analysis

The ML tree (Fig. 2) was nearly identical to the BI tree (not shown), and there was no inconsistency between the two analyses in the topology of the ingroup nodes, with significant support (i.e., BP ≥ 70% or BPP ≥ 0.95). The results showed the monophyly of *T. granuliferus*, with complete support (100% BP and 1.00 BPP; Fig. 2). The specimens of *T. granuliferus* were divided into four major clades, which corresponded to their geographic distributions: the main island of Japan and the Korean peninsula (C-I; 82%, 1.00), the Ryukyu Archipelago (C-II; 88%, 1.00), Kyushu, Shikoku, and the northeastern of Honshu (C-III; 90%, 1.00), and a narrow area in central eastern Honshu (C-IV; 100%, 1.00). Both analyses indicated the monophyly of an assemblage of C-I and C-II (83%, 0.98) and that of C-III and C-IV (98%, 1.00). Clade-I was further divided into three subclades: samples from the northern part of Kyushu, northwestern Honshu, Hokkaido, and west part of the Korean Peninsula (C-Ia; 79%, 1.00); along the Seto Inland Sea (C-Ib; 97%, 1.00); and from southern Kyushu and east Shikoku (C-Ic; 78%, 1.00). Clade-Ia and C-Ib seemed to constitute a monophyletic group (71%, 0.83), and leaving C-Ic as the most basal lineage within C-I. All of the clades and subclades were distributed allopatrically, except for the samples of C-I and C-III from Shikoku and Kyushu Islands. The distributions of C-I and C-III seemed to overlap in Shikoku Island, and the coexistence of these two clades was observed along the southeastern coast of Kyushu Island (sites 24 and 28 in Fig. 1 and Table 1).

Haplotype network analysis

The haplotype networks and their geographic distributions are shown in Figs. 3 and 4. A star-like network was found for C-Ia, with the most common haplotype H001, which was shared by 32 individuals collected from 10 different sites, and several other haplotypes that were shared between the Pacific coast and the Sea of Japan coast. A haplotype group (H017, H028–029, H031–033, H035–036, and H039–042) in C-Ia was confined to Kyushu and the western part of the main islands of Japan.

All members of C-II occurred in the Ryukyu Archipelago clade, which showed relatively high genetic divergence among specimens. The analysis resolved three separate networks within C-II with

no shared haplotypes among the islands, and two independent haplotypes.

Although some individuals within C-III shared common haplotypes, such as H085, H091 and H097, many others did not share. Although the network of C-IV had four haplotypes, the number and size of samples examined are too small to detect any definite patterns.

Estimation of divergence times and demographic population histories

Figure 5 gives divergence times and their 95% credibility intervals (CIs). The initial divergence of *T. granuliferus* into two major lineages (C-I–II and C-III–IV) was estimated to have occurred during the Tortonian (late) Miocene (9.0 Ma, CI: 7.3–10.9 Ma). The divergence times between C-I and C-II and between C-III and C-IV were estimated to have occurred near from boundary of the Pliocene and Miocene (ca. 5.3 Ma), approximately at 5.0 Ma (CI: 4.0–6.3 Ma) and 4.8 Ma (CI: 3.6–6.1 Ma), respectively, whereas those among the subclades of C-I occurred during Calabrian (early) Pleistocene–Piacenzian (late) Pliocene, C-Ia–Ib and C-Ic approximately at 2.6 Ma (CI: 2.0–3.3 Ma), and C-Ia and C-Ib at 1.7 Ma (CI: 1.2–2.3 Ma). The other intra-clade divergences were assumed to have begun during the Calabrian Pleistocene–Piacenzian Pliocene (C-II: 2.3 Ma, CI: 1.7–3.0 Ma), Ionian–Calabrian Pleistocene (C-III: 0.9 Ma, CI: 0.6–1.3 Ma), and Tarantian–Ionian Pleistocene (C-IV: 0.2 Ma, CI: 0.1–0.4 Ma), whereas intra-subclade divergences of C-I occurred in the Ionian–Gelasian Pleistocene (C-Ia, 0.9 Ma, CI: 0.6–1.2 Ma; C-Ib: 0.6 Ma, CI: 0.3–1.0 Ma; and C-Ic, 1.8 Ma, CI: 1.3–2.3 Ma).

The results of F_{ST} showed significant population differentiation (Table 2). Tajima's D and Fu's Fs showed signifi-
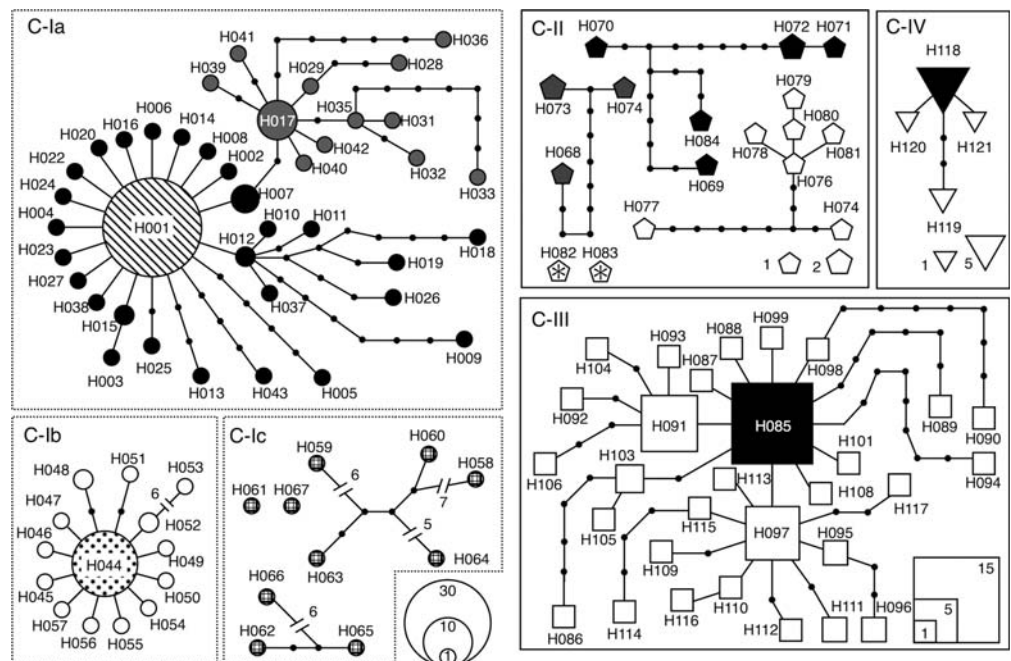

Fig. 3. Statistical parsimony networks of *COI* haplotypes of *Tylos granuliferus*. Haplotype groups were separated from one another at steps exceeding the 95% confidence connection limit. Attached letters adjacent to the symbols indicate haplotype names, consistent with those used in Table 1. Symbol size is proportional to the sample size.
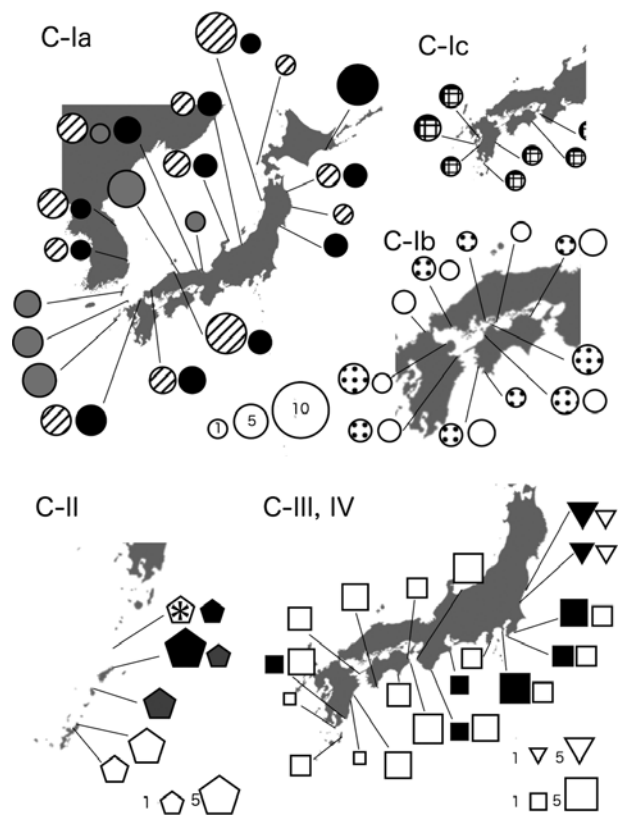
Fig. 4. Geographic distributions of *COI* haplotypes for each population. Symbol size is proportional to the sample size. Patterns for each symbol indicate those used in Fig. 3.

Table 2. Pairwise fixation indices (F_{ST}) for clades and subclades of *Tylos granuliferus*. Asterisk (*) indicates significant values ($P < 0.05$).

Sub/clades	C-Ib	C-Ic	C-II	C-III	C-IV
C-Ia	0.845*	0.811*	0.897*	0.962*	0.968*
C-Ib		0.806*	0.876*	0.970*	0.986*
C-Ic			0.710*	0.937*	0.903*
C-II				0.927*	0.891*
C-III					0.950*

Table 3. Results of haplotype diversity (h), nucleotide diversity (π), Tajima's D and Fu's Fs, and Harpending's Raggedness index (RI) and probablities of RI for sudden expansion and spatial expansion models. Asterisk (*) indicates significant values ($P < 0.05$).

Sub/clades	h	π	D	Fs	RI	sudden expansion	spatial expansion
C-I	0.895	0.019	−1.550*	−24.163*	0.017	0.513	0.860
C–Ia	0.809	0.005	−2.371*	−26.331*	0.014	0.979	0.975
C-Ib	0.677	0.002	−2.404*	−10.510*	0.057	0.671	0.643
C-Ic	1.000	0.029	−0.604	−1.991	0.026	0.895	0.887
C-II	0.988	0.025	−1.039	−3.857	0.015	0.595	0.732
C-III	0.914	0.005	−2.356*	−23.549*	0.021	0.715	0.705
C-IV	0.561	0.002	−0.299	−0.207	0.216	0.988	0.582

cant negative values in each clades except for C-Ic, C-II and C-IV (Table 3), whereas only a few populations demonstrated significant negative values (Table 1). Mismatch distribution analyses showed all of clades and subclades did not differ from sudden expansion and spatial expansion models significantly.

Fig. 5. Chronogram of *Tylos* inferred from BI of the *COI* gene. Numbers above each branch are estimated divergence times. The white boxes on the nodes indicate the 95% of the node age posterior probability distribution. Relationships within clades or subclades and their estimates are omitted. Se: Serravallian, To: Tortonian, Me: Messinian, Za: Zanclean, Pi: Piacenzian, Ge: Gelasian, Ca: Calabrian, Io: Ionian, and Ta: Tarantian.

DISCUSSION

Distribution patterns of populations and their relationships to current activities

Two hypotheses exist regarding the distribution patterns of littoral animals. Some authors have suggested a close relationship between the distribution patterns of populations and marine environmental factors, such as current activity, seawater temperature, and salinity (e.g., Hansen et al., 2007). However, several semiterrestrial coastal species do not show clear relationships between their distributions and local ocean currents (e.g., coastal tiger beetles: Satoh et al., 2004, *Ligia* spp.: Itani, 2000; Hurtado et al., 2010).

Our results from a substantially larger data set analyzed with methodological improvements show that the distributions of two lineages of *T. granuliferus* are strongly related to ocean currents, as reported for other coastal species (e.g., anadromous fish *Leucopsarion petersii*: Kokita and Nohara, 2011). Two
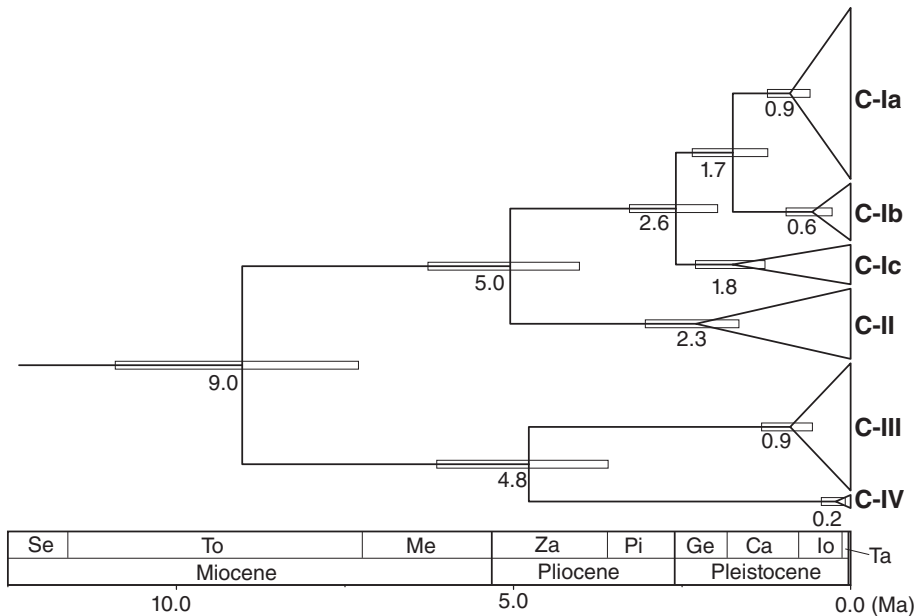
well-supported clades, C-I and the assemblage of C-III and C-IV, are distributed along the Tsushima and Kuroshio Currents, respectively. This strongly suggests that the present and past warm ocean currents have played a major role in the transportation and subsequent divergence of this semiterrestrial isopod. These distribution patterns also correspond to marine climatic zones, because the ocean currents determine the marine and coastal environments around the Japanese Archipelago (Nishimura, 1981; Fujikura et al., 2010).

Although our results show that *T. granuliferus* displays current-related distribution patterns, some other semiterrestrial coastal species display noncurrent-related patterns, as mentioned above. Members of *Ligia* are adapted to inland environments (Itani, 2000; Taiti et al., 2003; Lee, 1994), which suggests that *Ligia* has a tolerance for dry conditions and has diversified with inland dispersal. Inland dispersal has also been reported in some aquatic freshwater insects (Kovats et al., 1996). Indeed, species of the genus *Tylos* could have invaded inland regions and migrated to other beaches via sandy substrates (Brown and Odendaal, 1994). However, there are no large sand-hills in the Japanese Archipelago. Thus, the activities of warm currents along the continents and islands are considered to be a substantial factor in the formation of the distribution pattern of populations of this species. Although *T. granuliferus* lacks a planktonic stage and inhabits intertidal zone of sandy beaches seemingly avoiding water immersion (Imafuku, 1976), some members seem to have gradually extended their distributional ranges along coasts by ocean currents if powerful waves or rafting substrata were available (e.g., Kensley, 1974; Thiel et al., 2003), and such a transport mechanism is suggested in order to explain the wide geographic distribution of some terrestrial isopods (Taiti et al., 1992) or littoral amphipods (Myers, 1993).

Our results also suggest that the opening of the strait and the direction and bifurcation of the ancient ocean currents affected the divergence of these populations. The distribution of the Ryukyu Archipelago lineage (C-II) is restricted to the area south of the Tokara Strait where the Kuroshio Current branches off from the Tsushima Current and flows towards the Pacific Ocean at present, whereas the sister taxon (C-I) is distributed on the northwestern coasts, north of the strait, including on the main islands of Japan. Although the time of formation of this strait is still disputed (Nakagawa et al., 2001; Yamamoto et al., 2003; Odawara et al., 2005; see Iryu et al. [2006] for further discussion), Kizaki and Oshiro (1977, 1980) calculated that it dates back to no later than the Pliocene (2.6–5.3 Ma). Our gross estimate of the divergence time of C-I and C-II (5.0 Ma, CI: 4.0–6.3 Ma) is congruent with this period. This suggests that the divergence between C-I and C-II was initiated by the opening of the strait and the subsequent bifurcation of the Kuroshio Current.

The Tsushima Strait is located between Kyushu and the Korean Peninsula, and is presumed to have been established in the early (Calabrian) Pleistocene (ca. 1.7 Ma: Kitamura et al., 2001). Besides this establishment, inflows of the Tsushima Current into the Sea of Japan via another strait are suggested based on the presence of warm-water mollusks and planktonic foraminifers in the Pliocene and Gelasian Pleistocene (ca. 3.2, 2.9, 2.4, and 1.9 Ma; Kitamura and Kimoto, 2006). The divergence times among the subclades of C-I (2.6 and 1.7 Ma) are presumed to be consistent with these inflows of the Tsushima Current into the Sea of Japan.

The estimated divergence time between the common ancestor of C-I and C-II and that of C-III and C-IV (9.0 Ma, CI: 7.3–10.9 Ma) also seems corresponds to late (Tortonian) Miocene when a land bridge formed between the continent and the Japanese Archipelago through the Ryukyu Archipelago (Kizaki and Oshiro, 1977). This may have caused dramatic changes in paleogeographic and paleoenvironmental factors. In contrast, the occurrence of C-Ib along the Seto Inland Sea is attributed to complex seawater movements, including the ebb and flow of tides.

Historical biogeography

Our results suggest that the initial divergence within *T. granuliferus* corresponded to paleogeographic and paleoenvironmental changes during the late (Tortonian) Miocene (9.0 Ma, CI: 7.3–10.9 Ma). Based on our results, together with evidence that the genus *Tylos* is common within tropical/temperature regions (Kensley, 1974), avoids low-salinity conditions, and is inactive at low temperatures (Imafuku, 1976), the ancestor of *T. granuliferus* is assumed to have originated in a relatively low-latitude marine environment, somewhere on the subtropical coasts of East Asia, and to have then spread into both the Ryukyu and Japanese Archipelagos during the Miocene when these two archipelagos were connected to continental Asia (Kizaki and Oshiro, 1977). The paleo-East China Sea at this time was not a suitable habitat for marine and coastal species because it was dry land, so the ancestral populations must have been distributed only along the Pacific Ocean coast. The initial divergence of *T. granuliferus* might have been affected by the geological changes induced by the first phase of the formation of the Okinawa Trough (6–10 Ma; Shinjo, 1999).

At the beginning of the Pliocene (5.3 Ma), changes in the oceanic environments and the fragmentation of the Ryukyu Archipelago (Kizaki and Oshiro, 1977, 1980) could have affected the divergence between C-I and C-II (5.0 Ma, CI: 4.0–6.3 Ma), and also that between C-III and C-IV (4.8 Ma, CI: 3.6–6.1 Ma). The latter is presumed to have occurred allopatrically, with C-III in Kyushu, Shikoku and Honshu, and C-IV in the northeastern part of Honshu (Fig. 1). Interestingly, we observed a unique distribution pattern that has not been reported for other coastal species in Japan (C-IV in Fig. 1). Clade IV is distributed in a narrow restricted area located along the Pacific coast of northern Honshu, where the Kuroshio and Oyashio Currents meet. Pelc et al. (2009) have suggested that the areas in which ocean currents mix become barriers that inhibit gene flow among populations of coastal species. Therefore, it is probable that the integrity of C-IV has been maintained by the collision of two ocean currents. The opening of the strait between the Pacific Ocean and the Sea of Japan and the subsequent bifurcation of the ancient Kuroshio Current (ca. 3.2, 2.9, 2.4, and 1.9 Ma; Kitamura and Kimoto, 2006) and that of the present Tsushima Strait in the Pleistocene (ca. 1.7 Ma) should have enhanced the divergences among subclades of C-I (2.6 and 1.7 Ma). Considering relatively high genetic divergence of C-Ic (Table 3) and the direction of the Tsushima Current (Fig.

1), C-Ic distributed along the southern coast of Kyushu is supposed to be the ancestral lineage of C-I. This view is circumstantially supported by the findings that recent population expansions are expected in C-Ia and C-Ib, because these two subclades showed significant negative values in Tajima's D and Fu's Fs and because they did not differ from sudden expansion model in mismatch distribution analyses significantly. In order to confirm of validity of our scenario, relationships between ocean activities and divergences within each subclade should be seriously tested in future in the light of independent evidence with accumulation of detailed paleogeographic and climatic data.

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REFERENCES

Avise JC (2000) Phylogeography: The History and Formation of Species, Harvard University Press, Massachusetts

Brown AC, Odendaal FJ (1994) The biology of oniscid isopoda of the genus *Tylos*. Adv Mar Biol 30: 89–153

Burton RS, Lee BN (1994) Nuclear and mitochondrial gene genealogies and allozyme polymorphism across a major phylogeographic break in the copepod *Tigriopus californicus*. Proc Natl Acad Sci USA 91: 5197–5201

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9: 1657–1659

Dawson MN (2001) Phylogeography in coastal marine animals: a solution from California? J Biogeogr 28: 723–736

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7: 214

Drummond AJ, Suchard MA, Xie D, Rambaut A (2011) Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 29: 1969–1973

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform 1: 47–50

Fernandes AM, Gonzalez J, Wink M, Aleixo A (2012) Multilocus phylogeography of the wedge-billed woodcreeper *Glyphorynchus spirurus* (Aves, Furnariidae) in lowland Amazonia: widespread cryptic diversity and paraphyly reveal a complex diversification pattern. Mol Phylogenet Evol 66: 270–282

Fitzpatrick SW, Brasileiro CA, Haddad CFB, Zamudio KR (2009) Geographical variation in genetic structure of an Atlantic Coastal Forest frog reveals regional differences in habitat stability. Mol Ecol 18: 2877–2896

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotech 3: 294–249

Fu Y-X (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147: 915–925

Fujikura K, Lindsay D, Kitazato H, Nishida S, Shirayama Y (2010) Marine biodiversity in Japanese waters. PLoS One 5: e11836

Gray JS (1997) Marine biodiversity: patterns, threats and conservation needs. Biodivers Conserv 6: 153–175

Hansen HJ, Nielsen EE, Grønkjaer P (2007) Evolutionary mechanisms shaping the genetic population structure of marine fishes; lessons from the European flounder (*Platichthys flesus* L.). Mol Ecol 16: 3104–3118

Heads M (2005) Dating nodes on molecular phylogenies: a critique of molecular biogeography. Cladistics 21: 62–78

Ho SYW, Phillips MJ, Cooper A, Drummond AJ (2005) Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. Mol Biol Evol 22: 1561–1568

Huelsenbeck JP, Hillis DM (1993) Success of phylogenetic methods in the four-taxon case. Syst Biol 42: 247–264

Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP (2001) Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294: 2310–2314

Hurtado LA, Mateos M, Santamaria CA (2010) Phylogeography of supralittoral rocky intertidal *Ligia* isopods in the Pacific region from Central California to Central Mexico. PLoS ONE 5: e11633

Imafuku M (1976) On the nocturnal behaviour of *Tylos granulatus* Miers (Crustacea: Isopoda). Publ Seto Mar Biol Lab 23: 299–340

Iryu Y, Matsuda H, Machiyama H, Piller WE, Quinn TM, Mutti M (2006) Introductory perspective on the COREF Project. Island Arc 15: 393–406

Itani Y (2000) Phylogeography of Japanese *Ligia* species (Isopoda: Crustacea) based on molecular phylogenetic analysis. Mon Kaiyo 32: 246–251 (in Japanese)

Kass RE, Raftery AE (1995) Bayes Factors. J Amer Statist Assoc 90: 773–795

Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform 9: 286–298

Kensley B (1974) Aspects of the biology and ecology of the genus *Tylos* Latreille. Ann S Afr Mus 65: 401–471

Ketmaier V, Argano R, Caccone A (2003) Phylogeography and molecular rates of subterranean aquatic stenasellid isopods with a peri-Tyrrhenian distribution. Mol Ecol 12: 547–555

Kitamura A, Kimoto K (2006) History of the inflow of the warm Tsushima Current into the Sea of Japan at 3.5–0.8 Ma. Palaeogeogr Palaeoclimatol Palaeoecol 236: 355–366

Kitamura A, Takano O, Takata H, Omote H (2001) Late Pliocene–early Pleistocene paleoceanographic evolution of the Sea of Japan. Palaeogeogr Palaeoclimatol Palaeoecol 172: 81–98

Kizaki K, Oshiro I (1977) Paleogegraphy of the Ryukyu Islands. Mar Sci Mon 9: 542–549 (in Japanese with English abstract)

Kizaki K, Oshiro I (1980) The origin of the Ryukyu Islands. In "Natural History of the Ryukyus" Ed by K Kizaki, Tsukiji-shokan, Tokyo, pp 8–37 (in Japanese)

Kokita T, Nohara K (2011) Phylogeography and historical demography of the anadromous fish *Leucopsarion petersii* in relation to geological history and oceanography around the Japanese Archipelago. Mol Ecol 20: 143–164

Kovats Z, Ciborowski J, Corkum L (1996) Inland dispersal of adult aquatic insects. Freshwater Biol 36: 265–276

Larget B, Simon DL (1999) Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. Mol Biol Evol 16: 750–759

Lee JD (1994) A new mountain slater, *Ligia taiwanensis* (Isopoda, Ligiidae) from Taiwan. Crustacea 66: 110–115

Myers AA (1993) Dispersal and endemicity in gammaridean Amphipoda. J Nat Hist 27: 901–908

Nakagawa H, Matsuda H, Sato T, Hiratsuka M, Oda M (2001) Preliminary study on sedimentary facies and depositional age of the Chinen Formation in the southern part of Okinawa Island. J Sedimentol Soc Japan 53: 99–101

Nishimura S (1981) Animal distribution in the Sea of Japan. In "Guide to Seashore Animals of Japan with Color Pictures and Keys Vol 1" Ed by S Nishimura, Hoikusha, Osaka, pp 11–19

Nunomura N (1990) Studies on the terrestrial isopod crustacean in Japan 5. Bull Toyama Sci Mus 13: 1–58

Odawara K, Kudo S, Iryu Y, Sato T (2005) Geologic ages of the Chinen Formation and the "Reddish Limestone" in Komesu and Giza areas in southern Okinawa-jima based on calcareous nannofossil biostratigraphy. J Geol Soc Jpn 111: 313–331

Pelc RA, Warner RR, Gaines SD (2009) Geographical patterns of genetic structure in marine species with contrasting life histories. J Biogeogr 36: 1881–1890

Pielou EC (1979) Biogeography. John Wiley and Sons, New York, New York, USA

Rambaut A, Drummond AJ (2007) TRACER, version 1.4. Available from: <http:// tree.bio.ed.ac.uk/software/tracer/>

Ramos-Onsins SE, Rozas J (2002) Statistical properties of new neutrality tests against population growth. Mol Biol Evol 19: 2092–2100

Rapson S, Goldizen AW, Seddon JM (2012) Species boundaries and possible hybridization between the black mongoose (*Galerella nigrata*) and the slender mongoose (*Galerella sanguinea*). Mol Phylogenet Evol 65: 831–839

Rocha LA, Robertoson D, Rocha CR, Tassell JL, Craig MT, Bowen BW (2005) Recent invasion of the tropical Atlantic by an Indo-Pacific coral reef fish. Mol Ecol 14: 3921–3928

Ronquist F, Teslenko M, Mark P, Ayres DL, Darling A, Höhna S, et al. (2012) Mrbayes 3.2: efficient Bayesian phylogenetic inference and model selection across a large model space. Syst Biol 61: 539–542

Satoh A, Sota T, Ueda T, Enokido Y, Paik C, Hori M (2004) Evolutionary history of coastal tiger beetles in Japan based on a comparative phylogeography of four species. Mol Ecol 13: 3057–3069

Schmalfuss H (2003) World catalog of terrestrial isopods (Isopoda: Oniscidea). Stuttg Beitr Naturkd A 654: 1–341

Shaffer HB, Meylan P, McKnight ML (1997) Tests of turtle phylogeny: molecular, morphological, and paleontological approaches. Syst Biol 46: 235–268

Shinjo R (1999) Geochemistry of high Mg andesites and the tectonic evolution of the Okinawa Trough–Ryukyu arc system. Chem Geol 157: 69–88

Sota T, Hayashi M (2007) Comparative historical biogeography of *Plateumaris* leaf beetles (Coleoptera: Chrysomelidae) in Japan: interplay between fossil and molecular data. J Biogeogr 34: 977–993

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690

Taiti S, Ferrara F, Kwon DH (1992) Terrestrial Isopoda (Crustacea) from the Togian Islands, Sulawesi, Indonesia. Invertebr Taxon 6: 787–842

Taiti S, Arnedo MA, Lew SE (2003) Evolution of terrestriality in Hawaiian species of the genus *Ligia* (Isopoda, Oniscidea). In "The biology of terrestrial isopods, V. Crustaceana monographs" Ed by S Sfenthourakis, PB De Araujo, E Hornung, H Schmalfuss, S Taiti, K Szlavecz, Brill, Leiden, pp 85–102

Tajima F (1989) Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. Genetics 123: 585–595

Tanabe AS (2011) Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional and separate models for combined molecular phylogenetic analyses of multilocus sequence data. Mol Ecol Resour 11: 914–921

Thiel M, Guerra-Garcia JM, Lancellotti DA, Vásquez N (2003) The distribution of littoral caprellids (Crustacea: Amphipoda: Caprellidea) along the Pacific coast of continental Chile. Rev Chil Hist Nat 76: 297–312

Yamamoto K, Iryu Y, Nakagawa H, Sato T, Matsuda H (2003) Responses of coral reefs to increased amplitude of sea-level changes at the Mid-Pleistocene Climate Transition. Quatern Res 42: 279–294