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Intra-Individual Polymorphism in the Internal Transcribed Spacer 1 of Ribosomal DNA in the Polymorphic Lepturine Beetle *Leptura Mimica* Bates, 1884

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ABSTRACT

The lepturine beetle *Leptura mimica* Bates, 1884, occurs in Japan and presents polymorphic elytral patterns unique to Japan. Analysis of this polymorphism will contribute to our understanding of biodiversity and ecosystems in Japan. Molecular phylogenetic analysis of *L. mimica* was conducted to clarify the evolutionary aspect of the polymorphism. Mitochondrial DNA data showed two geographically divided clades, without polymorphic relations. Next, ribosomal DNA（rDNA）, a multigene family involved in concerted evolution, was investigated. Intra-individual polymorphisms in trinucleotide repeats were detected in the internal transcribed spacer 1（ITS1）region of rDNA. Such a polymorphism in ITS1 can be recognized as incomplete homogenization among rDNA cistron units, possibly due to hybridization between different lineages. In addition, an elytral pattern-dependent clade was formed in the phylogenetic tree deduced from all rDNA clones analyzed in this study. These results suggest that the polymorphism in the elytral pattern of *L. mimica*, in the context of rDNA data, is due to the genetically mixed status of some lineages after the establishment of each elytral pattern adapted to the ecosystem.

INTRODUCTION

Leptura mimica Bates, 1884 is a longicorn species belonging to the genus *Leptura*（Coleoptera: Cerambycidae）and is occasionally regarded as a subspecies of *L. annularis* Fabricius, 1801, or a member of the *L. annularis* group. The group occurs widely in the palearctic region, including Japan（Löbl & Smetana, 2010）. In Japan, the species presents polymorphic elytral patterns of roughly three types: macular, blackish, and brown. A macular type is universal to the group, so its distribution area is not limited, while the two other types are unique to Japan. In particular, the brown type has sometimes been controversial in taxonomic status, partly because its distribution shifts in western Japan. Taxonomic studies have dealt with some phenotypic information about male genitalia, elytral character, and hindwing, resulting in a

degree of specificity in the Japanese population, but an explanation as to elytral types remains elusive（Makihara & Saito, 1985; Makihara *et al*., 1991; Rossa *et al*., 2017; Fujita *et al*., 2018）.

In general, an elytral pattern may play a role in reproductive and/or survival strategies. In some butterflies, male wing patterns are useful for conspecific male recognition in the territorial contest, while female wing patterns are useful for conspecific mate recognition. Male wing patterns are also possibly useful for the female' s conspecific mate evaluation（Nijhout, 1991; Rutowski & Rajyaguru, 2013; Hoshino, 2020）. In wasp-mimicking moths and longicorns, the high-contrast aposematic pattern is mimicked to participate in the unpalatable insects' mimicry ring, so that predators avoid highly efficient patterns even though they are not unpalatable（Chittka & Osorio, 2007; Stevens & Ruxton, 2012）. Similarly, distributions of blackish and brown types of *L. mimica* correspond to those of some exemplary unpalatable insects of soldier beetles（Cantharidae）with corresponding colors, respectively（Imasaka, 1991; Hoshino, 2018）. Thus, in this context, the change to the blackish or brown type in *L. mimica* is presumed to be a transfer to a more efficient mimicry ring of unpalatable insects in each habitat or ecosystem. Based on the same context, the universal macular type, which is probably a wasp mimic, is widely fit; thus, it will not be advantageous to change other types. In addition, coloration of thermoregulatory functions is also indicated in insects（Ohsaki, 1986; Williams, 2007）. Therefore, the analysis of polymorphic elytral patterns may contribute to our understanding of biodiversity and ecosystems unique to Japan.

Phylogenetic analysis is an effective method for clarifying the origin and evolution of a character as an elytral pattern. Molecular phylogenetic analysis based on nucleotide sequences has recently been used. Phylogenetic analysis based on mitochondrial gene sequences has already been conducted in *L. mimica*. This resulted in two geographically divided clades of eastern and western Japan without any direct relationship with elytral polymorphism（Saito *et al*., 2002）. The mitochondrion genome is maternally inherited, which suits phylogenetics, reflecting biogeographical aspects（Avise, 2008）, while variable regions of nuclear genes seem to provide higher resolution within species or near-related species, possibly reflecting lineage or kindred. Ribosomal DNA（rDNA）can be such a target, possessing two internal transcribed spacer（ITS）regions of internal transcribed spacer 1（ITS1）and internal transcribed spacer 2（ITS2）flanked by 18S-, 5.8S-, and 28S-ribosomal RNA（rRNA）genes, respectively（Nei & Rooney, 2005）. Although rDNA is a multigene family under concerted evolution by which nucleotide sequences among rDNA cistron units are homogenized, the ITS region has been used to estimate phylogenetic relationships within closely related groups or species（e.g., Miller *et al*., 1996; Alvarez & Hoy, 2002）. Furthermore, incomplete homogenization among rDNA units has also been detected in various taxa, suggesting differentiation at the population, species, or ecotypic level（Fama *et al*., 2000; Alaranta *et al*., 2011）. Therefore, this approach will shed light on how *L. mimica* differentiates two novel elytral types in Japanese forests.

In this study, a molecular phylogenetic analysis was conducted based on mitochondrial genes for multiple samples of *L. mimica*

from localities in Japan to confirm the phylogeographic status and the range of genetic diversity in sympatric populations, followed by the analysis of rDNA to clarify elytral pattern evolution. As a novel approach, polymorphisms of short tandem repeats detected in the ITS1 region of rDNA of *L. mimica* was used to speculate phylogenetic relationships with respect to each elytral pattern.

MATERIALS & METHODS

Samples

Thirty samples were collected from nine sites in Japan. The sample code, collection data, and elytral type are presented in Table 1. Collection sites are shown as abbreviations and full addresses are as follows: Sapporo(Mt. Teine, Sapporo C., Hokkaido); Okutama (Mt. Kintai, Okutama T., Tokyo); Anayama (Anayama T., Nirasaki C., Yamanashi Pref.) Yatsugatake (Mts. Yatsugatake, Hokuto C., Yamanashi Pref.) Mt. Fuji (Mt. Fuji, Narusawa Vill., Yamanashi Pref.) Yokosuka (Mt. Miura-Fuji, Yokosuka, Kanagawa Pref.); Oki (Oki Is. (Dogo), Okinoshima T., Shimane Pref.) Tsushima (Tsushima Is. Mitsushima T. Tsushima C., Nagasaki Pref.) Yakushima (Yakushima Is. Yakushima T. Kagoshima Pref.). Samples were stored at -20 ° C in 100% ethanol until use.

Nucleotide sequencing

DNA extraction, polymerase chain reaction (PCR), and sequence reaction for nucleotide sequencing were conducted as described by Hoshino *et al*. (2015). Briefly, DNA was extracted from insect legs using the DNeasy blood and tissue kit (QIAGEN, Hilden, Germany). DNA samples were stored at -80℃ until use. The two partial regions of mitochondrial genes encoding NADH dehydrogenase subunit 5 (ND5) and cytochrome oxidase subunit 1 (CO1), and one region of nuclear rDNA were amplified by PCR using Quick Taq HS Dyemix (Toyobo Co. Ltd., Tokyo, Japan). All primers used for the amplification were as follows: ND5-lep-FW (5′ -GGWGCTAATTTTGAATTTGA-3′) and ND5-lep-RV (5′ -CATARCCAAAYCATATACCA-3′) for mitochondrial ND5, CO1-cer-FW (5′ -CCCGGATTTGGRATAATYTC-3′) and CO1-cer-RV (5′ -TCAGAATATCTRTGTTCDGC-3′) for mitochondrial CO1, and 18S-rRNA-univ-FW (5′ -ACACACCGCCCGTCGCTACTA-3′) and 28S-rRNA-cer-RV (5′ -GCTCWTCCCT KTTCGCTCGCA-3′) for rDNA. The PCR amplicons were purified, and the purified DNA fragments were directly sequenced using the ABI Prism Big Dye Terminator Cycle Sequence Kit version 3.1 (Applied Biosystems, Foster City, CA, USA). The obtained sequence data were processed for the phylogenetic analysis.

For rDNA, the PCR amplicons were not homogeneous, which inhibited direct sequencing. Therefore, cloning was conducted by ligating the purified amplicons into the T-vector pMD20 (Takara Bio, Shiga, Japan), recombinant plasmids were transfected into DH5-alpha competent *Escherichia coli* cells (Toyobo), and 1–14 cloned inserts were sequenced as described above. Sequencing was performed only on the 5′ -side using the forward primer (Fig. 1).

All gene sequence data in this paper have been submitted to the DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank databases under accession numbers shown in Table 1.

Molecular phylogenetic analysis

The phylogenetic relationships among the

Table 1. List of samples used in this study and the GenBank accession numbers

Collection site[*1]	Sample code	Collection record	GenBank accession nos.[*2]			Elytral pattern[*3]	Microsatellite repeat type[*4]
			Mitochondorial DNA		Ribosomal DNA [18S-ITS1]		
			CO1	ND5			
Sapporo	SAP-1	23 July, 2013	LC650583	LC650455	LC650626-LC650633	M	R6(3), R7(4), R8(1)
Oki Is.	OKI-1	12 June, 2010	LC650584	LC650456	LC650634-LC650637	M	R5(1), R6(2), R7(1)
	OKI-2	4 June, 2013	LC650585	LC650457	LC650638-LC650644	M	R6(1), R7(6)
	OKI-3	4 June, 2013	LC650586	LC650458	LC650645-LC650651	M-Bl	R6(7)
	OKI-4	5 June, 2013	LC650587	LC650459	LC650652-LC650659	M-Bl	R6(6), R7(2)
	OKI-5	5 June, 2013	LC650588	LC650460	LC650660	M-Bl	R7(1)
	OKI-6	5 June, 2013	LC650589	LC650461	ND	M-Bl	ND
	OKI-7	5 June, 2013	LC650590	LC650462	ND	M-Bl	ND
Okutama	OKU-1	11 August, 2012	LC650591	LC650463	LC650661-LC650666	Bl	R7(6)
	OKU-2	31 July, 2013	LC650592	LC650464	LC650667-LC650672	Bl	R7(6)
Yokosuka	YOK-1	22 May, 2013	LC650593	LC650465	LC650673-LC650680	Bl-Br	R4(4), R6(4)
	YOK-2	24 May, 2013	LC650594	LC650466	LC650681-LC650688	Bl	R7(5), R8(1), R10(2)
Mt. Fuji	FUJ-1	23 June, 1996	LC650595	LC650467	LC650689-LC650694	Bl-Br	R6(3), R9(3)
	FUJ-2	6 August, 2006	LC650596	LC650468	LC650695-LC650698	Bl	R5(1), R7(3)
Anayama	ANA-1	14 May, 2011	LC650597	LC650469	LC650699-LC650703	Br	R6(3), R7(2)
	ANA-2	5 May, 2013	LC650598	LC650470	LC650704-LC650711	Br	R5(6), R8(2)
Yatsugatake	YAT-1	12 August, 2011	LC650599	LC650471	LC650712-LC650713	Bl-Br	R6(2)
	YAT-2	12 August, 2011	LC650600	LC650472	LC650714-LC650720	M-Bl	R7(1), R8(6)
	YAT-3	12 August, 2011	LC650601	LC650473	LC650721-LC650734	M-Br	R6(14)
	YAT-4	12 August, 2011	LC650602	LC650474	LC650735-LC650738	M	R7(4)
	YAT-5	12 August, 2011	ND	ND	LC650739-LC650744	M-Br	R6(4), R7(2)
	YAT-6	August, 2012	LC650603	LC650475	LC650745-LC650751	Br	R6(6), R8(1)
Tsushima Is.	TSU-1	30 May, 2006	LC650604	LC650476	LC650752-LC650758	Br	R4(3), R6(1), R7(3)
	TSU-2	May, 2008	LC650605	LC650477	LC650759-LC650764	Br	R4(6)
	TSU-3	May, 2008	LC650606	LC650478	LC650765-LC650772	Br	R4(2), R7(6)
	TSU-4	24 June, 2008	LC650607	LC650479	LC650773-LC650780	Br-Bl	R6(1), R7(7)
	TSU-5	May, 2013	LC650608	LC650480	LC650781-LC650789	Bl	R4(1), R8(8)
	TSU-6	May, 2011	LC650609	LC650481	ND	Br	ND
	TSU-7	May, 2011	LC650610	LC650482	ND	Br	ND
Yakushima Is.	YAK-1	9 July, 2013	LC650611	LC650483	LC650790-LC650793	Br	R4(1), R7(3)
Leptura dimorpha Bates, 1873[*5]							
Okutama	LD-OKU-1	4 May, 2008	LC650612	LC650484	ND	ND	ND

[*1] Detals of the sites are described in the materials & methods section.
[*2] ND: not determined.
[*3] M: macular type, Br: brown type, Bl: blackish type.
[*4] The numbers of each repeat clones is described in parentheses.
[*5] This related species was used as an outgroup in the phylogenetic analysis.

28 samples were analyzed by comparing mitochondrial nucleotide sequences. Two partial gene sequences of CO1 and ND5 were concatenated and used as mitochondrial sequences. Sequences were aligned using the ClustalW program and then analyzed using

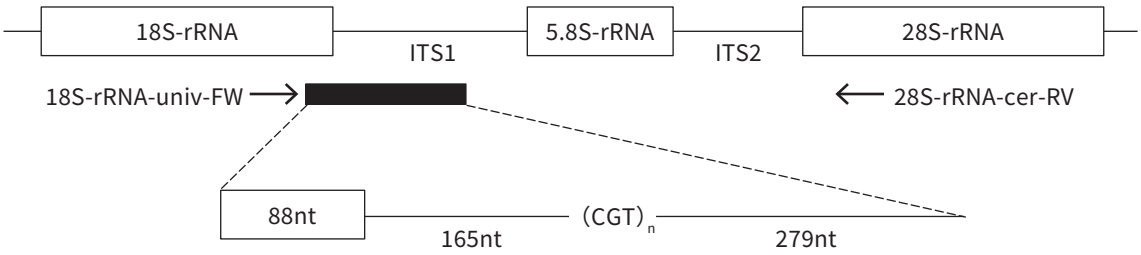

Figure 1. Schematic representation of ribosomal DNA (rDNA) structure (the upper part) and the analyzed region (the lower part). The arrows show the position of primer sets and the black bar indicates the sequenced region in which 88 nucleotides (nt) of the 18S-rRNA gene and trinucleotide (CGT) repeats in internal transcribed spacer 1 (ITS1) are included.

the MEGA program ver.7 (Kumar *et al*., 2016). The phylogenetic dendrogram was determined using the neighbor-joining (NJ) method.

The repeat numbers of trinucleotides (TN) detected in the ITS1 clones were characterized. Molecular phylogenetic analysis was applied for 88 nucleotides (nt) in the 3′-terminus of the 18S-rRNA gene, as described above.

RESULTS

Molecular phylogenetic analysis

To study the phylogeny of *Leptura mimica* in Japan, 28 mitochondrial gene sequences were aligned using the ClustalW protocol. The alignment data were tested by the bootstrap method, and a NJ tree was constructed (Fig. 2A). The tree showed that *L. mimica* was divided into two clades with a high bootstrap value of 99 using *L. dimorpha* as an outgroup. One clade included samples from Yokosuka, Oki, Tsushima, and Yakushima, and the other included all the remaining samples. Geographically, the western-southern and eastern-northern site samples were positioned in the upper and lower clades in Fig. 2A, respectively. Samples from the same collection site fell into the same clade.

Genetic characterization of rDNA

To clarify the phylogenetic relationships among the three elytral types of *L. mimica*, rDNA sequences of 26 samples were analyzed. However, it was impossible to determine the sequences, likely because of mixed amplicons originating from multigenic rDNA. Sequencing by cloning method revealed that there was polymorphism in the trinucleotide (TN) of CGT repeats within an individual. The numbers of TN repeats were in the range of 4-10, counting slightly changed TN, such as CTT, CGC, and CGA. The repeat types are demonstrated by adding the repeat numbers to R(repeat), and the numbers of clones read in this study are shown in parentheses for each repeat type (Table 1). Of all 168 clones, R6 and R7 occupied 60 and 62 clones, respectively, and R4 had 17 clones. Geographically, both R6 and R7 are universal to all sites, while R4 is concentrated at the western-southern sites of Tsushima and Yakushima. Focusing on the elytral types, R4 was also detected in brown and brown-mixed types, such as Anayama and Yokosuka samples, and R4 tended to be linked to the brown type. For the blackish type, only R7 clones were found in both Okutama samples in this study.

The NJ tree constructed based on 168 clones of 88 nt in the 18S-rRNA gene formed one dim but detectable clade, including all R4 repeat

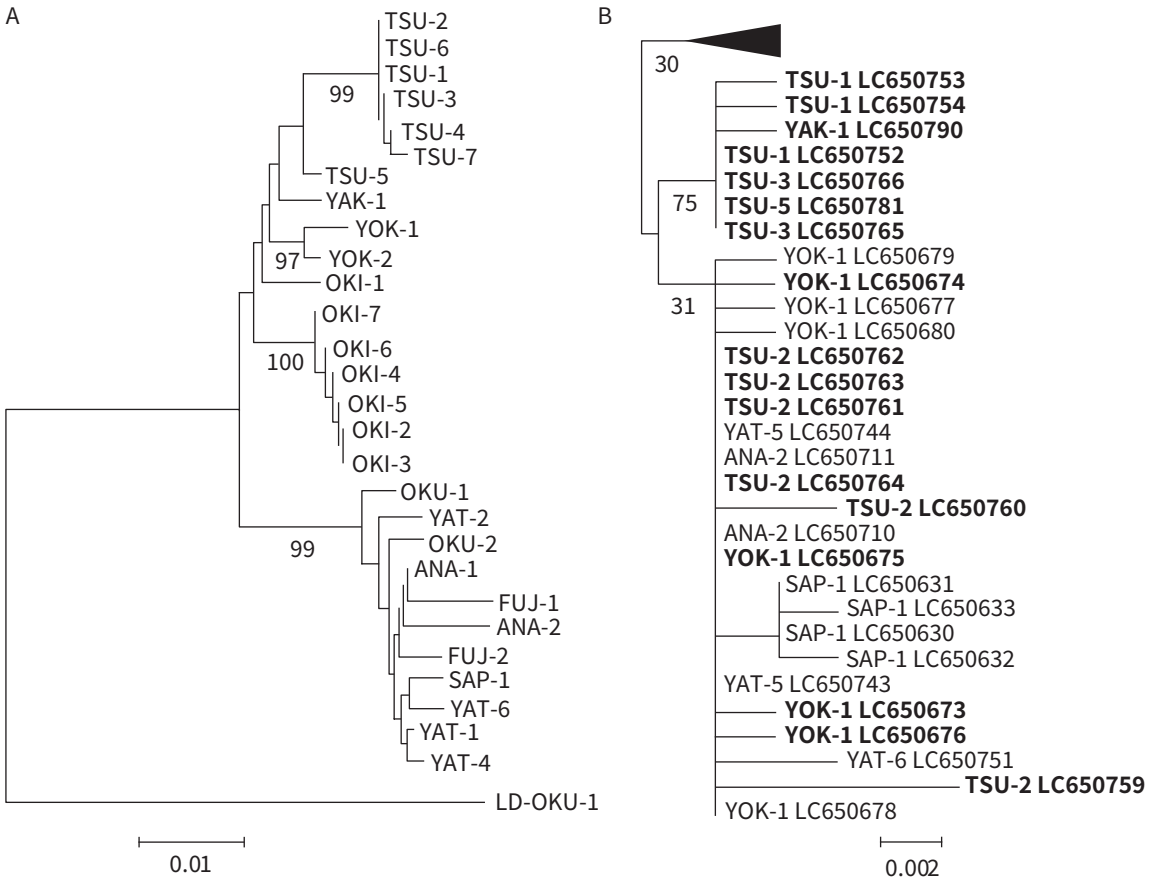

Figure 2. (A) Neighbor-joining (NJ) dendrogram showing phylogenetic relationships of *Leptura mimica* based on mitochondrial gene sequences. Abbreviations of each sample are shown in Table 1. (B) NJ dendrogram showing phylogenetic relationships based on 18S-rRNA gene sequences from all clones read in this study. Each clone is represented by its sample code with accession number and the bold letter shows the R4 type in Table 1. The black triangle shows one clade in which all other clones are included. Bootstrap values correspond to 500 replications. The black bar demonstrates the genetic distance.

clones (Fig. 2B). Remarkably, except for R4 clones, some clones from the brown or brown-mixed type from Anayama and Yatugatake were also included in the above-mentioned clade, regardless of the difference of clade in the mitochondrial gene-based tree.

DISCUSSION

In this study, two approaches with molecular data of 1) the mitochondrial gene and 2) nuclear ribosomal DNA (rDNA) explored the evolutionary aspect of polymorphic elytral patterns of *L. mimica*. For the first time, three collection sites on the distributional border (Oki Is., Tsushima Is., and Yakushima Is.) were added to the molecular phylogenetic analysis, and multiple samples from one collection site were examined in this study. Although the population of Oki has been recognized as a remarkable macular type (Makihara *et al*., 1991), Oki samples fell into a western-southern clade where the brown type was concentrated. Such a geography-dominant result was consistent with that of the phylogenetic tree shown by Saito *et al*. (2000). Multiple samples showed slight phylogenetic

variables, which probably reflect the genetic diversity in each population.

During the direct sequencing process, a region was found in which TN repeats occurred. Sequencing with the cloning method revealed that intra-individual polymorphism arose from the repeat region located in ITS1 of rDNA, possibly due to incomplete homogenization in the process of concerted evolution. Mating with different lineages or kindred may result in incomplete homogenization. Repeat sequences in ITS are also recognized as microsatellites that can be genetic markers for lineages within species (Alaranta *et al*., 2011). Such an intra-individual polymorphism of ITS was discovered in the polymorphic insects, which must be reminiscent of phylogenetic relationships. Therefore, this microsatellite region in *L. mimica* may be helpful in clarifying the evolutionary aspects of the elytral polymorphism.

Since it is known that non-parental repeat types can be generated by unequal crossing over and rearrangement (Alaranta *et al*., 2011), an analysis was conducted by focusing on major repeat types of R6, R7, and R4 in this study. R7 is a universal type as well as R6, but the blackish type from Okutama has only R7, which indicates that R7 seems to be linked more closely to the blackish type. Similarly, R6 tends to be linked with the macular type (Sapporo, Yatsugatake, Oki), while R4 is specific to the brown (Tsushima, Yakushima) and brown-mixed (Yokosuka) samples. Consequently, the three repeat types tend to reflect some degree of the elytral type.

Considering the role of elytral patterns in ecosystems, these three types must have been selected for adaptation to each habitat. If these genetic characteristics are co-maintained as genetic diversity, adaptability to environmental changes can be reinforced, which is in agreement with the wider distribution of *L. mimica* in Japan. As for the collection site of Okutama, there is an old-growth forest (Hoshino, 2018); therefore, no other genetic diversity is needed except for R7, which has been best fitted to the sustainable environment. Although further analysis of the repeat types for Eurasian continent samples will be needed, R7 is interpreted to be a Japanese-specific genetic type, accompanied by adaptive events such as participating in a novel mimicry ring and converting a host plant to a broadleaf tree.

In general, the 18S-rRNA gene is a core region and is suitable for phylogenetic analysis at the genus or higher taxonomic level. Nevertheless, in the phylogenetic tree of the 18S-rRNA sequence, all sequences from R4 clones formed a single clade, and some sequences that were not derived from R4 clones but from brown and brown-mixed elytral type samples also fell into the same clade. The results indicate that the lineage of R4 had been generated as a degree of independent lineage in the past. The brown type has a unique ecological aspect of inhabiting broadleaf trees of the lower altitudinal forest, and thus R4 type was probably induced by such an ecosystem universal in western Japan. These results suggest that the polymorphism in the elytral pattern of *L. mimica*, in the context of ITS repeat type, is due to the genetically mixed status of some lineages after the establishment of the type adapted to each ecosystem, rather than the expression control of pattern formation. Further exploration will be needed if the current status of intra-individual polymorphisms is under evolution or one complete genetic form.

In this study, the evolutionary aspect of the

polymorphic lepturine beetle *L. mimica* was suggested for the first time from the viewpoint of molecular characterization of rDNA. Notably, it was found that at least two novel lineages had been generated or introduced in past Japan; that is, the brown type is unique to western Japan and the R7 lineage may be positioned phylogenetically between the other two types. Currently, these genetic factors are co-maintained and may be characterized as part of the biodiversity of Japan. The intra-individual polymorphism in ITS may be linked not only with the elytral pattern but also to other characteristics and will be a valuable marker to speculate lineage generation, hybridization, and sorting. However, identification of more repetitive sites will be needed to address this issue in detail in the future.

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