mtDNA Phylogeny of Japanese Ant Crickets (Orthoptera: Myrmecophilidae): Diversification in Host Specificity and Habitat Use

by

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ABSTRACT

Ant crickets (Myrmecophilidae, Orthoptera) are typical ant guests. Although ten species (all belonging to genus *Myrmecophilus*) have recently been described from Japan, their phylogeny and the extent of host specificity are not known. Here, we reconstruct mtDNA phylogeny of 48 individuals from six species to examine their host specificity, habitat use, and congruence of mtDNA lineages with the morphological species. The cyt*b* phylogeny reveals seven well-supported lineages that in part do not corroborate morphological taxonomy. *M. kubotai* was split into two distinct mtDNA lineages which differ in their host specificity: one (lineage F) mainly parasitizes *Tetramorium tsushimae* (Myrmicinae) and the other (lineage E) parasitizes several species of Formicine ants. Five out of the seven *Myrmecophilus* lineages did not have significant host-specificity, although lineages C and the above mentioned F both preferably parasitized *T. tsushimae*. Preference for light environment was significant for three cricket lineages. Although ant crickets are not diverse in their morphology, these results demonstrate that they have diversified in host specificity and habitat use.

Key words: cryptic mtDNA lineages, cyt*b*, habitat specificity, host specificity, Mymecophilidae.

INTRODUCTION

In almost all ant nests, a variety of creatures live together with ants and are called 'ant guests' or 'myrmecophiles' (Hölldobler & Wilson 1990). The *Myrmecophilus* ant crickets are the only orthopteran ant guests that live in ant

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nests (Kistner 1982). They steal food from ants and/or eat food that is left in ant nests (Hölldobler 1947; Hölldobler & Wilson 1990). One particularly interesting adaptation to this life style is that ant crickets acquire the ant's cuticular hydrocarbons (CHCs) and coat their body surface with them, which allows them to live undetected in ant host's nests (Akino *et al.* 1996).

Historically, as few as four Japanese *Myrmecophilus* crickets have been known from mainland Japan (Maruyama 2004). Among them, '*M. sapporensis*' were recorded from nests of 26 ant species belonging to three ant subfamilies, and thus the ant cricket host-specificities were considered low (Sakai & Terayama 1995). Furthermore, the crickets collected from *Lasius japonicus* ant nests and experimentally introduced into non-host ants (*Formica japonica* and *Lasius nipponensis*) were readily accepted by the new hosts (Akino *et al.* 1996). Thus, it has been widely accepted that *Myrmecophilus* crickets are generalist in their host use (cf. Hölldobler & Wilson 1990).

Maruyama (2004) provided a new basis for studying the cricket specificity. Based on the structure of the body surface, he classified Japanese *Myrmecophilus* crickets into ten species. He used the character because other characters (e.g. leg spurs, body size, and body color) are unstable and variable within species (Maruyama 2004). As *Myrmecophilus* crickets are deficient in significant distinguishing morphological features among species, and are diverse in body color and size within species, this newly found taxonomic character is useful in detecting morphological grouping and offers the basis for discussing host specificity. However, the character can only be observed in freshly collected, mature individuals, and more importantly, the relevance of such 'morphospecies' should be assessed using other characters, such as DNA.

Recently, a cryptic species was found in European *Microdon* myrmecophilous hoverfly, where the two sister cryptic species are significantly different in host-specificity (Schönrogge *et al.* 2002). Thus, there is a possibility that some *Myrmecophilus* crickets also include cryptic species that differ in their host specificities.

In this study, we reconstruct the molecular phylogeny of Japanese *Myrmecophilus* crickets using mtDNA sequences to determine whether the phylogeny corroborates morphological taxonomy, and to examine host- and habitat specificities of each of thecricket lineages.

MATERIALS & METHODS

Collection of samples

Adult or nymph crickets were collected from host ant nests in or around hardwood stands. The collecting sites were extended all over Japan (Table 1). At each sampling site, ant nests were located in 5-20 randomly placed plots, each 2×5m, with the number of plots proportional to stand area. Once an ant nest was located, we tried to collect as many crickets as possible by digging it when it was subterranean, or by spraying insect rejectant into it when it was arboreal. The collected cricket samples were immediately preserved in 100% ethanol.

Specimens were identified by using field-emission scanning microscopy (JEOL, JSM-6390). The specimens were digitally micrographed without coating. Juvenile crickets were not identifiable because structures of the adult body surface are the key features (Maruyama 2004). The *Myrmecophilus* sp. in Table 1 represents unidentifiable juveniles. Voucher specimens are deposited in the Faculty of Science, Shinshu University, Matsumoto, Japan.

In total, 48 specimens including 6 putative species were analyzed (Table 1). *Ornebius bimaculatus* (Mogoplistidae) and *Gryllus rubens* (Gryllidae) (Gray *et al.* 2006, Genbank accession No. AY234789) were used as outgroups.

DNA isolation, polymerase chain reaction, and sequencing

DNA was extracted from hind legs of the crickets using DNeasy Blood & Tissue Kit (QUIAGEN), with other body parts being preserved for morphological identification. A 434 bp fragment of the mitochondrial gene cytochrome b (cyt*b*) corresponding to positions 10933-11367 in *Drosophila yakuba* mtDNA genome was amplified by polymerase chain reaction (PCR) with the primers CB1 (5'- TAT GTA CTA CCA TGA GGA CAA ATA TC -3') and CB2 (5'- ATT ACA CCT CCT AAT TTA TTA GGA AT -3') (Crozier *et al*. 1993), using the following temperature profile: 35 cycles of 95°C for 30s, 51°C for 30s and 72°C for 90s. After amplification, PCR products were purified using QIAquick PCR purification kit (QUIAGEN). Cycle sequencing reactions were performed with BigDye Terminator Ver 1.1 Cycle Sequencing kit on an ABI 3100 automated sequencer.

Table 1. Overview of the sampled specimens, mtDNA lineages (see Fig.1), and their cyt*b* Genbank accession numbers. Host ant taxon codes; F: Formicinae, M: Myrmicinae, P: Ponerinae, Aj: *Aphaenogaster japonica*, Cj: *Camponotus japonicus*, Co: *C. obscuripes*, Ds: *Diacamma* sp., Fj: *Formica japonica*, Lf: *Lasius flavus*, Lj: *L. japonicus*, Lsa: *L. sakagamii*, Lsp: *L. spathepus*, Mk: *Myrmica kotokui*, Pc: *Pachycondyla chinensis*, Pf: *Pheidole fervida*, Ps: *Polyergus samurai*, Tt: *Tetramorium tsushimae*. *This cricket walked on the ground.

Species	mtDNA lineage	Sample No.	Environment (Shaded or Open)	Host ant subfamily	Host ant species	Locality	Genbank accession No.
M. formosanus	A	48	no data	P	Ds	Naha, Okinawa	in prep.
	B	46	no data	P	Pc	Ogasawara, Tokyo	in prep.
M. tetramorii	C	18	O	M	Tt	Kai, Yamanashi	in prep.
M. kinomurai	D	4	S	F	Lsp	Akeno,Yamanashi	in prep.
	G	1	S	F	Lsp	Oki, Shimane	in prep.
	G	3	S	F	Lsp	Neo, Gifu	in prep.
	G	8	S	F	Ps	Shokawa, Gifu	in prep.
	G	9	S	F	Ps	Shokawa, Gifu	in prep.
	G	10	S	F	Lsp	Shokawa, Gifu	in prep.
	G	13	S	F	Lsp	Matsumoto, Nagano	in prep.
	G	15	S	F	Lsp	Matsumoto, Nagano	in prep.
	G	16	O	F	Cj	Matsumoto, Nagano	in prep.
	G	22	S	F	Cj	Matsumoto, Nagano	in prep.
	G	37	S	F	Lj	Mishima, Niigata	in prep.
	G	43	S	F	Lf	Akashina, Nagano	in prep.
M. gigas	G	23	S	F	Co	Matsumoto, Nagano	in prep.
M. sapporensis	G	33	O	F	Lj	Aomori, Aomori	in prep.
	G	34	O	F	Lj	Aomori, Aomori	in prep.
	G	35	O	F	Lj	Mimmaya, Aomori	in prep.
M. kubotai	E	2	O	F	Lj	Fujinomiya, Shizuoka	in prep.
	E	6	O	F	Lj	Koganei, Tokyo	in prep.
	E	11	O	F	Lj	Machida, Tokyo	in prep.
	E	12	O	F	Fj	Machida, Tokyo	in prep.
	E	14	O	F	Fj	Matsumoto, Nagano	in prep.
	E	20	O	F	Ps	Matsumoto, Nagano	in prep.

Table 1 (continued). Overview of the sampled specimens, mtDNA lineages (see Fig.1), and their cyt*b* Genbank accession numbers. Host ant taxon codes; F: Formicinae, M: Myrmicinae, P: Ponerinae, Aj: *Aphaenogaster japonica*, Cj: *Camponotus japonicus*, Co: *C. obscuripes*, Ds: *Diacamma* sp., Fj: *Formica japonica*, Lf: *Lasius flavus*, Lj: *L. japonicus*, Lsa: *L. sakagamii*, Lsp: *L. spathepus*, Mk: *Myrmica kotokui*, Pc: *Pachycondyla chinensis*, Pf: *Pheidole fervida*, Ps: *Polyergus samurai*, Tt: *Tetramorium tsushimae*. *This cricket walked on the ground.

Species	mtDNA lineage	Sample No.	Environment (Shaded or Open)	Host ant subfamily species	Locality	Genbank accession No.
M. kubotai	E	24	O	F Cj	Matsumoto, Nagano	in prep.
	E	40	O	F Lj	Matsumoto, Nagano	in prep.
	E	41	O	*	Matsumoto, Nagano	in prep.
	E	42	O	F Fj	Matsumoto, Nagano	in prep.
	E	45	S	M Pf	Matsumoto, Nagano	in prep.
	F	5	O	M Tt	Koganei, Tokyo	in prep.
	F	7	O	M Tt	Ichikawa, Chiba	in prep.
	F	26	O	F Cj	Matsumoto, Nagano	in prep.
	F	39	O	F Fj	Kai, Yamanashi	in prep.
M. sp.	C	32	O	M Tt	Kai, Yamanashi	in prep.
	E	27	O	M Tt	Matsumoto, Nagano	in prep.
	E	29	S	F Lj	Matsumoto, Nagano	in prep.
	E	30	O	F Fj	Numazu, Shizuoka	in prep.
	E	31	O	F Lsa	Numazu, Shizuoka	in prep.
	F	17	O	M Tt	Numazu, Shizuoka	in prep.
	F	19	O	M Tt	Matsumoto, Nagano	in prep.
	F	21	O	M Tt	Kai, Yamanashi	in prep.
	F	25	O	M Tt	Ueda, Nagano	in prep.
	F	47	O	M Tt	Fukuoka, Fukuoka	in prep.
	G	28	S	M Aj	Matsumoto, Nagano	in prep.
	G	36	S	F Co	Abashiri, Hokkaido	in prep.
	G	38	S	M Aj	Matsumoto, Nagano	in prep.
	G	44	S	M Mk	Matsumoto, Nagano	in prep.

Phylogenetic analysis

Sequences were aligned using Clustal X (Thompson *et al.* 1997). We reconstructed maximum likelihood (ML) tree using PHYML (Guindon and Gascuel 2003). Modeltest 3.06 (Posada & Crandall 1998) was used for hierarchical likelihood ratio tests for significant differences among increasingly complex substitution models. The GTR+G model was selected by the Akaike information criterion. Clade support was assessed with 1000 bootstrap replicates using PHYML.

Statistics

Preference of cricket lineage to ant taxon or habitat openness was determined by one-way chi-square tests. The proportion of the host taxon among the cricket lineage's total hosts (observed proportion) was compared to the expected proportion (the proportion of that host taxon's nests among the total ant nests sampled). If the crickets inhabit that host taxon significantly more often than the expected proportion, they are judged to 'prefer' that host. So, the 'preference' is relatively defined here, with regard to all the other cricket lineages. As such, host specificity and preference for habitat openness of cricket lineages C, E, F and G (sample size, $n \geq 2$) was examined. The statistical analysis was performed using R (version 2.3.1; R Development Core Team 2005).

RESULTS

Phylogenetic analysis

A matrix of 48 sequences of 434 bp mitochondrial cytb gene contained 191 parsimony-informative sites. The cricket phylogeny revealed seven lineages (Fig. 1), with lineages A and B corresponding to *M. formosanus*, lineage C to *M. tetramorii* and an unidentified species, lineage D to *M. kinomurai,* lineages E and F to *M. kubotai*, and lineage G to *M. gigas, M. kinomurai,* and *M. sapporensis.* The monophyly of the lineages A+B, C, E, F, G, and F+G were each supported although the bootstrap value for each lineages was relatively low. Because our sampling was carried out randomly for ant hosts, the disparity in lineage size may reflect the natural abundance of the cricket lineages except for lineages A and B, which were sampled in a short period of time on remote islands.

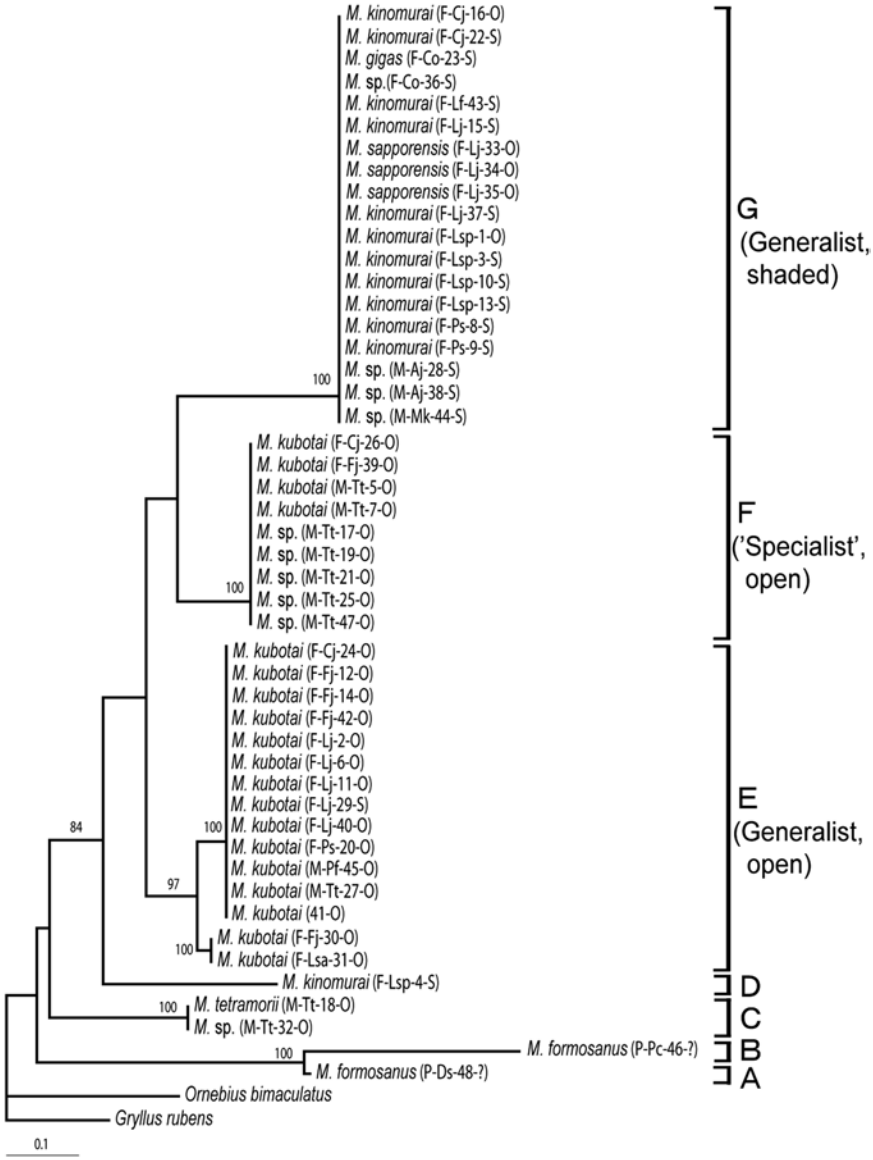

Fig.1. ML tree of the *Myrmecophilus* crickets, as estimated from cyt*b* sequences (434bp). The host ant taxon and the canopy openness of the collection sites are indicated by codes (host ant subfamily - host ant species - sample number - light environment). Host ant taxon code: see Table 1; Canopy openness code: O = open habitat, S = shaded habitat. *Gryllus rubens* (Grilidae) and *Ornebius bimaculatus* (Mogoplistidae) was used as an outgroup. *M.* sp: samples unidentifiable because they are juveniles and lack the body surface characters. Bootstrap values (>50%) are shown above branches from 1000 replicates. Branches are drawn to scale, with the bar representing 10% sequence divergence.

In *M. kinomurai*, one lineage G was a generalist which parasitized a wide variety of host ant species and was distributed in western Japan (Table 1), while the other lineage D (one sample) parasitized *L. spathepus* and was collected in Yamanashi Prefecture, eastern Japan. In *M. kubotai*, one lineage E was a generalist and the other lineage F preferred to parasitize *Tetramorium* (Fig. 1 & Table 2). The two lineages both distributed in central Japan and they were sympatric at a locality in Tokyo (Table 1), yet distinct host specialization is maintained.

Host- and habitat preferences

We tested host preferences for four cricket lineages (C, E, F and G), and found that lineage F showed significant preference for *Tetramorium tsushimae* (Table 2). Although lineage C showed significant preference for *T. tsushimae* (Table 2), we need to take caution for this result due to scarcity of samples. For habitat preference, lineages E and F preferred open environment such as forest edges, and G for shaded environment like forests (Table 2).

Table 2. One-way chi-square test for biased host- and habitat preferences of the ant crickets. If the crickets inhabit that host/habitat in significantly higher proportion than the expected proportion, i.e., the proportion of that host/habitat among total available host nests with any crickets sampled, they are judged to 'prefer' that host/habitat. See text for details. *: $P < 0.05$, **: $P < 0.005$, ns: not significant.

Cricket lineage (sample size)	Host taxon / Canopy openness tested	Expected proportion	Observed proportion	*P*
		Host preference		
C (2)	*Tetramorium tsushimae*	10 / 44 (0.23)	2 / 2 (1.00)	ns
E (15)	*Formica japonica*	5 / 44 (0.11)	4 / 15 (0.27)	ns
	Lasius japonicus	10 / 44 (0.23)	5 / 15 (0.33)	ns
	Formicinae	31 / 44 (0.70)	12 / 15 (0.80)	ns
F (9)	*Tetramorium tsushimae*	10 / 44 (0.23)	7 / 9 (0.78)	**
G (19)	*Aphaenogaster japonica*	2 / 44 (0.05)	2 / 19 (0.11)	ns
	Camponotus obscuripes	2 / 44 (0.05)	2 / 19 (0.11)	ns
	Camponotus japonicus	4 / 44 (0.09)	2 / 19 (0.11)	ns
	Lasius japonicus	10 / 44 (0.23)	5 / 19 (0.26)	ns
	Lasius spathepus	4 / 44 (0.09)	4/ 19 (0.21)	ns
	Polyergus samurai	3 / 44 (0.07)	2 / 19 (0.11)	ns
	Formicinae	31 / 44 (0.70)	16 / 19 (0.84)	ns
		Habitat preference		
C (2)	Open	30 / 45 (0.67)	2 / 2 (1.00)	ns
E (15)	Open	30 / 45 (0.67)	14 / 15 (0.93)	*
F (9)	Open	30 / 45 (0.67)	9 / 9 (1.00)	**
G (19)	Shaded	15 / 45 (0.33)	16 / 19 (0.84)	**

DISCUSSION

Phylogeny

The mtDNA phylogeny revealed seven distinct lineages. Six of the seven lineages each corresponded to one morphospecies (Fig.1). Although gene trees do not always represent species trees, mitochondrial DNA trees have proven robust in inferring species boundaries or corroborating morphological hypotheses (e.g., Wiens & Penkrot 2002). The basically congruent feature of mtDNA lineages with morphological groupings shown here (Fig.1) suggests that the mtDNA tree of the crickets reflects species phylogeny for the most part.

We detected two sets of cryptic mtDNA lineages in *M. kinomurai* (lineages D and G) and in *M. kubotai* (lineages E and F, Fig.1). Based on the fact that the lineages showed distinct host specificities and/or distributions (Table 1), they, especially the extensively-sampled E and F, appear to represent cryptic species. Although ecological and distributional traits may not definitively form the basis of a species concept, they are often informative for delimiting species in the absence of a robust morphological framework (Quek et al. 2004).

Three morphospecies, *M. gigas*, *M. kinomurai*, and *M. sapporensis*, established by Maruyama (2004) based on structures of body surface, were all included in a single lineage G. The absence of sequence variation among the three morphospecies may indicate 1) incomplete lineage sorting of the mitochondrial gene, 2) introgression of mtDNAs among the three morphospecies, and/or 3) that the three morphospecies are invalid and are simply morphological variants of a single species. Incomplete lineage sorting and/or introgression of mtDNAs across species boundaries both might be the case because the three species are close relatives (Maruyama 2004), and because *M. kinomurai* and *M. gigas* are often sympatric. Alternatively, if the intraspecific morphological variation ever explains the identical DNA sequences, the variation of the structures of body surface and body size may reflect 'postimaginal development' as reported for termitophilous beetles, which adjust their own body size and shape to host termite species in response to a termite-derived chemical substance (Kistner 1979; Ballerio 2000). Comprehensive sampling combined with phylogenic analyses with more (especially nuclear) DNA markers will clarify whether the cryptic mtDNA lineages are cryptic species,

and whether incomplete lineage sorting and/or introgression of mtDNA occurs (e.g. Sota & Vogler 2001; Shaw 2002).

Host preferences

Crickets of lineage F significantly preferred *T. tsushimae*. In general, specific association of myrmecophiles to their host ants are maintained partly by the myrmecophile's adaptation to the behavior of its host ants. For example, the staphylinid beetle *Pella comes* (*Zyras comes*) which lives together with *Dendrolasius* ants adapts behaviorally to the ants (Akino 2002). They intrude in ant trails, walk actively, make contact with the ants using antennae, and obtain regurgitated food from the host ants. In the case of the lineage F crickets, they move more slowly than other *Myrmecophilus* cricket species (T. K., personal observation). Several species of *Tetramorium* are known as the slow-moving ants (Javier & Xim 1994; Fielder 1990), and so is *T. tsushimae*. Thus, the slow behavior of lineage F crickets appears to be an adaptation to the host ant's slow behavior. Such behavioral similarity of *Myrmecophilus* crickets to their host ant species was also reported for European *Myrmecophilus* cricket species (Hölldobler 1947). Accordingly, the specificity of the cricket lineage F may partly be due to behavioral adaptation to *T. tsushimae.* Another possible factor maintaining the specificity is the unique shape of scales covering the cricket's body surface, which have been used for the morphological classification (Maruyama 2004). The shape of scales differs between cricket morphospecies, and this might be important in absorbing CHCs of their specific ant host species.

Lineages E and G were generalists. This finding is consistent with Akino *et al.*'s (1996) finding that ant crickets parasitizing a natural colony of *Lasius japonicus* could live in experimentally-introduced colonies of *Formica japonica* and *Lasius nipponensis*. These generalist *Myrmecophilus* cricket lineages can flexibly change their CHC profiles by acquiring CHCs from host ants (Akino *et al.* 1996), and they may therefore readily be accepted by different ant hosts.

Preferences for habitat openness

The cricket lineages C, E and F tended to be collected in open forest edges, whereas lineage G was collected in shaded forests (Table 2). Thus, their habitat preference may depend on environmental factors such as light intensity and/

or humidity. Judging from this, they may first select habitat by its openness, and then colonize preferable or dominant ant taxa available at the habitat. For example, in the Kanto area (central Japan), dominant ant species include *T. tsushimae* (Myrmicinae, openland), *F. japonicus* (Formicinae, openland) and *L. japonicus* (Formicinae, open and shaded land) (Yamaguchi 2004). So, if we assume that crickets of lineage F prefer openland-dwelling myrmicines as their potential hosts, *T. tsushimae* is the most likely choice for them because *T. tsushimae* is the only dominant myrmicine in openland. So, lineage F may have the potential to be a host-generalist, but due to the poverty of host fauna in central Japan, lineage F is forced to be a 'specialist'. In this sense, it is not surprising that the formicine-preferring cricket lineages (E and G) are generalists, because formicines include a diversity of dominant ground dwellers in central Japan.

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