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# Differentiation of the Dragonfly Genus *Davidius* (Odonata: Gomphidae) in Japan Inferred from Mitochondrial and Nuclear Gene Genealogies

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**To infer the differentiation of Japanese *Davidius* dragonflies, we investigated the genealogies of the mitochondrial cytochrome oxidase subunit I gene (COI) and the nuclear ribosomal RNA gene region encompassing 18S, ITS1, 5.8S, and ITS2 sequences for three species endemic to Japan—*Davidius nanus*, *D. fujiama*, and *D. moiwanus*—as well as *D. lunatus* from the Korean Peninsula. According to the mitochondrial and nuclear gene genealogies, *D. nanus* and *D. moiwanus* are closely related and are sister to the continental species *D. lunatus*, whereas *D. fujiama* differentiated from an ancestor of the other three species. Although the mitochondrial DNA data did not resolve the relationships between *D. nanus* and three *D. moiwanus* subspecies, the nuclear DNA data indicate the monophyly of *D. moiwanus* and its subspecies. The nuclear gene genealogy suggests that isolated wetlands used by larval *D. moiwanus* derive from the ancestral riverine habitats of *D. nanus* and other *Davidius* species. The COI sequence divergence among local populations was much greater in *D. moiwanus* than in *D. nanus*, which may be the result of differences in the dispersal ranges associated with the habitat types of these species.**

**Key words:** colonization, habitat shift, diversification, phylogeography

## INTRODUCTION

The colonization and subsequent ecological diversification of organisms on islands have contributed greatly to global species diversity (Grant, 1998). Although radiations of colonizing organisms on oceanic islands, such as Hawaii and the Galapagos, are well known, major islands adjacent to continents also provide novel sites for species radiation. The Japanese Archipelago was formed in the mid Miocene (Ichikawa *et al*., 1970; Yonekura *et al*., 2001) and encompasses various climatic regions, from subtropical to subarctic. Since the mid Miocene, extensive geographic changes, including connection with and disconnection from continental areas, and climatic changes are thought to have contributed to the faunal diversity of the Japanese islands. Especially for insects, colonization of the Japanese islands by ancestral stocks from the continent and their subsequent diversification seem to have contributed to the rich fauna of Japan (Hirashima, 1989). The historical process of faunal diversification can be explored within the framework of phylogeography (Avise, 2000) by analyzing the molecular phylogeny of species distributed within Japan as well as closely related continental species. Nevertheless, few molecular phylogenetic studies have investigated the relationships among insects inhabiting the Japanese islands and the Asian continent (*e.g.*, Tominaga *et al*., 2000; Sota and Hayashi, 2004; Sota *et al*., 2004).

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Dragonflies (Odonata) are flying insects with potentially great mobility. The order Odonata consists of three suborders: two large groups, Zygoptera and Anisoptera, and one small group, Anisozygoptera, containing only two species worldwide (Tsuda, 2000; Silsby, 2001). Of these, the suborder Zygoptera (the so-called damselflies) consists of relatively weak fliers, closely associated with small streams and ponds. Recent molecular phylogenetic studies of Zygoptera have revealed differentiation at various geographical scales, such as among local habitats and among isolated islands (Chippindale *et al*., 1999; Brown *et al*., 2000; Misof *et al*., 2000; Turgeon and McPeek, 2002; Jordan *et al*., 2003; Wong *et al*., 2003; Hayashi *et al*., 2004, 2005; Watts *et al*., 2004). In contrast, few phylogeographic studies of the suborder Anisoptera, which includes many active fliers, have been conducted (Artiss, 2004; Hovmöller and Johansson, 2004). Although anisopteran groups with wide distributions generally show relatively low genetic differentiation among regions (*e.g.*, Artiss, 2004), some are endemic to different continental regions and islands and have diversified ecologically to different habitats like small streams and ponds (*e.g.*, Silsby, 2001). Therefore, some groups of Anisoptera are well suited for studies of insect diversification on islands.

In this paper, we examined the differentiation of anisopteran dragonflies in the genus *Davidius* in Japan by investigating the genealogies of the mitochondrial cytochrome oxidase subunit I (COI) gene and the nuclear ribosomal RNA gene (18S, ITS1, 5.8S, and ITS2) for four species in Japan and Korea. Three *Davidius* species (*D. nanus*, *D. moiwanus*, and *D. fujiama*) are endemic to the Japanese mainland (Okumura, 1935; Ishida *et al*., 1988; Sugimura *et al*., 1999); one species (*D. lunatus*) occurs on the Korean

Peninsula and in Primorsky Krai, Russia (Bartenef, 1914; Okumura, 1937; Asahina, 1989). In addition, 18 species have been described from other parts of East Asia (Tsuda, 2000). Because no *Davidius* species are distributed on the Ryukyu Archipelago, in Taiwan, or in most of Siberia, the ancestral population of Japanese *Davidius* species likely colonized Japan from the Korean Peninsula. In this study, we assessed the phylogenetic relationships among four *Davidius* species and their subspecies and thereby inferred the colonization and differentiation of the three Japanese species.

## MATERIALS AND METHODS

### Sampling and DNA sequencing

We examined 32 individuals belonging to four *Davidius* species from 11 localities in Japan and Korea (Table 1, Fig. 1). Identification of the species followed Asahina (1989), Asahina and Inoue (1973), Ishida *et al*. (1988), Ishida (1996) and Sugimura *et al*. (1999). *Lanthus fujiacus* was used as the outgroup. This species belongs to the same tribe Octogomphini (*sensu* Carle, 1986) with the genus *Davidius*. Adults and larvae collected in the field for DNA analysis were preserved in 99% ethanol.

To extract genomic DNA, muscle tissue of adults or larvae preserved in 99% ethanol was digested with protein kinase in CTAB (hexadecyltrimethylammonium bromide) buffer, and total DNA was extracted using standard phenol-chloroform extraction and ethanol precipitation methods. Polymerase chain reaction (PCR) was used to amplify a sequence of 880 base pairs (bp) of the mitochondrial COI gene from total DNA template using two newly designed primers: COS2265 (forward): 5'-GCA CAA GAA AGA GGG AAA AAA GA-3' and COA3145 (reverse): 5'-TT ATG GTG TGG TCA TGA AAG TA-3'.

To amplify about 1100 bp of the region encompassing ITS1, 5.8S ribosomal RNA (rRNA), and ITS2, we used the primers: 5'-TCA ACA CGG GAC CCA GGC CC-3' (forward, in 18S rRNA; Pilgrim *et al*., 2002) and 5'-GCT TAA ATT CAG CGG-3' (reverse, in 28S rRNA; Weekers *et al*., 2001). We also used the internal primers; 5'-TAG AGG AAG TAA AAG TCG-3' (forward, in 18S rRNA; Weekers *et al*., 2001) and 5'-CGA TGA TCA AGT GTC CTG CA-3' (reverse, in 5.8S rRNA; Pilgrim *et al*., 2002).

A dye terminator cycle sequencing reaction was performed with ABI PRISM BigDye Terminator (PerkinElmer), and the products were electrophoresed on an ABI 377 sequencer. Direct sequencing of the ITS region can result in overlapping of signals owing to multiple copies or heterozygosity, such that subcloning of PCR products is necessary to determine exact sequences (*e.g*., Hovmöller and Johansson, 2004). However, we encountered no signal overlap for *Davidius* specimens, although the ITS region could not be sequenced for the outgroup species *Lanthus fujiacus* for an unknown reason. The sequence data are available in GenBank (accession numbers: for COI, AY841170–AY841193; for ITS, DQ010043–DQ010052).

### Phylogenetic analysis

DNA sequences of the COI gene were aligned using ClustalX (Thompson *et al*., 1997) with default settings. The sequenced region corresponded to positions 2292 to 3118 of the *Drosophila yakuba* mitochondrial DNA sequence (GenBank accession X03240; Clary *et al*., 1985), encompassing 721 bp of COI, 62 to 64 bp of leucine transfer RNA (tRNA), and 36 bp of cytochrome oxidase subunit II. Alignment of the sequences required gaps of 1 to 2 bp in the leucine tRNA region for *D. fujiama* and *L. fujiacus*. The final aligned sequence was 821 bp long.

For COI data, maximum parsimony, maximum likelihood, and Bayesian analyses were used to reconstruct the phylogenetic relationships among the taxa. Parsimony analysis was performed using PAUP* version 4.0b10 (Swofford, 2002). The tree search was a heuristic search of 100 random addition analyses of tree bisection-reconnection (TBR) branch swapping (MulTrees option activated). Gaps were considered missing data. The confidence in each node was assessed using bootstrap replications (1,000 replicates of random addition analysis) and Bremer support (Bremer, 1994) using TreeRot version 2 (Sorenson, 1999).

The Tamura-Nei model (including the proportion of invariable sites and the gamma distribution for rate variation among sites [TrN+I+G]; Tamura and Nei, 1993) was selected as the best-fit model by a likelihood ratio test implemented in Modeltest 3.06 (Posada and Crandall, 1998) for the COI data. With the parameter setting provided by Modeltest, maximum likelihood analysis was performed using PAUP*; the tree search used 100 random addition analyses of TBR branch swapping (MulTrees option activated). Bayesian analysis was performed using MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001). Following the available settings in MrBayes, Bayesian Markov Chain Monte Carlo (MCMC) analysis was performed under the general time reversible (GTR) +I+G

**Table 1.** Specimens used in the phylogenetic analysis and haplotype/allele codes of COI and ITS sequences.

| Locality[a] | Taxon | No. specimens | COI haplotype (*n*)[b] | ITS allele (*n*)[b] |
|---|---|---|---|---|
| 1. Kibune, Kyoto, Japan | *Davidius nanus* (Selys) | 2 | D7(1), D8(1) | A7 (1), A8 (1) |
| " | *D. fujiama* Fraser | 3 | D31(1), D33(1), D34(1) | A31 (3) |
| " | *Lanthus fujiacus* (Fraser) (outgroup) | 1 | D32(1) | — |
| 2. Yamanouchi, Nagano, Japan | *D. nanus* (Selys) | 5 | D7(2), D40(1), D42(1), D43(1) | A7 (4), A8 (1) |
| " | *D. moiwanus moiwanus* (Okumura) | 2 | D5(1), D6(1) | A5 (2) |
| 3. Kashima, Saga, Japan | *D. nanus* (Selys) | 1 | D144(1) | A7 (1) |
| 4. Gimhae, Busan, Korea | *D. lunatus* (Bartenef) | 3 | D147(1), D148(1), D149(1) | A147 (3) |
| 5. Sapporo, Hokkaido, Japan | *D. m. moiwanus* (Okumura) | 4 | D15(1), D16(2), D17(1) | A15 (1), A16 (3) |
| 6. Tomata, Okayama, Japan | *D. m. taruii* Asahina et Inoue | 4 | D44(4) | A3 (4) |
| 7. Ohara, Sakyo, Kyoto, Japan | *D. m. taruii* Asahina et Inoue | 2 | D19(1), D25(1) | A3 (2) |
| 8. Geihoku, Hiroshima, Japan | *D. m. sawanoi* Asahina et Inoue | 4 | D1(1), D2(2), D47(1) | A1 (4) |
| 9. Tottori, Japan | *D. m. sawanoi* Asahina et Inoue | 2 | D145(2) | A145 (2) |

[a] Locality numbers are those used in Fig. 1.
[b] Haplotype/allele codes correspond to those used in Figs. 3–5.

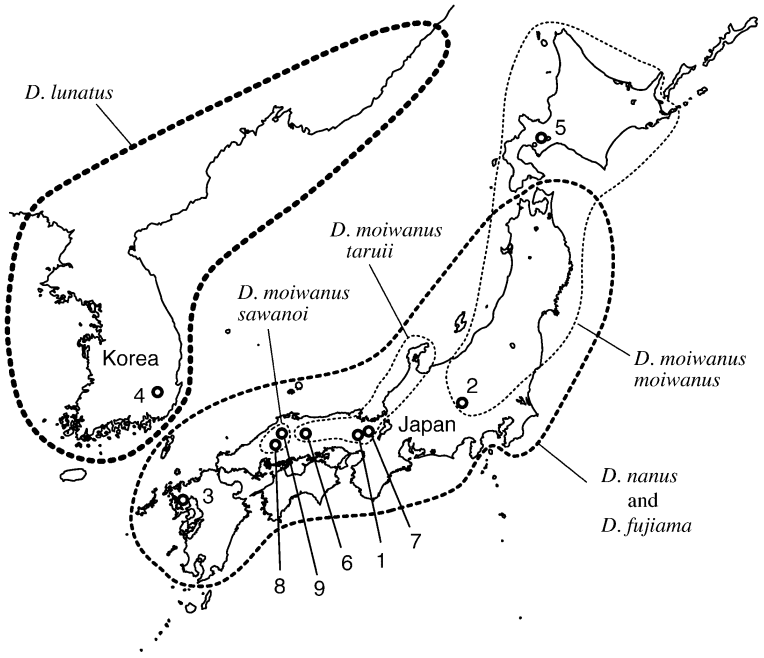


**Fig 1.** Geographic distribution and sample localities of the four *Davidius* species. See Table 1 for locality names corresponding to the numbers on the map.

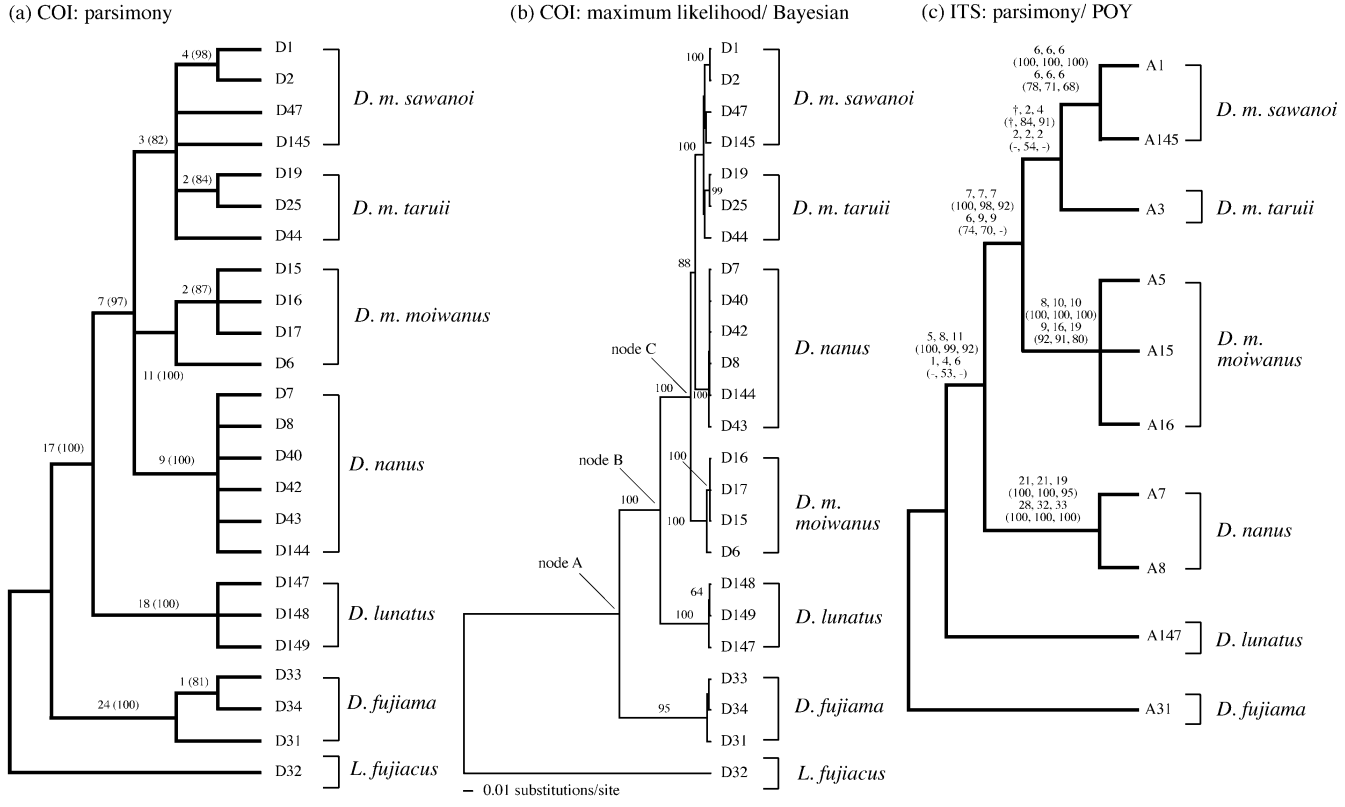


**Fig. 2.** (a) Strict consensus of the shortest trees reconstructed by unweighted parsimony analysis. Numerals above branches indicate the Bremer support value, with the bootstrap percentage (when >50%) in parentheses. (b) Maximum-likelihood tree with molecular clock assumption. The numerals above the branches are the posterior probabilities (percentages) of nodes shared with the 50% majority-rule consensus tree resulting from Bayesian analysis. Nodes A, B, and C were considered in the estimation of divergence times. (c) Strict consensus of optimal (shortest or lowest cost) trees from maximum parsimony and POY analyses of the ITS data set. The results were identical for the two methods and for three gap/change cost settings, except that maximum parsimony analysis with gap/change cost=1:1 did not reveal the node with *D. m. taruii* and *D. m. sawanoi*. Numerals above the branches: upper two rows, Bremer support values, with bootstrap percentages (when>50%) in parentheses, from maximum parsimony analysis; lower two rows, Bremer support values, with jackknife percentages (when>50%) in parentheses, from POY analysis. Triplet numerals are for gap/change costs of 1:1, 2:1, and 3:1, respectively.

model. The MCMC analysis was run for 1.5 million generations, and every 100th tree was sampled. The burn-in period was determined graphically, and the first 5,000 trees were discarded when we obtained a 50% majority consensus tree and the posterior probabilities of nodes in the tree.

To examine the extent of haplotype diversity of COI sequences in *D. moiwanus* and *D. nanus*, a statistical parsimony tree (network) was calculated using TCS version 1.13 (Clement *et al.*, 2000). Haplotypes were connected parsimoniously using 95% confidence limits.

In the analyses of the nuclear gene, the DNA sequence encompassing partial 18S, ITS1, 5.8S, and partial ITS2 from each specimen was used without partitions (hereafter called the *ITS sequence*). We first aligned the sequences using ClustalX with default settings. The aligned sequences were 1030 bp in total length, with 538 bp of 18S, 205 bp of ITS1, 140 bp of 5.8S, and 147 bp of ITS2. Because of many informative gaps required in the alignment of ITS1 and ITS2 regions, model-based phylogenetic analyses were not applied to the aligned ITS data. For the aligned ITS data, we performed parsimony analysis with gap/change cost ratios of 3:1, 2:1, and 1:1. The tree search used 100 random addition analyses with TBR branch swapping. The confidence in each node was assessed using bootstrap replications (1,000 replicates of random addition analysis) and Bremer support. Because tree construction by using the highly variable ITS sequences would be sensitive to the alignment of the data matrix, we also used POY 2.0 (Gladstein and Wheeler 1997) to directly find optimal trees for the ITS data without prior alignment. This tree search used 20 random addition analyses with TBR branch swapping and kept a maximum of 20 shortest trees from each analysis. To explore the different weighting schemes, we performed tree searches with gap/change cost ratios of 3:1, 2:1, and 1:1. Node support was evaluated by calculating Bremer support, and 200 jackknife pseudoreplicates were performed for each gap/change cost ratio.

We also performed simultaneous analyses of the combined COI and ITS sequence data using the same parsimony and POY analyses as for the ITS data. For the parsimony analysis, we calculated the incongruence length difference (ILD: Farris *et al.*, 1994, 1995) between COI and ITS data partitions with its significance level obtained by partition homogeneity test (1000 permutations) as implemented in PAUP*. Here we used ILD as a heuristic measurement of congruence between COI and ITS data partitions but not as a supporting evidence for data set combinability following the criticisms to ILD (Yoder *et al.*, 2001; Barker and Lutzoni, 2002). It is considered that a simultaneous analysis of multiple loci can improve phylogenetic reconstruction through accumulation of concordant phylogenetic signals even when conflicting signals exist in parts of data partitions (Gatesy *et al.*, 1999a, 1999b).

To estimate divergence times, we entered the COI data into PAUP* and obtained a maximum likelihood tree with the molecular clock assumption. A likelihood ratio test was performed to show that the data conformed to a molecular clock ($2\Delta$=33.3, $df$=22, $P$>0.05). We calibrated the clocked tree using the postulated time of disconnection between western Japan and the continent (opening of the Tsushima Strait) 3.5 million years ago (Ma; Tada, 1994). To obtain the standard error of the estimation, we used PAUP* to obtain 20 bootstrap trees with topological constraints of the maximum likelihood tree with the molecular-clock assumption. Each bootstrap tree was calibrated to obtain node ages.

## RESULTS

### Mitochondrial gene genealogy

The unweighted parsimony analysis yielded 20 shortest trees of 317 steps (consistency index [excluding uninformative sites; CI]=0.71; retention index [RI]=0.88). The consensus tree showed a monophyly of all species except *D. moiwanus* (Fig. 2a). The maximum likelihood and Bayesian analyses resulted in a topology that revealed the paraphyly of *D. moiwanus* (Fig. 2b). These analyses suggest that *D. nanus* and *D. moiwanus* are sister to *D. lunatus* and that these three species form a monophyletic group with respect to *D. fujiama*. Of the three *D. moiwanus* subspecies, *D. m. moiwanus* is clearly different from *D. m. taruii* and *D. m. sawanoi*, whereas the latter two do not exhibit reciprocal monophyly. Of the 20 shortest trees from the unweighted parsimony analysis, 16 recovered the monophyly of *D. moiwanus*. The other shortest trees showed the sister relationship of *D. nanus* and *D. m. sawanoi*+*D. m. taruii*, as did the maximum likelihood/Bayesian analysis. These different results from the two analyses are largely due to the different ways of evaluating different types of substitutions: weighted parsimony analysis (in which transversions are given twice the weight of transitions) resulted in the same topology as

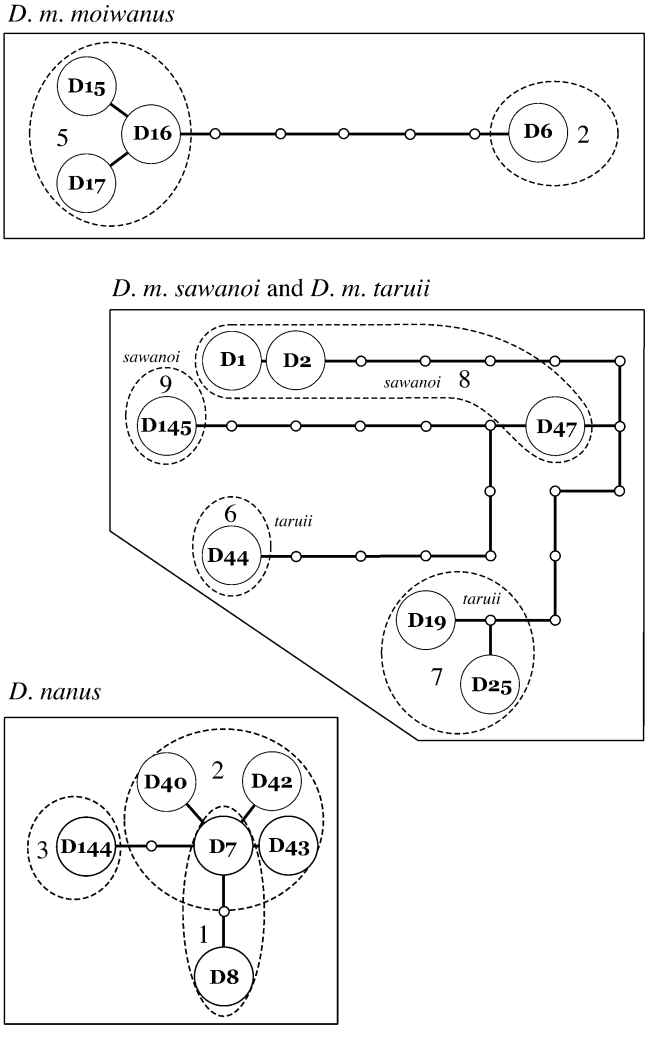


**Fig. 3.** Statistical parsimony tree of the haplotypes from *D. nanus* and *D. moiwanus*. Haplotypes are indicated by large open circles with haplotype codes (see Table 1 and Fig. 2). Mutational steps are represented by thick lines with small open circles representing intermediate haplotypes that did not appear in the samples. Haplotypes from the same locality are encircled by a dashed line, with the locality number indicated (see Table 1).

the maximum likelihood/Bayesian analysis (data not shown). However, the alternative topologies were not mutually exclusive: the Templeton test implemented in PAUP* on the weighted data did not reject alternative topologies (monophyly vs. non-monophyly of *D. moiwanus*; $P>0.05$).

The relationship among the *D. nanus* and *D. moiwanus* haplotypes was represented using statistical parsimony trees (Fig. 3). Seven *D. m. taruii* and *D. m. sawanoi* haplotypes were connected within the 95% confidence limit of 12 steps, as were four *D. m. moiwanus* haplotypes and six *D. nanus* haplotypes. The three groups (*D. m. moiwanus*, *D. m. sawanoi+taruii*, *D. nanus*) were disconnected beyond the 95% confidence limit. Compared to the divergence of haplotypes from distant localities, seen in *D. m. moiwanus* and in *D. m. sawanoi+taruii*, differentiation among *D. nanus* haplotypes from distant localities was small. These patterns indicate isolation and differentiation among local *D. moiwanus* populations and recent range expansion by *D. nanus*.

## Nuclear gene genealogy

For ITS, ten alleles were obtained from 32 *Davidius* specimens. The ITS sequence of *Lanthus fujiacus* was not

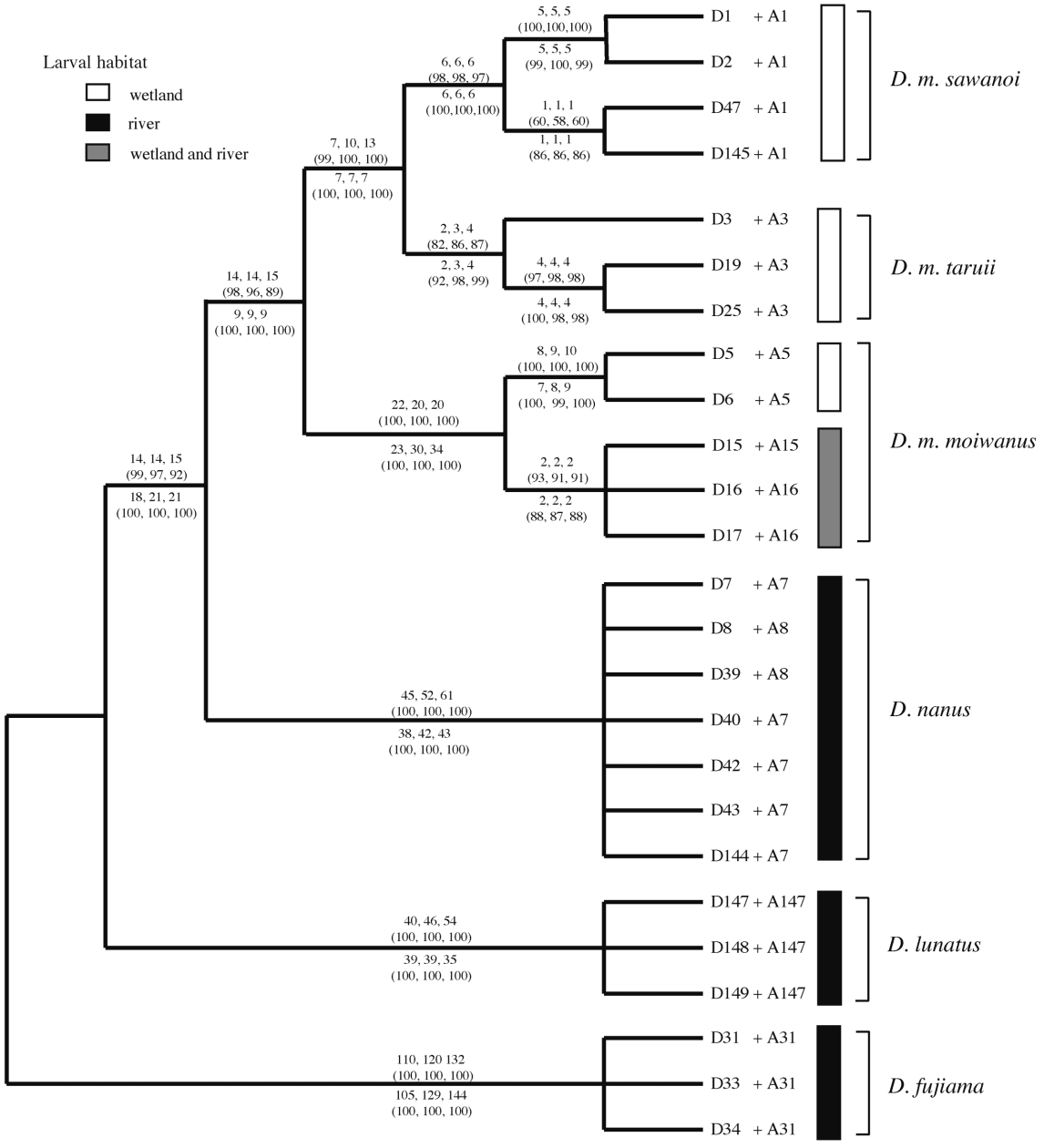


**Fig. 4.** Strict consensus of optimal (shortest or lowest cost) trees resulting from maximum parsimony and POY analyses of the combined COI+ITS data set. Results were identical for the two methods and three gap/change cost settings. The combination of COI haplotype and ITS allele is indicated at the terminal node (see Table 1 for haplotype/allele codes). Numerals above branches: Bremer support values, with bootstrap percentages in parentheses, from maximum parsimony analysis. Numerals below branches: Bremer support values, with jackknife percentages in parentheses, from POY analysis. Triplet numerals are for gap/change costs of 1:1, 2:1, and 3:1, respectively. Larval habitats are indicated by bars beside the taxon names.

obtained. Although the ITS regions showed high variability, sequence variation within populations as well as among populations within subspecies or species was rather low. Standard parsimony analysis of the aligned data yielded shortest trees of 210 steps (4 trees; CI=0.86; RI=0.85), 257 steps (2 trees; CI=0.81; RI=0.65), and 304 steps (2 trees; CI=0.78; RI=0.76) for gap/change cost ratios of 1:1, 2:1, and 3:1, respectively. The consensus trees under the three weighting schemes had the same topology (Fig. 2c) except that the sister relationship of *D. m. sawanoi* and *D. m. taruii* was not revealed under 1:1 gap/change cost ratio.

Tree searches with different gap costs (1, 2, and 3) using POY 2.0 also resulted in the same topology as in Fig. 2c; the tree length was 203, 269, and 333 for gap costs of 1, 2, and 3, respectively. The ITS tree revealed *D. moiwanus* monophyly, with *D. nanus* sister to *D. moiwanus*. Within *D. moiwanus*, *D. m. moiwanus* was monophyletic, and the western subspecies *D. m. sawanoi* and *D. m. taruii* formed another clade. Each of the latter two subspecies was also monophyletic; two *D. m. taruii* populations exhibited only one allele.

**Simultaneous analyses of combined data**

In parsimony analysis, the combined data of COI and ITS resulted in 20 shortest trees of 407, 448, and 487 steps for gap/change cost ratios of 1:1, 2:1, and 3:1, respectively (CI=0.85, RI=95 for gap cost=1; CI=0.84, RI=0.95 for gap cost=2 and 3). The topology of the strict consensus tree (Fig. 4) was congruent with the ITS tree (Fig. 2 c). The node support was high although there was a small incongruence between COI and ITS data partitions as reveled by significant ILD (incongruence length difference) (ILD=5, 5, and 7 for gap cost=1, 2,and 3, with $P$=0.006, 0.013, and 0.015, respectively). The small incongruence between the two partitions (% ILD=1.1–1.4% of the tree length of the combined data) was attributable to the placements of *D. moiwanus* samples (see Fig. 2 a and c). However, the combined data resolved the relationships among the subspecies of *D. moiwanus* with high node support values. The POY analysis gave almost the similar results with the parsimony analysis; the tree length was 377, 411, and 443 for gap cost of 1, 2, and 3, respectively, and the node supports were generally high (Fig. 4).

**Timing of divergence: COI data**

The maximum likelihood tree with the molecular-clock assumption, which did not differ statistically from the tree without this assumption, was used to estimate divergence times (Fig. 2b). To calibrate age, the branching between *D. fujiama* and the other three species (Fig. 2b; node A) was assumed to be 3.5 Ma. With this calibration, the branching between *D. lunatus* and *D. nanus*+*D. moiwanus* (node B) represents 1.92±0.01 (mean±SE) Ma, and differentiation within the *D. nanus*+*D. moiwanus* lineage (node C) occurred after 0.77±0.04 Ma. Alternatively, if node B were assumed to be 3.5 Ma, node A would represent 6.37±0.03 Ma, and node C would represent 1.41±0.07 Ma.

## DISCUSSION

**Species relationships**

Although analysis of mitochondrial DNA sequence data did not clearly reveal the monophyly of *D. moiwanus*, nuclear DNA sequence data did. Species-level polyphyly or paraphyly is a common phenomenon for gene genealogies, especially those of mitochondrial genes (*e.g.*, Funk *et al.*, 2003; Ballard and Whitelock 2004). Theoretically, this paraphyly can result from incomplete or random lineage sorting and introgressive hybridization. Our results suggest that three groups (*D. nanus*, *D. moiwanus moiwanus*, and *D. m. taruii*+*D. m. sawanoi*) differentiated over a short time, which could lead to an ambiguous (polytomous) differentiation pattern. Because the sequence divergence between *D. nanus* and *D. moiwanus* subspecies is large and there has been no evidence of interspecific hybridization, the effects of introgressive hybridization can be excluded. Thus, monophyly of *D. moiwanus* and *D. nanus* was supported by unweighted parsimony trees, whereas the accounting differences for different types of substitutions in weighted parsimony and maximum likelihood/Bayesian analysis resulted in paraphyly.

Although the ITS region is one of a few available nuclear markers in odonates, it has been used mainly for analyzing geographic distribution patterns of alleles among intra- and inter-specific populations (*e.g.*, Pilgrim *et al.*, 2002; Hayashi *et al.*, 2004); its use in phylogenetic analysis has been limited (Hovmöller and Johansson, 2004). The high variability of ITS sequences can cause difficulty in alignment and hamper their use in phylogenetic inference. However, we used dynamic optimization in POY 2.0 as well as standard parsimony analysis to obtain consistent results with different gap cost-weighting schemes. The phylogenetic hypothesis resulting from the ITS data (Fig. 2 c) coincided with the taxonomy of species and subspecies, and this hypothesis was enhanced by the simultaneous analysis of the combined COI and ITS data. Following these results, we conclude that of the three Japanese *Davidius* species, *D. nanus* and *D. moiwanus* are sister to the continental congener *D. lunatus*, whereas *D. fujiama* differentiated earlier from the ancestor of *D. lunatus*, *D. nanus*, and *D. moiwanus*. Further, within *D. moiwanus*, the eastern subspecies *D. m. moiwanus* differentiated from the western population, which subsequently differentiated into two subspecies: *D. m. taruii* and *D. m. sawanoi*.

**Ecological differentiation**

The larvae of *D. nanus*, *D. fujiama*, and *D. lunatus* inhabit rivers. However, larvae of *D. moiwanus* inhabit narrow wetland streams in Honshu, and both wetland streams and rivers in Hokkaido, where no other *Davidius* species occurs (Ishida *et al.*, 1988; Sugimura *et al.*, 1999).

The phylogenetic hypothesis resulting from the combined data of COI and ITS (Fig. 4) suggests that the riverine habitat is ancestral larval habitat of *Davidius* and the wetland habitat is derived. Part of the ancestral population (ancestral *D. moiwanus*) may have adapted to wetlands, and subsequently, the range of the riverine type (ancestral *D. nanus*) may have extended to encompass localized wetlands. The recent range expansion by *D. nanus* is supported by the low sequence divergence over the distribution range compared to that of *D. moiwanus* (Fig. 3), although more extensive sampling is needed.

*Davidius nanus* and *D. fujiama* deposit eggs in the sur-

face waters of rivers, co-occurring over a wide range in Japan. Although further field study is needed for their habitat use, adult *D. nanus* emerge in the middle reach of the river, far below their oviposition sites, whereas *D. fujiama* emerge not far below the oviposition sites. Moreover, *D. nanus* reproduce earlier than *D. fujiama* (Ishida *et al*., 1988; Odonatological Society of Osaka, 1998). *Davidius lunatus* occurs only in Korea and Primorsky Krai, Russia; therefore, a detailed study of its larval habitat use is indispensable to understand the extent of the *Davidius* riverine niche in the absence of sympatric species.

**Timing of divergence**

The gene genealogies suggest that the continental *Davidius* lineage colonized Japan at least twice; the first colonizer was the ancestor of *D. fujiama*; the second was the ancestor of *D. nanus* and *D. moiwanus*. If, however, the four *Davidius* species studied here are monophyletic, it is also possible that the ancestor of the four species colonized Japan, and the ancestor of *D. lunatus* migrated from Japan to the continent. These two hypotheses require two dispersal/colonization events and are equally parsimonious reconstruction of the historical biogeography. We cannot determine which is the most likely hypothesis because we could not examine continental *Davidius* species other than *D. lunatus*. In either case, we can assume that the Tsushima Strait has acted as the major geographic barrier promoting differentiation of the continental and Japanese lineages.

Since no fossil record is available to calibrate the COI sequence divergence of *Davidius*, we assumed that either of two nodes that led to *D. fujiama* and *D. lunatus* corresponds to the time when the western channel (Tsushima Strait) of the Sea of Japan opened 3.5 Ma (Tada, 1994). The Tsushima Strait was closed from 10 to 3.5 Ma (Tada, 1994); during this period, faunal exchange between the continent and Japan would have been frequent. After 3.5 Ma, the Tsushima Strait was open except from 2.5 to 1.7 Ma and later regression periods during glacial ages (Iijima and Tada 1990; Tada 1994; Kitamura *et al*., 2001).

The estimated substitution rate for insect mitochondrial DNA sequence data, including the COI region, ranges from 1.5% (Farrell, 2001; Quek *et al*., 2004) to 2.3% (Brower, 1994) per million years. The uncorrected pair-wise sequence divergence at node A (Fig. 3) is 11.0±0.3% (mean ±SD), implying a substitution rate of 3.1% per million years, which is much faster than previously reported. When node B is assumed to be 3.5 Ma, the substitution rate is 2.3% (uncorrected pair-wise sequence divergence, 7.7±0.2%), equal to the highest proposed rate. Under this assumption, the *D. fujiama* lineage that gave rise to *D. lunatus, D. nanus,* and *D. moiwanus* may have existed in Japan in the Pliocene. At present, we have no evidence to support either calibration. However, either calibration yields estimated divergence times for the *D. nanus+D. moiwanus* clade in the Pleistocene (1.4–0.8 Ma). Differentiation among *D. nanus* and *D. moiwanus* subspecies was likely promoted by the rapid changes in climate and geography in Japan during the Pleistocene.

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