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Phylogenetic Analyses of Fat Body Endosymbionts Reveal Differences in Invasion Times of Blaberid Wood-feeding Cockroaches (Blaberidae: Panesthiinae) into the Japanese Archipelago

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ABSTRACT—Cockroaches have endosymbiotic bacteria in their fat bodies. Recent molecular phylogenetic analyses on both hosts and endosymbionts have revealed that co-evolution has occurred throughout the history of cockroaches and termites. Co-cladogenesis was also shown among closely related taxa (woodroach genus *Cryptocercus*; Cryptocercidae), and thus endosymbiont data are likely to be useful for biogeographical analyses. To test the possibility of co-cladogenesis among inter- and intraspecific taxa, as well as the utility of endosymbiont data for inferring biogeographical scenarios, we analyzed rRNA genes of endosymbionts of Japanese and Taiwanese Panesthiinae (*Salganea* and *Panesthia*; Blaberidae), on which phylogenetic analyses previously had been performed based on the mitochondrial genes. Statistical analyses on the topologies inferred from both endosymbiont and host mitochondria genes showed that co-cladogenesis has occurred. The endosymbiont sequences examined appear to have evolved in a clock-like manner, and their rate of evolution based on the host fossil data showed a major difference in the time of invasion of the two Japanese genera, that is congruent with the recent analyses of their mitochondrial genes.

Key words: endosymbiotic bacteria, wood-feeding cockroaches, *Blattabacterium*, 16S rRNA, molecular clock, COII, Tokara Strait

INTRODUCTION

Invertebrates commonly live together with micro-organisms, such as bacteria or fungi. For example, the termites have special protozoa and bacteria in their intestines, which aid in wood digestion. The explosive propagative power of aphids is supported by receiving nutritional assistance of essential amino acids and other materials from symbiotic bacteria in the body. Cockroaches are similar, with symbiotic bacteria (*Blattabacterium* sp.) existing in the special bacteriocytes of their fat bodies. These bacteria were suggested to be involved in the recycling or storage of nitrogen (Wren and Cochran, 1987). Based on rDNA sequence data, cockroach and termite endosymbionts were placed among the *Flavobacteria-Bacteroides* (Bandi *et al.*, 1995).

It is believed these symbiotic bacteria have been passed from the mother to the offspring over several millions of years, via infection of ovaries (Sacchi *et al.*, 2000). Recently molecular phylogenetic analyses at the family level of symbiotic bacteria of cockroaches and termites have been performed, and they are in agreement with the phylogenetic relationships among hosts (Lo *et al.*, 2003). Thus far, few analyses at lower taxonomic levels have been performed. Clark *et al.* (2001) showed that relationships among bacteria of *Cryptocercus* spp. were congruent with their

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hosts.

In this study, we tested the hypothesis that co-cladogenesis has occurred at the species level in wood-feeding cockroaches (*Salganea* and *Panesthia;* Panesthiinae, Blaberidae), on which molecular analyses based on the mitochondrial COII gene (hereafter COII) have already been performed (Maekawa *et al.*, 1999). We used 16S rRNA genes (hereafter 16S) of endosymbionts, because, as suggested by previous works (Moran *et al.*, 1993; Clark *et al.*, 2001; Lo *et al.*, 2003), a relatively constant substitution rate (molecular clock) for the bacterial 16S appears to have operated over evolutionary time.

Clark *et al.* (2001) and Maekawa *et al.* (2005) have shown that endosymbiont 16S data are useful for testing hypotheses about the historical biogeography of *Cryptocercus* spp. woodroaches. The mobility of the Panesthiinae used in this study is expected to be relatively low, since these cockroaches often spend their lifetime in the same patch of decayed wood. Although their actual dispersal potential of adults has not been analyzed yet, their distributional patterns are expected to have been strongly influenced by geological events (Maekawa *et al.*, 1999, 2001, 2003). Analyses of COII data of Japanese/Taiwanese Panesthiinae suggest differences in the timing when *Salganea* and *Panesthia* became established in Japan. *Salganea* spp. is estimated to have entered the north of the Tokara Strait, which is known as a border of the Oriental and the Palaearctic faunal regions (see Fig. 1), approximately 6-14 million years ago (MYA), while Panesthia entered approximately 1-3 MYA (Maekawa and Matsumoto, 2003). Here we estimate the time of divergence time of these groups using a bacterial 16S molecular clock, and discuss their biogeography.

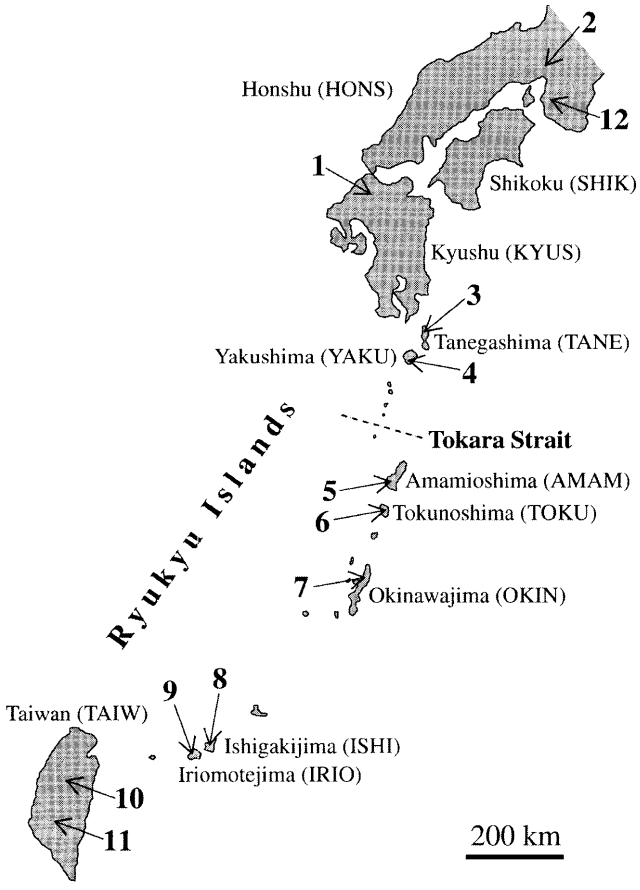

Fig. 1. A map showing the Ryukyu Islands and Taiwan. The Tokara Strait is also shown. The numbers indicate the sampling localities of *Salganea* and *Panesthia* used in this study.

MATERIALS AND METHODS

Cockroaches for the analyses of the fat body endosymbionts were collected in the various regions of Japan (Honshu, Kyusyu and Ryukyu Islands) and Taiwan (Maekawa *et al.*, 1999). The individuals used in this study are shown in Table 1 and Fig. 1. All data of the mitochondrial COII gene are already present in GenBank (Maekawa *et al.*, 1999). Accession numbers of each sequence are also shown in Table 1.

The endosymbiont DNA were extracted from the fat body using the DNeasy Tissue Kit (QIAGEN). The endosymbiont 16S were amplified using 4 primers (33f, 660r, 660f and 1294r) designed by Clark *et al.* (2001). The temperature profile for PCR was described in Clark *et al.* (2001). PCR products were purified using the MagExtractor Kit (TOYOBO) and used as templates for sequencing, performed using a DNA sequencer (ABI310).

Bacterial 16S were aligned manually based on the previously reported alignment (Lo *et al.*, 2003). We included published data for endosymbionts of *Pycnoscelus surinamensis* (Blaberidae, Pycnoscelinae) and *Blattella germanica* (Blattellidae) (Bandi *et al.* 1995). These sequences were used as outgroups, because the endosymbiont of *B. germanica* was shown to be the sister group of a monophyletic clade consisting of those of blaberid taxa (Lo *et al.*, 2003), and because *Py. surinamensis* is the only blaberid taxon for which both endosymbiont 16S and mitochondrial COII data are currently available.

Phylogenetic analyses were performed under maximum parsimony (MP), maximum likelihood (ML) and Bayesian criteria. For likelihood analysis, the most appropriate model of sequence evolution was determined using Modeltest 3.06 (Posada and Crandall, 1998). To verify that each of the datasets contained significantly more phylogenetic structure than random data, we measured the skew (g1) in the distributions of tree lengths for each gene, based on 1000 random generated trees ('generate trees option' in PAUP*4.0b10 (Swofford 2000)). The significance of g1-values was assessed using the critical values for four-state character data listed previously (Hillis and Huelsenbeck, 1992). To check for potential variations in base composition among the sequences in each dataset, the chi-squared test for stationarity in Tree-Puzzle 5.0 was used (Strimmer and Haeseler, 1996). For tree-topology estimation we performed Bayesian inference of phylogeny using the program MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). Parameters for the selected model of substitution were estimated from the data. A total of 50000 trees were obtained (ngen=500000, samplefreq=10), and the first 5000 of these were considered as the 'burn in' and discarded. A 50% majority-rule consensus tree of the remaining trees was produced. Branch lengths for this consensus tree were calculated in PAUP* under ML criteria, estimating parameters from the data. Two independent runs under the same model of sequence evolution were performed. For MP analysis, 50% majority-rule bootstrap trees were obtained using PAUP* (1000 replicates, 10 random addition replicates per bootstrap replicate). For endosymbiont 16S data, all characters were weighted equally, and gaps were treated as a fifth base. Meanwhile, for mitochondrial COII data, saturation

Table 1. Samples used in this study.

Species	Location	Abbreviation of location	GenBank Accession numbers		Sample no.
			mitochondrial COII	endosymbiont 16S	
Salganea esakii Roth	Kyushu (Mt. Hikosan)	KYUS (HIK)	AB007514	AB231588	1
	Tanegashima (Onigasawa)	TANE (ONI)	AB007517	AB231589	3
	Yakushima [Mt. Mochomudake (COII), Yudomari (16S)]	YAKU (MOC, YUD)	AB007518	AB231590	4
S. taiwanensis ryukyuanus Asahina	Amamioshima [Mt. Yuwandake (COII, 16S), Mt. Yuidake (16S)]	AMAM (YUW, YUI)	AB005459, AB007519	AB231591, AB231592	5
	Tokunoshima (Mt. Mikyodake)	TOKU (MIK)	AB007520	AB231593	6
	Okinawajima (Mt. Yonahadake)	OKIN (YON)	AB007521, AB007522	AB231594, AB231595	7
S. taiwanensis taiwanensis Roth	Ishigakijima (Mt. Omotodake)	ISHI (OMO)	AB007523	AB231596	8
	Iriomotejima (Komi)	IRIO (KOM)	AB007524	AB231597	9
	Taiwan [Nanzankei (COII), Mt. Kantosan and Mt. Rosan (16S)]	TAIW (NAN, KAN, ROS)	AB007525, AB007526	AB231598, AB231599	10
S. gressitti Roth	Taiwan [Mt. Habonsan (COII, 16S), Mt. Kantosan (16S)]	TAIW (HAB, KAN)	AB007528, AB007529	AB231600, AB231601	10
S. raggei Roth	Taiwan [Santimon (COII), Nanzankei (16S)]	TAIW (SAN, NAN)	AB007530, AB007531	AB231602, AB231603	10, 11
Panesthia angustipennis spadica (Shiraki)	Honshu [Shimizura (COII), Kawanishi (16S)]	HONS (SHI, KAW)	AB007532	AB231604	2, 12
	Taiwan [Mt. Habonsan (COII), Mt. Rozan (16S)]	TAIW (HAB, ROS)	AB011236	AB231608	10
P. angustipennis yayeyamensis Asahina	Ishigakijima [Mt. Omotodake (COII, 16S), Mt. Bannadake (COII)]	ISHI (OMO, BAN)	AB007539, AB007540	AB231606, AB231607	8
	Iriomotejima [Urauchi (COII), Shirahama (16S)]	IRIO (URA, SHIR)	AB007541	AB231605	9
P. angustipennis baluensis Hanitsch	Borneo (Malaysia, Sabah, Mt. Kinabalu)	–	AB007542	AB231609	–
Pycnoscelus surinamensis (Linnaeus)	(for outgroup)	–	AB193345	X75623	–
Blattella germanica (Linnaeus)	(for outgroup)	–	AB011235	X75626	–

of 3rd codon transitions was observed among taxa used in this study (Maekawa *et al.*, 1999). Thus we downweighted (1/4 to other substitutions) or excluded 3rd codon transitions. Clock-like evolution in the bacterial 16S examined was tested for using the option in Tree-Puzzle 5.0.

Bacterial and mitochondrial tree topologies were compared using the following three statistical tests (Lo *et al.*, 2003). First, using Component Lite (R. Page, University of Glasgow, UK), tree comparison metrics (partition, path-length difference, triplets and quartets) were calculated with 1000 randomized trees. The other two tests examined the null hypothesis that the endosymbionts have undergone co-cladogenesis with the mitochondria of their hosts based on MP (tree-lengths; Templeton test) and ML criteria (likelihood scores; Shimodaira-Hasegawa test) as implemented in PAUP*. Significantly different scores can be interpreted as rejection of the null hypothesis. These two tests were performed using either the bacterial or the mitochondrial datasets, with full optimization and 1000 RELL bootstrap replicates.

RESULTS

Phylogenetic trees

Endosymbiont 16S sequences obtained in this study were about 1280 bp. There was very little length variation among the sequences. Significant phylogenetic signal was found, with skew values below the critical values for significance at the $P<0.01$ level (Hillis and Huelsenbeck, 1992). All data sets contained sequences that did not significantly differ in base composition. The most appropriate model of nucleotide substitution for likelihood analyses selected by Modeltest 3.06 was HKY+I+G. Fig. 2A shows the 50% majority-rule consensus tree obtained from Bayesian analysis, along with posterior probabilities (PP) and bootstrap values (BV) from Bayesian and MP analysis respectively. The topologies found in Bayesian and MP analysis were consistently in agreement with each other. Endosymbionts harbored in Taiwanese taxa (locality no. 10) were basal to those in Japanese taxa (locality no. 1–9). In Japanese taxa, strong support was found for the monophyly of each taxa in the same islands or neighboring region, with the exception of the difference between bacteria of *S. taiwanensis* in Ishigakijima (no. 8) and Iriomotejima (no. 9).

Fig. 2B shows the 50% majority-rule consensus tree obtained from Bayesian analysis using mitochondrial COII gene sequences (685 bp). The model selected by Modeltest was TrN+I+G. Although 50% majority-rule consensus trees based on the weighted MP analysis (3rd codon transition downweighted) are essentially consistent with the Bayesian tree, with the exception of decreased resolution at some tips (asterisks in Fig. 2B). As shown previously (Maekawa *et al.*, 1999; Maekawa and Matsumoto, 2003), Japanese taxa of both genera were shown to be monophyletic groups respectively.

Clock test in endosymbiont 16S

To check for clock-like evolution in bacterial 16S examined, we performed the likelihood-ratio test available in TreePuzzle 5.0 using the HKY+I+G model of substitution. The lnL of the tree without clock was –2482.97, while that with clock was –2489.22. Based on a likelihood ratio test, the null hypothesis that the sequences have evolved in a

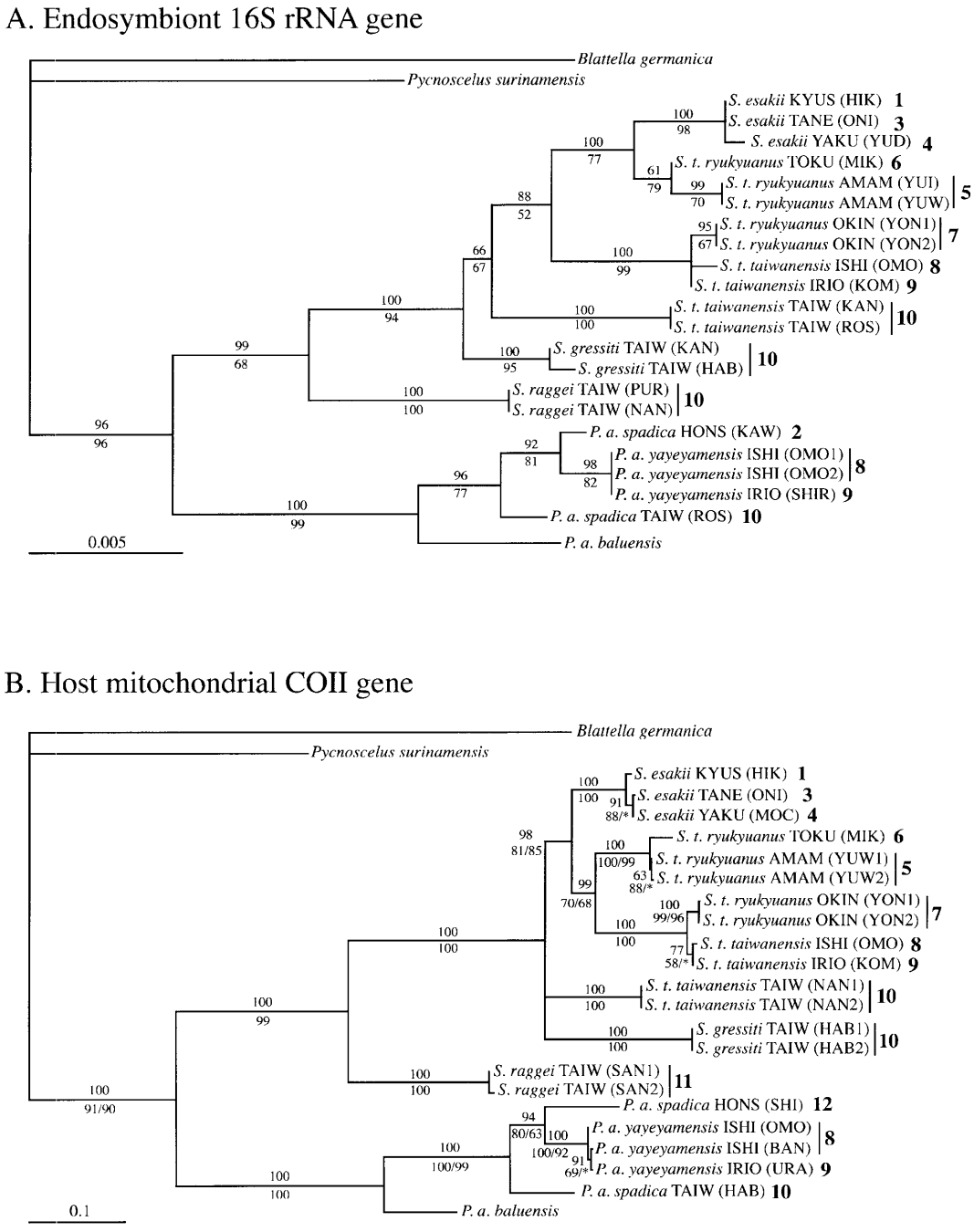

Fig. 2. Molecular phylogeny of (A) endosymbionts inferred from 16S rRNA genes and (B) hosts inferred from mitochondrial COII genes of *Salganea* and *Panesthia*. The topology and branch lengths shown were obtained using Bayesian inference of phylogeny, with the (A) HKY+I+G and (B) TrN+I+G models of substitution, respectively. Posterior probabilities (PP), expressed as percentages, are shown above branches to indicate the level of support for each node. Branches with less than 50% PP were collapsed to form polytomies. Bootstrap values (expressed as percentages) from a MP analysis are shown below nodes. The values in the COII tree (B) are from downweighting (1/4 to other substitutions) and excluding 3rd codon transitions, respectively (only one number is given if bootstrap support was identical at that node). An asterisk indicates that a node that was not supported in greater than 50% of MP bootstrap replicates. Each location number (shown in Fig. 1) is also shown. The scale bar indicates number of inferred substitutions per site. *Pycnoscelus surinamensis* and *Blattella germanica* were not forced to be outgroups.

clock-like manner could not be rejected at the significance level of 5% (P=0.946).

Comparing 2 tree topologies

The topologies of bacterial and mitochondrial trees were nearly identical, although there was one notable difference between these trees (Fig. 3). Monophyly of Japanese

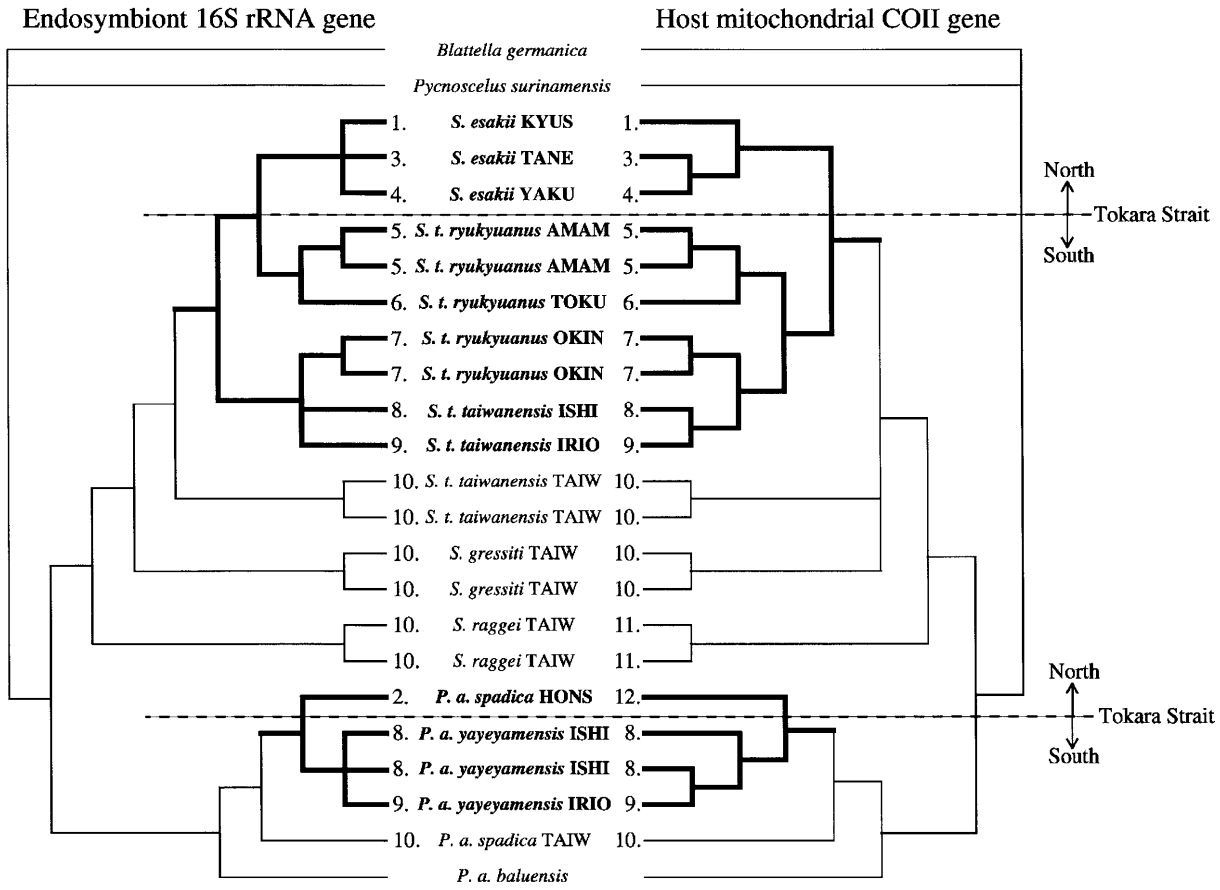

Fig. 3. Topological comparison between endosymbionts and hosts. The thick lines indicate the phylogeny of Japanese taxa. One conflict was shown between the two trees among Japanese *Salganea* spp., but the difference was not found to be significant by the several statistical tests (see RESULTS).

S. taiwanensis was found in the mitochondrial phylogeny, but not in the endosymbiont one, where *S. esakii* is more closely related to *S. taiwanensis* in Amamioshima (no. 5) and Tokunoshima (no. 6) than to other Japanese taxa. Analyses in Component Lite, however, showed that there was a significant level of similarity between these 2 tree topologies, with a very low probability of obtaining observed tree distances by chance ($P<0.001$ for all 4 metrics). Moreover, the results of statistical comparisons of parsimony tree-lengths and likelihood scores, when the best topology of the COII dataset was forced onto the endosymbiont dataset and vice versa, also did not support the significant differences between topologies. Tree-lengths based on the endosymbiont dataset were 110 (bacterial tree topology; Fig. 3 left) and 112 (mitochondrial tree topology; Fig. 3 right). Templeton test showed that these differences were not found to be significant ($P=0.32$). Similarly, tree-lengths based on the COII dataset (3rd TIs excluded) were 667 (bacteria) and 662 (mitochondria) ($P=0.25$). Likelihood scores based on the endosymbiont dataset were –2470.32 (bacteria) and –2476.50 (mitochondria), and those based on COII were –3671.16 (bacteria) and –3662.39 (mitochondria). The SH-test suggested a nonsignificant difference between 2 tree topologies ($P=0.17$ and 0.06, respectively). We were therefore unable to reject the null hypothesis that co-evolution has occurred throughout the evolutionary history of symbionts and the mitochondria of their hosts.

DISCUSSION

The resultant phylogenetic relationships inferred from the endosymbiont 16S were highly congruent with those from mitochondrial COII. One disagreement was found between these two phylogenies, although this topological difference was not found to be statistically significant. Therefore it appears that strict co-evolution has taken place throughout the history of the cockroach and bacterial taxa, even among closely related taxa. However further analyses on host nuclear genes are required to test this hypothesis further. Slight disagreements between the host and *Blattabacterium* trees were also shown not only among the closely related taxa (intra-species; Clark *et al.*, 2001) but also among the higher taxonomic groups (family-level; Lo *et al.*, 2003). A wider range of taxa and multiple genes from the endosymbionts are needed to test whether these disagreements are found only in the 16S phylogeny. It is possible that the disagreements are the result of the phylogenetic methods used, as these may not be capable of describing

with 100% accuracy the true relationships among the sequences.

Maekawa and Matsumoto (2003) suggested that Japanese taxa north and south of the Tokara Strait diverged from each other 6-14 MYA (*Salganea*) and 1-3 MYA (*Panesthia*), based on the COII transversion divergence. Consequently, it is suggested that the timing of immigration to the north of the Tokara Strait substantially differed in both genera. Based on the clock test on endosymbiont 16S data, and the fact that the phylogeny of *Blattabacterium* sp. has been shown to be highly congruent with mitochondrial data, it was deemed appropriate to infer divergence dates for these taxa using a molecular clock method. For calibration, we used 57 MYA (Eocene) and 90 MYA (Gallic-Turonian) as the latest possible time of first appearance of the family Blaberidae and Blattellidae in the fossil record, respectively (Labandeira, 1994; Vršanský *et al.*, 2002). The family Blaberidae was shown to be most closely related to the Blattellidae by the all phylogenetic hypotheses proposed to date (Roth, 2003). The average maximum likelihood distance between 16S from *B. germanica* (Blattellidae) and the Blaberidae species examined was 3.13±0.3% [average±standard deviation (SD), n=23]. Thus, with the assumption of a molecular clock, 0.0156 substitutions per site have occurred in each of these lineages since their last common ancestor. Based on a minimum and maximum divergence date of 57 and 90 MY, we can estimate that the maximum and minimum rate of evolution is 0.0137 and 0.0087 substitutions per site per 50 MY. These rates are within the range of 0.0076 to 0.0232 substitutions per site per 50 MY previously reported for the 16S sequences of aphid symbionts (Moran *et al.*, 1993). The rate of 16S evolution used here partly overlaps, but is slightly higher than the rate (0.0084–0.0111 substitutions/site/50 MY) based on a minimum estimate for the divergence time of the ancestors leading respectively to the woodroach *Cryptocercus* (Cryptocercidae) and termites (180-135 MYA), though the same 16S regions were used for the phylogenetic analyses (Maekawa *et al.*, 2005). This result is consistent with the hypothesis of a speed-up in the evolutionary rate of *Blattabacterium* 16S in the cockroach lineage leading to the Blattellidae and Blaberidae (Lo *et al.*, 2003). Using the rate of 0.0087–0.0137 substitutions per site per 50 MY, the divergence of the lineages leading to Japanese taxa north and south of the Tokara Strait [which have a mean ML distance of 0.54±0.09% (*Salganea*, n=9) and 0.24±0.00% (*Panesthia*, n=3)] is estimated to have occurred between 8.6-15.6 MYA (*Salganea*) and between 4.4-6.9 MYA (*Panesthia*). These values are fairly consistent with the estimated divergence times based on COII transversions, although the divergence times of *Panesthia* north and south of the Tokara Strait are slightly older than those based on mitochondrial COII (1-3 MYA). These data confirm the hypothesis that these two genera invaded the north of the Tokara Strait at very different times, probably during two periods of land-expansions from the initial formation of the Ryukyu archipelago [early Pliocene (~10-5 MYA) and the early Pleistocene (~1.5-1 MYA) (Kizaki and Oshiro, 1980; Ota, 1998; Otsuka and Takahashi, 2000)]. Present study suggests that genetic exchange among *Salganea* north and south of the strait might not occurred during the recent land-expanding period. We are currently expanding our study to address this issue by analyzing many individuals and other suitable genetic markers.

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