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MACRO- AND MICROSCALE DISTRIBUTION PATTERNS OF TWO CLOSELY RELATED JAPANESE *MNAIS* SPECIES INFERRED FROM NUCLEAR RIBOSOMAL DNA, ITS SEQUENCES AND MORPHOLOGY (ZYGOPTERA: CALOPTERYGIDAE)

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Much variation occurs in morphology and colouration among individuals of Japanese *Mnais* spp. It has been noted that 2 groups of *Mnais* often cohabit a stream in western Japan. There is, however, no clear morphological difference in ♂ appendages and penis between the 2 groups, and this makes it difficult to determine their taxonomic status. In this study, to clarify the relationships between the sympatric species on both small (along a stream) and large (across Japan) geographic scales, sequences of the internal transcribed spacers 1 and 2 (ITS1 and 2) of nuclear ribosomal DNA are compared. Base substitutions occurred at 4 sites of 223 bps of the ITS1 region, and by their combinations, the four sequence types could be distinguished among a total of 800 individuals. In the ITS2 region (total 411 bps including 5.8S rRNA region), all examined individuals had the same sequence. The geographical distribution of each ITS1 sequence type and morphological data of wings and a pterostigma suggest that Japanese *Mnais* includes 2 distinct spp., *M. strigata* Selys, 1853 and *M. costalis* Selys, 1869. Their distribution ranges overlap widely in western Japan, where *M. strigata* is usually found at smaller and upper streams than *M. costalis*.

INTRODUCTION

In Japan, the damselflies of the genus *Mnais* (Odonata: Calopterygidae) are widely distributed, excluding the Ryukyu Islands and several small islands (SUGIMURA et al., 1999). The Japanese *Mnais* group is also recorded from the Kurile Islands, Russia (HARITONOV & MALIKOVA, 1998), an uncertain record of *M. pruinosa* is known from Korea (cf. LEE, 2001), but no species has ever been found in continental Russia.

Thus, Japanese *Mnais* is an isolated group in the distribution range of *Mnais* (Fig. 1). *Mnais* damselflies are stream-dwellers with low dispersal ability (TAGUCHI & WATANABE, 1992). This genus has been intensively studied from the view point of behavioural ecology. It has been shown, copulating males remove rival sperm stored in the female (SIVA-JOTHY & TSUBAKI, 1989a) and two wing-colour forms of males coexist within a population usually corresponding to their mating tactics, territorial males and non-territorial, sneaking males (HIGASHI, 1976, 1981; UBUKATA, 1979; SUZUKI et al., 1980; NOMAKUCHI et al., 1984; NOMAKUCHI & HIGASHI, 1985; NOMAKUCHI, 1988, 1992; SIVA-JOTHY & TSUBAKI, 1989b; WATANABE & TAGUCHI, 1990, 1997; TSUBAKI et al., 1997; HOOPER et al., 1999; PLAISTOW & TSUBAKI, 2000; HIGASHI & NOMAKUCHI, 2002; TSUBAKI, 2003). It seems interesting to search for an evolutionary pattern of mating behaviour of *Mnais* using a phylogenetically comparative method.

However, a great variability in body size, wing colouration, and other marking patterns of individuals make it difficult to distinguish them taxonomically. A consensus view has not yet been obtained to distinguish inter- or intraspecific populations. At present, four taxonomic treatments coexist; (1) one species including three subspecific groups (e.g., ASAHINA, 1975b, 1976; HAMADA & INOUE, 1985), (2) two species, one of which separating into several subgroups or two subspecies (e.g., HIURA, 1972; ISHIDA et al., 1988; SUGIMURA et al., 1999), (3) three species that recognize the three groups in (1) and (2) as all different species (e.g., SUZUKI, 1980, 1981), and (4) four species by adding another undescribed species to (3) (e.g., SUZUKI, 1984b, 1985). Despite the difference in their taxonomic treatments, all agreed with a phenomenon that two groups of *Mnais* often cohabit a stream in western Japan, segregating their microhabitats, one prefers the upper reaches of streams and the other lives in the lower reaches (YAMAMOTO, 1955, 1956; HIROSE & ROKUYAMA, 1966; ISHIDA, 1969; SUZUKI & EGUCHI, 1979), and probably being isolated reproductively because field-caught mating pairs were always intra-group members (SUZUKI et al., 1980). Difficulty in taxonomic conclusions may result not only from a great morphological variation across all Japanese populations but also due to no clear morphological difference in male terminalia and penis (ASAHINA, 1975b).

DNA sequencing techniques and their comparison contribute to understand spatial and temporal patterns of speciation of animals, in particular the closely related species (reviewed by CATERINO et al., 2000). The nuclear ribosomal internal transcribed spacers 1 and 2 (ITS1 and 2) are noncoding, rapidly evolving DNA regions and have proved useful for comparing closely related insect species, subspecies, or populations (ALVAREZ & HOY, 2002). WEEKERS et al. (2001), SAMRAOUI et al. (2002, 2003) and PILGRIM et al. (2002) sequenced ITS regions for phylogenetic analysis of some damselflies and dragonflies. In this study, we analyze populations of Japanese *Mnais* damselflies by sequencing ITS1 and 2 and by comparing some morphological characters of specimens taken (1) throughout the Japanese Archipelago (macro-scale distribution) and (2) taken along a single or neighboring river systems (micro-scale distribution).

MATERIAL AND METHODS

A total of 800 *Mnais* specimens, 556 adult males, 140 adult females and 104 larvae, were used for DNA analysis. Most adults were preserved in pure ethanol, but about a quarter were dried. Larvae were all preserved in ethanol. Specimens were collected to cover their entire distribution range in Japan. On the other hand, to examine the micro-scale genetic structure, intensive collections were made in the four river systems; the Yodo-gawa and Kumozu-gawa Rivers (Shiga and Mie prefectures), the Waidani-gawa River (Wake-gun, Okayama prefecture), the Tsukitani-gawa River (Soja-shi, Okayama prefecture), and the Chikugo-gawa and Yamakuni-gawa Rivers (Oita prefecture) (Fig. 1). In the Yodo-gawa and Kumozu-gawa Rivers, which flow in opposite direction from a common watershed, *Mnais* were collected at four sampling sites (Sites 1-4; Oishiodawara in the branch Uji-gawa, and Maruyama, Kozu and Nishiaoyama in the branch Kizu-gawa) and at two sites in the latter (Sites 5-6; Higashiaoyama and Isegi). The minimum distance between Sites 1 and 6 is 55 km. Sampling was made at 1 km intervals in the Waidani-gawa River (five sites) and 200-400 m intervals in the Tsukitani-gawa Rivers (five sites). In the Chikugo-gawa and Yamakuni-gawa Rivers, each flowing in an opposite direction from the same mountain, *Mnais* were collected at Uchikawano (Site 1) in the Chikugo-gawa, and at Nakama, Kanayoshi and Nishiyakata (Sites 2-4) in the Yamakuni-gawa. Site 4 locates 35 km northeast from Site 1.

Muscular tissue was isolated from the thorax in adult specimens and from basal segments of legs in the larvae. The tissue was homogenized in a sterile 1.5-ml centrifuge tube containing 500 μl of lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl), 50 μg of Proteinase K, and 10 μg of RNase. The mixture was incubated 3 h at 55°C. Following a series of phenol-chloroform and chloroform-isoamylalcohol

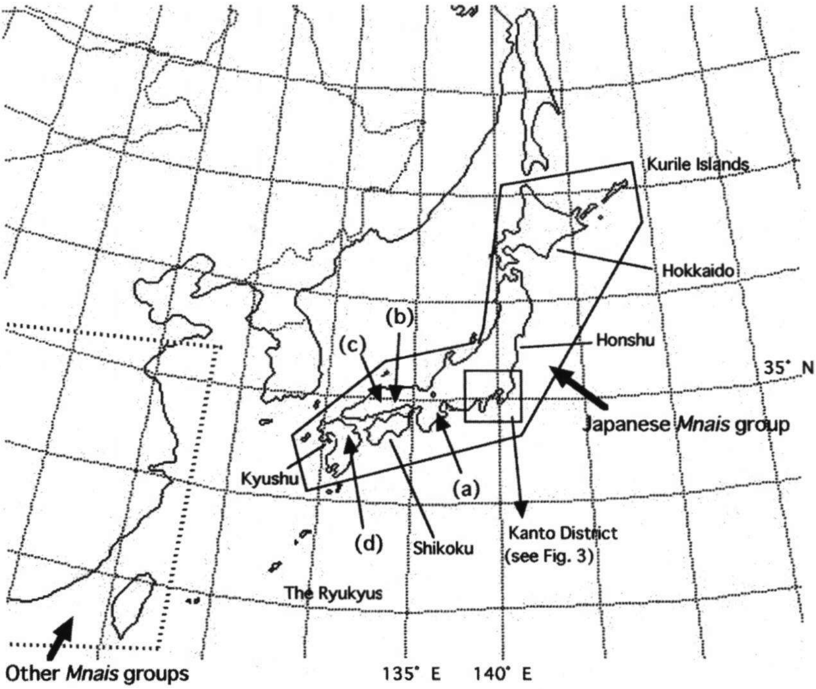

Fig. 1. The distribution ranges of Japanese and the other groups of *Mnais* damselflies and the four river systems (a-d) where small scale genetic structures were examined based on ITS1 sequences (see Fig. 4).

extractions, DNA was precipitated with an equal volume of pure ethanol. The pellet was rinsed with 70% ethanol, dried, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA).

Ribosomal spacers (ITS1 and ITS2) and the ribosomal 5.8S rDNA were amplified by use of the polymerase chain reaction (PCR). The primers 5'-TAGAGGAAGTAAAAGTCG-3' and 5'-GCTTAAATTCAGCGG-3' (WEEKERS et al., 2001) were used to amplify the entire ITS1, 5.8S, and ITS2 regions. The reverse internal primer in the conserved regions of the 5.8S rDNA was used for confirmation (5'-CCGAGTGATCCACCGTTTAGGAT-3'). The reaction mix was a total volume of 30 µl in a 500-µl tube, composed of 19.7 µl of distilled water, 3.0 µl of 10× PCR buffer, 2.4 µl of 25 mM dNTP mix, 1.5 µl of each primer (10 pM), 0.3 µl

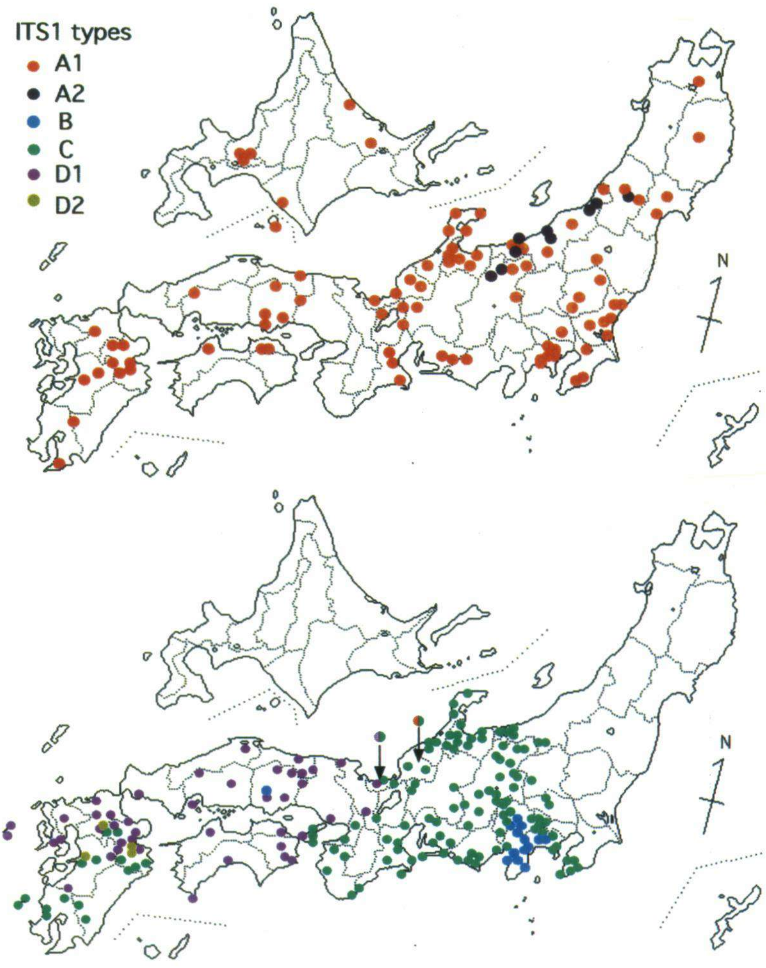

Fig. 2. Geographical distributions of ITS1 sequence types of *Mnais* damselflies in Japan. Dotted lines indicate prefectural boundaries. Two groups, Type A1/2 (upper) and the others (lower), are revised as *M. costalis* and *M. strigata*, respectively. Arrows indicate two hybrid F1 individuals of Types A1/C and Types C/D1.

of Taq polymerase (5 U/µl; Takara, Japan), and 1.5 µl of template DNA. The reaction mix was layered with a drop of mineral oil (Sigma) and the tubes were placed in a thermal cycler (PTC-100TM; MJ Research, Inc.). Cycling conditions were 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min for 30 cycles. PCR products, after purified by a centrifugal filter device (Microcon YM-100; Millipore Corporation), were used for direct sequencing with the BigDye technology, the protocol of the ABI Prism BigDye terminator cycle sequencing ready reaction kit, and thereafter analyzed on an ABI Prism 377 DNA sequencer (PE Applied Biosystems).

The width of heads (between the outer margins of eyes) and the total length of the left forewing (right one if the left was broken) were measured with a slide caliper to the nearest 0.05 mm. A pterostigma of the forewing was also measured for its length along the costa and width at the widest part (including vein's width), under a binocular microscope with an ocular micrometer, to the nearest 0.01 mm (10× magnification).

RESULTS

DISTRIBUTION PATTERNS

There were six sequence types of the 223-bp ITS1 region (accession numbers in the GenBank database, AB125623–AB125628) among the 800 individuals studied. Base replacements occurred at 77th, 116th, 132nd, and 156th sites (5'-3') and represented as AGTA (Type A1, N=270), GGTA (Type A2, N=53), AGTG (Type B, N=35), AGCA

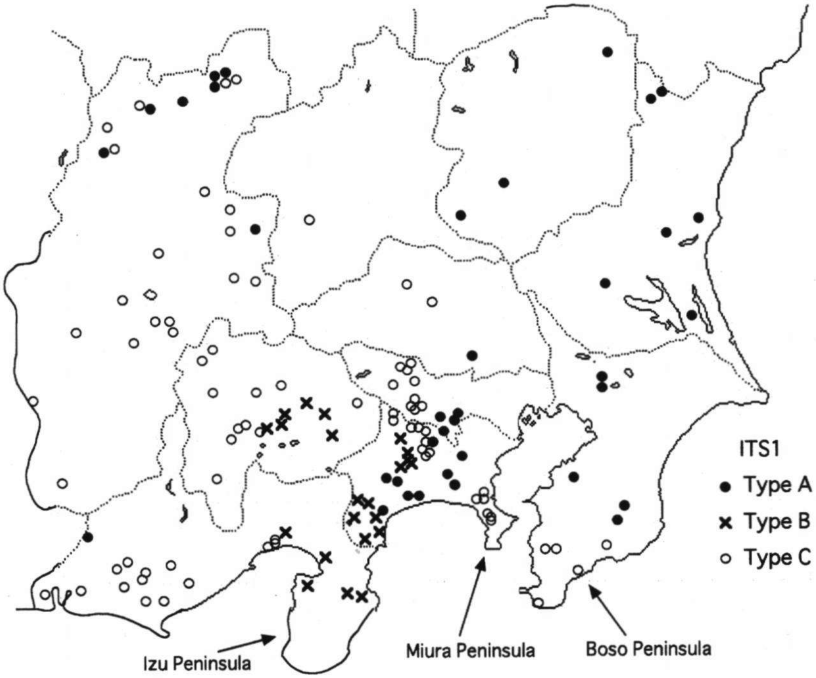

Fig. 3. Distributions of ITS1 sequence types of *Mnais* damselflies in Kanto District and adjacent areas, central Japan (for location, see Fig. 1). Dotted lines indicate prefectural boundaries. Individuals of Type A are revised as *M. costalis* and those of Types B and C are *M. strigata*.

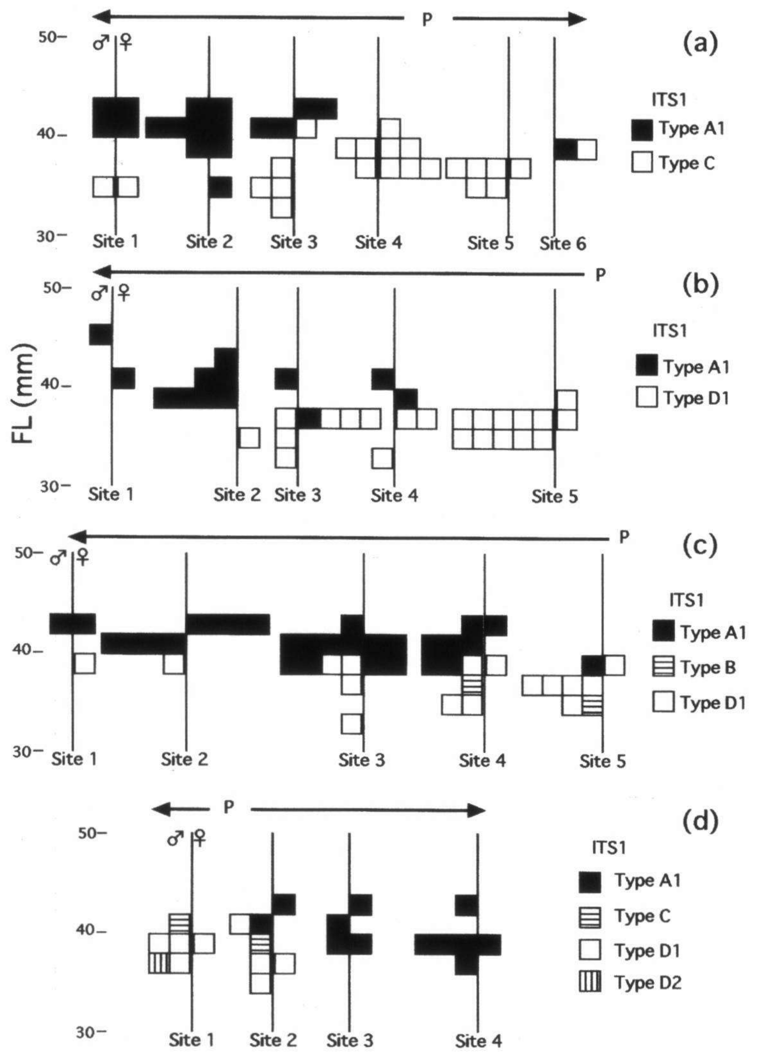

Fig. 4. Genetic structures of *Mnais* damselflies collected at four to six sites along a single stream or between two adjacent streams with a common watershed at the four river systems: (a) the Yodo-gawa and Kumozu-gawa Rivers; – (b) the Waidani-gawa River – (c) the Tsukitani-gawa River; and – (d) the Chikugo-gawa and Yamakuni-gawa Rivers. For location of the studied river systems, see Fig. 1. Each square represents a single damselfly with information of the forewing length (FL), sexes, and ITS1 sequence types. Arrows indicate the direction of stream flows from a peak (p). Two groups, Type A1 and the others, are revised as *M. costalis* and *M. strigata*, respectively.

(Type C, N=328), AGCG (Type D1, N=104), and ACCG (Type D2, N=8). One female and one male adults had hybrid DNA of Types A1 and C, AG(T/C)A, and of Types C and D1, AGC(A/G), respectively. The base replacement of A to G at the 77th bp occurred only in Type A2, and the replacement of G to C at the 116th bp occurred only in Type D2. Therefore, Type A2 was closely related to Type A1 (so that Type A group), and Type D2 was close to Type D1 (Type D group).

The sequence including the 5.8S rDNA and ITS2 region (411 bps, accession number, AB125622) was read for a total of 45 individuals, but did not differ among the six types of ITS1; Types A1 (N=9), A2 (N=4), B (N=5), C (N=21), D1 (N=4), and D2 (N=2).

The geographical distribution of Type A1 was widest, while A2 appeared in a narrow area, Ngano, Niigata, and Yamagata Prefectures (Fig. 2 upper). The other types were found more or less allopatrically in western Japan (Fig. 2 lower). The distribution range of Type C was separated by the range of Types D1 and D2, and Type B was found in a small range in central Japan and also in one place of western Japan that was occupied by Type D1. Figure 3 shows more detailed distribution patterns of the ITS1 types in and around Kanto District, central Japan, where there is the east limit of the range of the Types B-D group. The populations of Type C in the Miura Peninsula and the southern part of the Boso Peninsula were isolated from the identical one by Type A population.

Along the single river and between the neighboring rivers, individuals of Type A (all subtype A1) and those of Types C or D (subtypes D1/D2) coexisted (Fig. 4). Type A individuals had usually larger wings and dominated in a lower reach than the other ITS1 type(s).

MORPHOLOGICAL CHARACTERS

Of 556 adult males whose ITS1 types were determined, a data set of forewing length/head width and pterostigma length/width was obtained for 548 individuals (8 males lacked head/wings). The relationships between the head width and forewing length were similar among the males of Types B, C and D, but males of Type A had a relatively longer forewing than those males (Fig. 5). The regression equation was $Y=7.810X-9.144$ ($N=213$, $r=0.907$, $P<0.001$) for the males of Type A and $Y=6.334X-2.467$ ($N=335$, $r=0.781$, $P<0.001$) for those of Types B, C and D. The slopes of these regression lines differed significantly (ANCOVA; $F_{1,544}=14.6$, $P<0.0001$). The mean ratio of forewing length/head width was 6.35 ($N=213$, $SD=0.18$) in Type A, 6.00 ($N=18$, $SD=0.23$) in Type B, 5.94 ($N=229$, $SD=0.21$) in Type C, and 5.92 ($N=88$, $SD=0.20$) in Type D.

The relationships between the pterostigma length and width were similar among the males of Types B, C and D (Fig. 6 upper). However, the pterostigma was more elongated in Type A males than other types of males (Fig. 6 lower). The regression equation was $Y=0.121X+0.550$ ($N=213$, $r=0.524$, $P<0.001$) for the males of Type A and $Y=0.130X+0.634$ ($N=335$, $r=0.379$, $P<0.001$) for those of Types B, C and D. The slopes did not differ between these regression lines (ANCOVA; $F_{1,544}=0.15$, $P=0.70$), but their

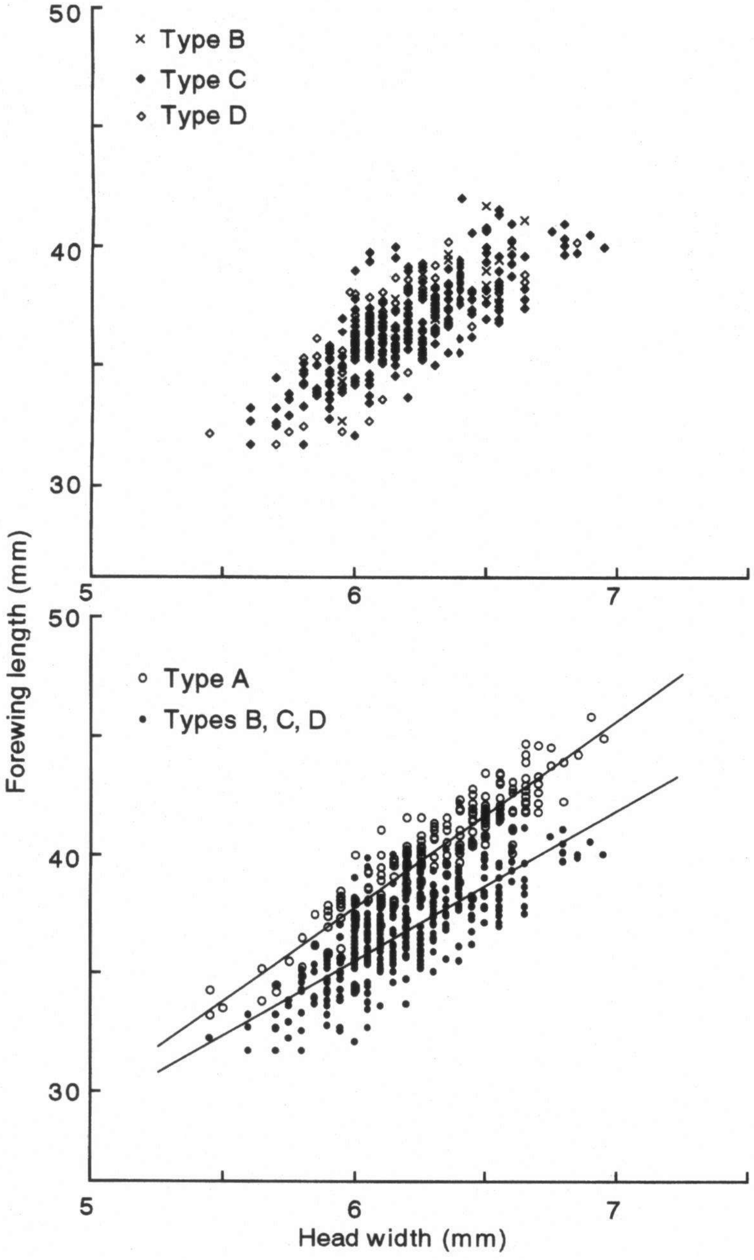

Fig. 5. The relationships between the head width and forewing length among three types of ITS1 sequences (upper), and between the two ITS1 groups (lower) of *Mnais* damselflies. The two groups, Type A and the others, are revised as *M. costalis* and *M. strigata*, respectively.

intercepts differed significantly ($F_{1,545}$=122.8, P<0.0001). The mean length/width ratio of the pterostigma was 2.56 (N=213, SD=0.36) in Type A, 1.83 (N=18, SD=0.26) in Type B, 1.76 (N=229, SD=0.28) in Type C, and 1.82 (N=88, SD=0.28) in Type D.

DISCUSSION

Sequencing of ITS1 revealed that Type A1 is widely distributed in Japan with its subtype, Type A2. The other types (Types B, C, and D1 with subtype D2) occupy western Japan, replacing each other. In western Japan where the populations of Type A and the other types usually coexist along a single stream, the former was found more frequently in lower reaches and the latter in upper reaches. Several researchers pointed out that the thorax size and the pterostigma shape of males are key characters to distinguish these two groups in western Japan (e.g., YAMAMOTO, 1956; ASAHINA, 1975b; ISHIDA et al., 1988). Thoracic size may be correlated with wing size because flight muscles are contained within the thorax. In our measurements, the relative length of forewings and the shape of pterostigma differed between males of Type A and Types B, C and D. These results suggest that Japanese *Mnais* are divided into two groups, Type A and the other types. Only a single female of 800 individuals examined was confirmed a hybrid F1 between the two (Type A/C) despite examination of several apparent hybrids based on morphology. This fact suggests that the two species are well isolated reproductively.

Our conclusion differs from previous specific/subspecific groupings. The geographical boundaries observed among each ITS1 type do not coincide with any boundaries of previously proposed groups. Among the previously known three groups corresponding to subspecific/specific names, *pruinosa* (including *Mnais* sp. in sense of SUZUKI, 1984b, 1985), *costalis* and *nawai*, YAMAMOTO (1956) and ASAHINA (1975b) pointed out their morphological similarities between *costalis* and *nawai*, but HIURA (1972), SUZUKI (1984a), ISHIDA et. al. (1988), SUGIMURA et. al. (1999) and other researchers regarded *costalis* and *pruinosa* as more related taxa than *nawai*. In this respect, our conclusion is that *costalis* and *nawai* are not heterogenous but a single group, and *pruinosa* differs from it. The two populations of Type A and Types B-D are renamed *M. costalis* and *M. strigata*, respectively, without subspecific division.

MNAIS COSTALIS SELYS, 1869

Mnais costalis SELYS, 1869: 651. Lectotype (♂) designated by ASAHINA (1975a).

Mnais pruinosa f. *costalis*; SELYS, 1883: 128-129.

Mnais pruinosa costalis; ASAHINA, 1975b: 31-40.

Mnais strigata nawai YAMAMOTO, 1956: 19-22 with 2 pls. Holotype (♂) by original designation. New synonym.

Mnais pruinosa nawai; ASAHINA, 1975b: 34-36.

Both type specimens of *M. costalis* and *M. strigata nawai* have a much narrower pterostigma in forewings (YAMAMOTO, 1956; ASAHINA, 1975a); therefore, Type A

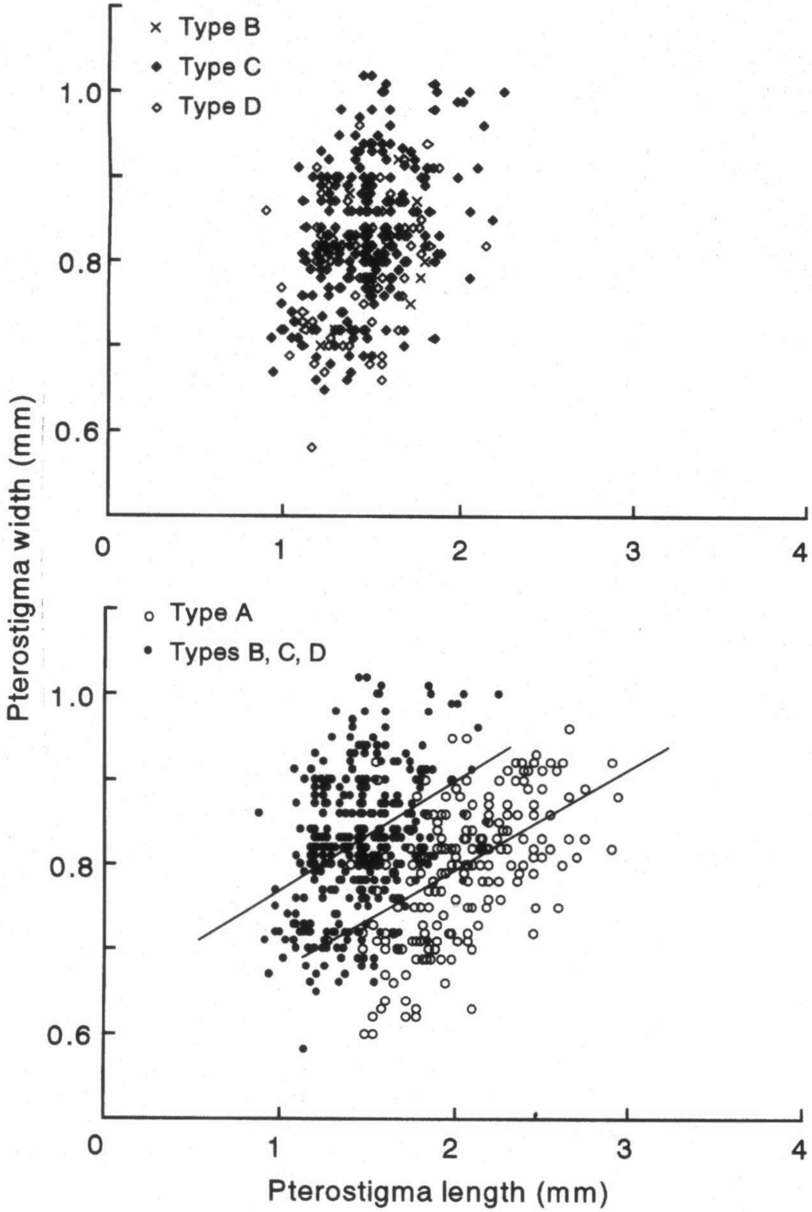

Fig. 6. The relationships between the pterostigma length and width among three types of ITS1 sequences (upper), and between the two ITS1 groups (lower) of *Mnais* damselflies. The two groups, Type A and the others, are revised as *M. costalis* and *M. strigata*, respectively.

group can be identified as *M. costalis*, and *M. strigata nawai* is synonymized with it. Its distribution ranges the four main islands (Hokkaido, Honshu, Shikoku, and Kyushu) and the island Oki-togo. However, some vacant areas are found in Honshu (mountainous regions of Chubu District, southern part of Tokai District, and southern part of Kinki District), Shikoku (a southern half), and Kyushu (Nagasaki prefecture). This species consists of populations so far identified as *M. nawai* (or *M. pruinosa nawai*) and *M. costalis* (or *M. pruinosa costalis*) excluding those known in central Japan (in particular Kanto District). *Mnais* from the Kurile Islands (under *M. pruinosa costalis* by HARITONOV & MALIKOVA, 1998) may be this species, judged from its range, but needs confirmation. Mating behaviour of adults and other related studies by UBUKATA (1979), TSUBAKI et al. (1997), HOOPER et al. (1999), PLAISTOW & TSUBAKI (2000), HIGASHI & NOMAKUCHI (2002), and TSUBAKI (2003) are made for this species.

MNAIS STRIGATA SELYS, 1853, STAT. REV.

Mnais strigata SELYS, 1853: 20. Lectotype (♂) designated by ASAHINA (1975a). Synonymized with *M. pruinosa* by ASAHINA (1975a).

Mnais pruinosa SELYS, 1853: 20-21. Lectotype (♂) designated by LIEFTINCK (1971). Synonymized by OGUMA (1913).

Mnais strigata was first described by HAGEN in SELYS (1853), but was dealt as a form of *M. pruinosa* by ASAHINA (1975a, 1975b). However, *M. strigata* appeared before *M. pruinosa* in SELYS's (1853) paper; so by page precedence *strigata* is the valid name for the group of Types B, C and D, in accordance with OGUMA (1913), who used the name *pruinosa* as one of the forms of *M. strigata*. Lectotype specimens of both *M. strigata* and *M. pruinosa* are believed to be collected in Nagasaki prefecture, Kyushu (ASAHINA, 1975a), where the population of Type A has never been obtained (also see HAMADA & INOUE, 1985). This species is distributed in western Japan, including an island Awaji-shima, Goto Islands, and Koshiki Islands. It corresponds to the populations so far identified as *M. pruinosa* (or *M. pruinosa pruinosa*), including *Mnais* sp. in the sense of SUZUKI (1984b, 1985), and *Mnais costalis* (or *M. pruinosa costalis*) in central Japan (especially in Kanto district). This species has not been known outside the Japanese archipelago (TSUDA, 2000). Studies on mating behaviour and on other related subjects, by HIGASHI (1976, 1981), NOMAKUCHI et al. (1984), NOMAKUCHI & HIGASHI (1985), NOMAKUCHI (1988, 1992), SIVA-JOTHY & TSUBAKI (1989a, b), and WATANABE & TAGUCHI (1990, 1997), are available for this species.

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