Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular Phylogenetics and Evolution 40 (2006) 484–500
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Molecular phylogeny of the *Drosophila virilis* section (Diptera: Drosophilidae) based on mitochondrial and nuclear sequences

Bao-cheng Wang [a,f], Jecheol Park [b], Hide-aki Watabe [c], Jian-jun Gao [d], Jing-gong Xiangyu [a], Tadashi Aotsuka [b], Hong-wei Chen [e], Ya-ping Zhang [a,d,*]

[a] *Laboratory of Cellular and Molecular Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China*
[b] *Department of Biology, Tokyo Metropolitan University, Hachioji-shi 192-0397, Japan*
[c] *Biological Laboratory, Sapporo College, Hokkaido University of Education, Sapporo 002-8502, Japan*
[d] *Laboratory for Conservation and Utilization of Bio-resource, Yunnan University, Kunming 650091, China*
[e] *Department of Entomology, South China Agriculture University, Guangzhou 510642, China*
[f] *Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*

Received 8 June 2005; revised 17 March 2006; accepted 18 March 2006
Available online 28 March 2006

Abstract

Regardless of the well-documented *virilis* species group, most groups of the *Drosophila virilis* section have not been completely studied at molecular level since it was suggested. Therefore, phylogenetic relationships among and within species groups of the *virilis* section are generally unknown. In present paper, the complete mitochondrial ND2 gene and fragment of COI gene in combination with a nuclear gene, Adh coding region, were used to derive the most extensive molecular phylogeny to date for the *Drosophila virilis* section. A total of 111 individuals covering 61 species were sampled in this study. Novel phylogenetic findings included (1) support for the paraphyly of the *melanica* and *robusta* species group and at least two subgroups of the *robusta* species group, the *lacertosa* and *okadai* subgroups, were distinguished as paraphyletic taxa. In addition, (2) present results revealed the sister relationship between *D. moriwakii* and the *robusta* subgroup, conflicting with current taxonomy regarding *D. moriwakii*, which was shifted from the *robusta* species group to the *melanica* group. (3) In contrast to the *robusta* and *melanica* species groups, monophyly of the *polychaeta* species group, the *angor* group and the *virilis* group was confirmed, respectively. However, the monophyletic *quadrisetata* species group was resolved with uncertainty. (4) Our analyses of combined data set suggested close relationship between the *quadrisetata* species group and the unpublished *clefta* group, and the *okadai* subgroup is sister to the clade comprising of the *quadrisetata* and *clefta* species groups. Within the *virilis* section, *D. fluvialis* and three tropical species groups, the *polychaeta* group, the *angor* group and the *repleta* group, are found to branch off earlier than other ingroup taxa. This suggests that the *virilis* section might have originated in the Old World tropics. Besides, the derived status of the close affinities of the *quadrisetata* group, the *clefta* group, and the *melanica* and *robusta* groups is probably the result of their adaptation to forests between subtropical and cool-temperate climate. Based on the consideration of the phylogenetic placement of the species of the *virilis* section, we suggest that at least five independent migrations occurred from the Old World to the New World.

Keywords: The *Drosophila virilis* section; Molecular phylogeny; Mitochondiral DNA; Adh gene

1. Introduction

The *Drosophila virilis* section of the subgenus *Drosophila*, which was established by Hsu (1949) on the basis of the similarity of male genitalia structures, is very large with many species groups, such as the *melanica*, the *polychaeta*, the *robusta*, the *virilis* groups, and so on (Sturtevant, 1942).

The *Drosophila virilis* section flies geographically spread different climatic zones, mainly in the Northern Hemisphere, except for some quasi-domestic species, *D. virilis* Sturtevant, *D. polychaeta* Sturtevant, *D. hydei* Spencer, and

* Corresponding author. Fax: +86 871 519 5430.
E-mail addresses: zhangyp@mail.kiz.ac.cn,zhangyp1@263.net.cn (Y. Zhang).

1055-7903/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2006.03.026

D. repleta Duda, the wide distribution ranges of which are due to artificial transportations in modern times (Dobzhansky, 1965; Watabe and Nakata, 1989). The *polychaeta* species group has been recorded from circum-tropical and-subtropical regions; most species from Asiatic tropics, *D. hirtipes* Lamb from the tropical Africa, and *D. illota* Williston from Neo-tropics. The well-known species groups of the *virilis* section, the *melanica* group, the *robusta* group and the *virilis* group, are distributed in middle geographic latitudes of both the Old and New Worlds. Most of the first two group flies dwell mainly on warm temperate regions. The *virilis* group flies prefer to rather cool climates and are distributed from cool temperate to cold regions, with its northern limits in circum-polar areas, such as Alaska, Siberia, and Scandinavia (Throckmorton, 1982; Toda et al., 1996).

With exception of the *polychaeta* species group, most species groups mentioned above have been well studied biogeographically, chromosomally, electrophoretically, or genetically, from the viewpoints of evolution and speciation (Baker, 1980; Narayanan, 1973; Spicer, 1992; Spicer and Bell, 2002; Stalker, 1966, 1972; Stone et al., 1960). However, the phylogenetic relationships between the species groups of the *virilis* section have not been explored since it was established, and only two closely related species groups, the *robusta* and *melanica* groups, were once considered together (for the review, see Levitan, 1982). In most cases, a single or very limited number of species were selected as representatives of their respective species groups for phylogenetic studies (Pelandakis and Solignac, 1993). Further, most species included in those articles were North American or European members of the *virilis* section (Narayanan, 1973; Stalker, 1966, 1972), and very few data sets were dealt with the Oriental species.

The faunal survey of the Drosophilidae in the past two decades have demonstrated that the *virilis* section flies are very abundant in the Oriental Region, especially in southern China, suggesting that lower geographic latitudes of Asia may be very important when we consider the emergence of species groups of the *virilis* section or adaptive radiation. Recently, a number of new species belonging to the *virilis* section were discovered from China (Chen and Watabe, 1993; Toda, 1988; Toda and Peng, 1989; Watabe et al., 1990a,b). Two new species groups of the *virilis* section, the *angor* group and the *quadrisetata* group, were established (Toda and Peng, 1989; Watabe and Peng, 1991), first of which has been found in the tropical to subtropical region of Asia and the latter of which mainly in middle and southwestern parts of China (Toda and Peng, 1989; Zhang and Liang, 1994; Watabe et al., 1990b). Further, several new species or new taxonomic groups are under consideration for descriptions (Table 1, our unpublished data).

Although morphological similarities have been mentioned in descriptions of the new species or new groups, phylogenetic relationships among and within the species groups or subgroups are still poorly understood on the whole. For a better understanding of the evolutionary history of the *virilis* section established by Hsu (1949) as well as the *virilis–repleta* radiation proposed by Throckmorton (1975), it is necessary to construct a reliable phylogeny of this taxonomic group by using the molecular data.

The nuclear Adh (alcohol dehydrogenase) gene has frequently been used for the molecular phylogeny of *Drosophila*, and it has been proven to be a reliable marker (Atrian et al., 1998; Nurminsky et al., 1996; Russo et al., 1995). Thus, we employed Adh coding region as genetic marker to explore the phylogenetic relationships among and within species groups in the *virilis* section. Further, we compared sequences of the mitochondrial (mt) NADH dehydrogenase subunit 2 (ND2) and the cytochrome oxidase subunit I (COI) gene region to estimate the phylogeny of the *virilis* section, especially phylogenic relationships among closely related species. Based on the molecular data, we discuss the origin of the *Drosophila virilis* section and the following adaptive radiation in different climatic zones.

2. Materials and methods

2.1. Sample

A total of 111 samples representing 61 species of the *virilis* section flies were included in this study, among which the basic taxonomic work of some undescribed species is lacking or uncertain. Several (from 2 to 6) individual samples of one species in 19 taxa were used to gauge the intraspecific variations. In addition, Adh gene has been proved to be a useful marker in the phylogenetic relationships of *Drosophila* species, and many sequences have been released in GenBank. Hence, we included some Adh sequences primarily the members of the *virilis* and the *repleta* species groups in our analyses in order to compare them with our experimental results. The detail information for each sample is listed in Table 1. *Drosophila* (*Sophophora*) *melanogaster* and *D*(*S*). *yakuba* were used as outgroup.

2.2. DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from single fly by the standard proteinase K and phenol/chloroform extraction method (Sambrook et al., 1989) with some modifications.

Three gene fragments were amplified via polymerase chain reaction (PCR). The primers used for PCR and/or sequencing are listed in Table 2. PCR was performed in 25 μl volumes, with a final concentration of 0.4 μM of each primer, 1 mM of each dNTP, 2.5 mM of Mg^{2+} and 1 U of *Taq* polymerase (*Sabc* Inc.). Thermal conditions were 5-min initial denaturation at 95 °C, then followed 35 thermal cycles including 1 min denaturation at 94 °C, 1 min annealing at 57 °C (ND2), 48 °C (COI) and 42 °C (Adh), respectively, and 1 min extension at 72 °C, the last cycle was run at 72 °C for 8 min as a terminal extension. For all amplifications, negative controls were employed to void possible contamination.

Table 1
The samples and sources used in this study

Species group	Species	Material sources	Adh	ND2	COI
polychaeta species group	*D. asper*	Mengyang, Yunnan	DQ471619	DQ471474	DQ471545
	D. daruma 1	Menglun, Yunnan	DQ471618	DQ471473	DQ471544
	D. daruma 2	Menglun, Yunnan	DQ471621	DQ471476	DQ471547
	D. daruma 3	Mengla, Yunnan	DQ471623	DQ471478	DQ471549
	D. daruma 4	Okinawa, Japan	DQ471632	DQ471487	DQ471558
	**D. polychaeta X*	Menglun, Yunnan	DQ471626	DQ471481	DQ471552
	D. latifshahi	Okinawa, Japan	DQ471634	DQ471490	DQ471561
	D. polychaeta 1	Bowling Green Stock Center	AY750126	AY750086	AY750100
	D. polychaeta 2	Bowling Green Stock Center	DQ471629	DQ471484	DQ471555
	D. hirtipes	Bowling Green Stock Center	—	DQ471489	DQ471560
angor species group	**D. angor A*	Mengla, Yunnan	DQ471612	DQ471456	DQ471538
	**D. angor B*	Mengla, Yunnan	DQ471613	DQ471468	DQ471539
	**D. angor C 1*	Shiwanda Mt., Guangxi	—	DQ471497	DQ471568
	**D. angor C 2*	Shiwanda Mt., Guangxi	—	DQ471498	DQ471569
	**D. angor C 3*	Shiwanda Mt., Guangxi	—	DQ471499	DQ471570
	**D. angor C 4*	Mengla, Yunnan	DQ471614	DQ471469	DQ471540
	**D. angor E*	Mengla, Yunnan	DQ471615	DQ471470	DQ471541
	D. angor 1	Shiwanda Mt., Guangxi	DQ471638	DQ471495	DQ471566
	D. angor 2	Shiwanda Mt., Guangxi	—	DQ471496	DQ471567
	D. angor 3	Mengla, Yunnan	DQ471616	DQ471471	DQ471542
	D. angor 4	Mengla, Yunnan	DQ471617	DQ471472	DQ471543
	D. angor 5	Okinawa, Japan	DQ471627	DQ471482	DQ471553
	D. angor 6	Taiwan, China	DQ471628	DQ471483	DQ471554
	D. hei	Wuliang Mt., Yunnan	DQ471622	DQ471477	DQ471548
	D. velox	Taiwan, China	DQ471637	DQ471494	DQ471565
quadrisetata species group	*D. barutani 1*	Qinling Mt., Shannxi	DQ471642	DQ471504	DQ471576
	D. barutani 2	Qinling Mt., Shannxi	DQ471644	DQ471506	DQ471579
	D. barutani 3	Leigong Mt., Guizhou	DQ471620	DQ471475	DQ471546
	D. barutani 4	Wuyi Mt., Jiangxi	DQ471648	DQ471513	DQ471586
	D. barutani 5	Taiwan, China	DQ471630	DQ471485	DQ471556
	D. barutani 6	Leigong Mt., Guizhou	DQ471639	—	DQ471571
	D. multidentata	Leigong Mt., Guizhou	DQ471624	DQ471479	DQ471550
	D. perlucida	Qinling Mt., Shannxi	DQ471625	DQ471480	DQ471551
	D. beppui 1	Simao, Yunnan	—	DQ471508	DQ471581
	D. beppui 2	Simao, Yunnan	DQ471645	DQ471509	DQ471582
	D. beppui 3	Taiwan, China	DQ471631	DQ471486	DQ471557
	D. karakasa	Simao, Yunnan	DQ471649	DQ471514	DQ471587
	D. potamophila 1	Simao, Yunnan	—	DQ471512	DQ471585
	D. potamophila 2	Taiwan, China	—	DQ471463	DQ471532
	D. pilosa	Taiwan, China	—	DQ471491	DQ471562
	D. quadrisetata	Kyushu, Japan	DQ471635	DQ471492	DQ471563
	**D. sp T*	Taiwan, China	DQ471636	DQ471493	DQ471564
	**D. sp IZU*	Izu, Japan	—	DQ471465	DQ471534
robusta species group					
lacertosa subgroup	*D. bai 1*	Gaoligong Mt., Yunnan	AY750141	AY750072	AY750115
	D. bai 2	Ailao Mt., Yunnan	AY750145	AY750085	DQ471578
	D. bai 3	Lugu Lake,Yunnan	DQ471647	DQ471511	DQ471584
	D. medioconstricta 1	Leigong Mt., Guizhou	AY750143	AY750075	AY750104
	D. medioconstricta 2	Menglun, Yunnan	AY750144	AY750074	AY750106
	D. medioconstricta 3	Mengyang, Yunnan	DQ471640	DQ471502	DQ471574
	D. medioconstricta 4	Taiwan, China	AY750135	AY750079	AY750107
	D. yunnanensis 1	Gaoligong Mt., Yunnan	—	DQ471455	DQ471537
	D. yunnanensis 2	Jizu Mt., Yunnan	AY750129	AY750059	AY750093
	D. yunnanensis 3	Daiyun Mt., Fujian	DQ471650	DQ471515	DQ471588
	D. yunnanensis 4	Taiwan, China	AY750131	AY750070	AY750102
	D. lacertosa 1	Qinling Mt., Shannxi	DQ471610	—	—
	D. lacertosa 2	Kunming, Yunnan	AY750116	AY750071	AY750090
	D. lacertosa 3	Wuyi Mt., Jiangxi	DQ471652	DQ417517	DQ471590
	D. lacertosa 4	Daiyun Mt., Fujian	DQ471651	DQ417516	DQ471589
	D. lacertosa 5	Tokyo, Japan	AY750122	AY750063	AY750095

Table 1 (*continued*)

Species group	Species	Material sources	Adh	ND2	COI
okadai subgroup	*D. gani 1*	Leigong Mt., Guizhou	AY750142	AY750073	AY750103
	D. gani 2	Qinling Mt., Shannxi	—	DQ471500	DQ471572
	D. gani 3	Aomori, Japan	AY750133	AY750077	AY750109
	D. gani 4	Taiwan, China	AY750134	AY750078	AY750108
	**D. bipatita*	Guan Mt., Jiangxi	DQ471646	DQ471510	DQ471583
	D. neokadai 1	Jizu Mt., Yunnan	—	AY750076	AY750105
	D. neokadai 2	Qinling Mt., Shannxi	DQ471641	DQ471503	DQ471575
	D. neokadai 3	Hokkaido, Japan	AY750130	AY750067	AY750098
	D. okadai	Hokkaido, Japan	AY750136	AY750080	AY750110
	D. unimaculata	Switzerland	AY750140	AY750084	AY750114
robusta subgroup	**D. borusta*	Gaoligong Mt., Yunnan	—	DQ471521	DQ471594
	**D. cheda*—like	Guan Mt., Jiangxi	DQ471609	DQ471467	DQ471536
	D. pseudosordidula	Aomori, Japan	AY750137	AY750081	AY750111
	D. robusta	Bowling Green Stock Center	AY750138	AY750082	AY750112
	D. sordidula	Tokyo Metropolitan Univ.	AY750139	AY750083	AY750113
melanica species group	*D. longiserrata 1*	Anshun, Guizhou	—	DQ471501	DQ471573
	D. longiserrata 2	Anshun, Guizhou	AY750117	AY750057	AY750091
	D. longiserrata 3	Anshun, Guizhou	AY750118	AY750061	AY750088
	D. tsigana 1	Anshun, Guizhou	AY750121	AY750058	AY750092
	D. tsigana 2	Anshun, Guizhou	AY750119	AY750060	AY750087
	D. tsigana 3	Anshun, Guizhou	AY750120	AY750062	AY750089
	D. tsigana 4	Hokkaido, Japan	DQ471654	DQ417519	DQ471592
	D. tsigana 5	Hokkaido, Japan	—	DQ417520	DQ471593
	D. tsigana 6	Hokkaido, Japan	AY750128	AY750069	AY750101
	D. melanica	Bowling Green Stock Center	AY750123	AY750064	AY750094
	D. micromelanica	Bowling Green Stock Center	AY750124	AY750065	AY750096
	D. paramelanica	Bowling Green Stock Center	AY750127	AY750068	AY750099
	D. euronotas	Bowling Green Stock Center	AY750132	—	—
	D. moriwakii	Hokkaido, Japan	AY750125	AY750066	AY750097
virilis species group	*D. virilis 1*	Qinling Mt., Shannxi	DQ471643	DQ471505	DQ471577
	D. virilis 2	Tokyo Metropolitan Univ.	DQ471668	DQ471466	DQ471535
	D. lummei	Tokyo Metropolitan Univ.	DQ471663	DQ471459	DQ471606
	D. kanekoi 1	Leigong Mt., Guizhou	DQ471653	DQ417518	DQ471591
	D. kanekoi 2	Hokkaido, Japan	DQ471661	DQ471457	DQ471604
	D. americana	Tokyo Metropolitan Univ.	DQ471655	DQ471524	DQ471597
	D. ezoana	Hokkaido, Japan	DQ471658	DQ471527	DQ471600
	D. littoralis	Sweden	DQ471662	DQ471458	DQ471605
	D. montana	Hokkaido, Japan	DQ471665	DQ471461	DQ471608
	D. borealis		U26839	—	—
	D. lacicola		U26840	—	—
	D. flavomontana		U26838	—	—
	D. texana		U26844	—	—
repleta species group	*D. anceps*	Bowling Green Stock Center	DQ471656	DQ471525	DQ471598
	D. eohydei	Tokyo Metropolitan Univ.	DQ471659	DQ471528	DQ471601
	D. hydei 1	Tokyo Metropolitan Univ.	DQ471660	DQ471529	DQ471602
	D. hydei 2	Unknown		DQ471530	DQ471603
	D. repleta	Tokyo Metropolitan Univ.	DQ471667	DQ471464	DQ471533
	D. buzzatii		M62743	—	—
	D. mayaguana		M62742	—	—
	D. wheeleri		M62851	—	—
	D. mettleri		M57300	—	—
	D. mojavensis		X12536	—	—
	D. mulleri		X03048	—	—
	D. navojoa		AY156524	—	—
	D. mercatorum	Tokyo Metropolitan Univ.	DQ471664	DQ471460	DQ471607
annulimana species group	*D. aracataca*	Bowling Green Stock Center	DQ471657	DQ471526	DQ471599
nannoptera species group	*D. nannoptera*	Bowling Green Stock Center	DQ471666	DQ471462	DQ471531
clefta species group	**D. spng*	Qinling Mt., Shannxi	—	DQ471522	DQ471595
	**D. cleftalike*	Lugu Lake, Yunnan	—	DQ471523	DQ471596
	**D. clefta*	Ailao Mt., Yunnan	—	DQ471507	DQ471580

(*continued on next page*)

Table 1 (*continued*)

Species group	Species	Material sources	Adh	ND2	COI
ungrouped species	*D. fluvialis*	Taiwan, China	DQ471633	DQ471488	DQ471559
melanogaster species group	*D. melanogaster*		M17833	NC 001709	NC 001709
	D. yakuba		X54120	X03240	X03240

*, tentative species names since they are undescrbed new species at present.

Table 2
Primers used for PCR and/or sequencing in this study

Primer name	Primer sequence (5′- - - - - - - -3′)	Utility
ND2-1	ATATTTACAGCTTTGAAGG	PCR/sequencing
ND2-2	AAGCTACTGGGTTCATACC	PCR/sequencing
ND2-M-1	TGATTTCCTAATTTAATAGAAGG	PCR/sequencing
ND2-M-2	TGGTAAAAATCCTAAAAATGGAGG	PCR/sequencing
COI-1	ATCGCCTAAACTTCAGCCAC	PCR/sequencing
COI-2	TCCATTGCACTAATCTGCCA	PCR/sequencing
COI-M-1	CAACATTTATTTTGATTTTTTGG	PCR/sequencing
COI-M-2	CCTGATTCTTGACTAATAATATG	PCR/sequencing
Adh-1	AACAAGAAYRTNRTNTTYGT	PCR
Adh-2	TAGATNYSNGARTCCCARTG	PCR
M13	AAGCTTGCATGCCTGCAGGTCGACGAT	Sequencing
RV-M13	CGGTACCCGGGGATCCTCTAGAGAT	Sequencing

N, A/C/G/T; R, A/G; Y, T/C; S, G/C.

PCR products were purified with a PCR purification kit (Watson Biomedicals, W5212). The purified PCR products of mt genes were sequenced directly. For nuclear Adh gene, the cleaned PCR products were ligated into pMD18-T vector (TAKARA Inc.) by using *Escherichia coli* JM109 as a host. At least three positive clones were selected for plasmids extracting, and these plasmids were used as templates for DNA sequencing reactions. The double-strand DNA was determined by ABI 3700 automated sequencer according to the instruction provided by the manufacturer. DNA sequences were edited with SeqMan and aligned with MegAlign in the DNASTAR package (DNASTAR Inc.) and checked manually. All sequences were deposited in GenBank and the accession numbers for each sequence are also shown in Table 1.

Owing to bad preserved condition of some samples, high quality of DNA is unavailable for them. Therefore, not all three genes of every sample were achieved successfully. For example, the Adh gene of a few samples and two mt genes of *D. euronotus* Petterson et Ward cannot be obtained in this study. Additionally, the ND2 sequences of *D. clefta* is incomplete. Although inclusion of incomplete data could increase taxa in the analyses, we exclude samples with missing genes from the combined datasets.

2.3. Data analysis

Aligned sequences were imported into PAUP* 4.0b4a (Swofford, 2000) for phylogenetic analysis. Base frequencies, sequence variation, and divergence values were determined via program MEGA 3.0 (Kumar et al., 2004). In order to determine if nucleotide composition bias occurred among taxa, a χ^2 goodness-of-fit test was performed on the sequence data for each codon position of each gene region. For two mt genes, potential saturation of transition at third codon positions were assessed by plotting the number of substitutions versus TN93 (Tamura and Nei, 1993) sequence divergence via the program DAMBE (Xia and Xie, 2001).

Unweighted MP analyses were performed using heuristic searches with 10 random additions of taxa and tree-bisection–reconnection (TBR) algorithm. For concatenated mt genes, transversion analysis under MP framework was also conducted in order to eliminate the effect of saturation. All indels were excluded from the analyses and those unavailable nucleotides were treated as missing characters. At most 100 trees were saved in order to save computation time. Bootstrap value was calculated to assess the level of support for each node of the parsimonious trees.

Besides MP analysis, maximum likelihood (ML) method was also employed to construct the phylogeny based on nuclear and mitochondrial genes, respectively. Prior to the performance of the ML analyses, the hierarchical likelihood ratio tests in Modeltest 3.06 (Posada and Crandall, 1998) implemented in PAUP* was employed to determine the most appropriate substitution model and parameters for the data matrices. MP trees were bootstrapped with 1000 replicates to evaluate the confidence for each node of the tree topology, the branches with less than 50% support are generally shown as unresolved polytomies. However, for ML analyses, we gave up bootstrap calculation since the computation was time-consuming.

To evaluate the sensitivity of the tree topology to method of analysis, Bayesian analysis with Markov Chain Monte Carlo (MCMC) searching was also used in this study via program MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). Four search chains involving one cold and three hot were set to run, and each chain was allowed to run 3,000,000 "generations." Trees were saved every 100

generations and the first 300,000 generations were discarded as burn-in and ensure that a stable likelihood had been reached. A strict consensus of remaining trees was computed and the posterior probability for each node was estimated to assess the robustness of the findings.

It has been proposed to combine all available data partitions in a simultaneous analysis by several researchers (O' Grady, 1999; Remsen and DeSalle, 1998; Rodriguez-Trelles et al., 1999). In this study, we analyzed mitochondrial and nuclear gene separately and then combined these genes for a multiple loci analysis. Before the genes were concatenated, the partition homogeneity, also referred to as the incongruence length difference (ILD) (Farris et al., 1995), was checked by using PAUP* to test whether the degree of incongruence among the actual loci in the data set was significantly different from random partitions of the same data. In this study, PHT was performed between transversional mt gene and Adh data set. Data partitions were regarded as being in conflict if they supported incongruent clades of the same taxa with bootstrap proportions of >70% or Bayesian posterior probabilities of >95%.

3. Results

3.1. Molecular characterization and sequence divergence

The characteristics of DNA sequences for each gene are listed in Table 3. For all individuals, aligned COI and Adh sequences are 1500 and 711 base pairs, respectively. As for ND2 sequence, the length heterogeneity was observed across different taxa: most of ND2 sequences are 1023 bp, the exceptions include *D. angor* B, *D. bai* Watabe et Liang, *D. angor* Lin et Ting and all the *polychaeta* group species, among which the length of ND2 gene are 1026 bp. A triplet nucleotides insertion occurs unambiguously at the 145-147th positions of the alignment of the sequences of the *polychaeta* group, and 241-243rd positions of *D. angor* B. However, the positions of the triplet nucleotide insertion in *D. angor* and *D. bai* are uncertain and we exclude all the insertions in the alignment of ND2 sequence in the subsequent analyses.

The base frequencies statistics indicated that high AT content was present in both COI and ND2, typical for insect mitochondrial DNA (Clary and Wolstenholme, 1985; Crozier and Crozier, 1993; Flook et al., 1995). For Adh, contents of AT and GC are almost equal, with a little higher GC contents than AT contents. The highest variable sites were found at the third codon positions, and the second codon position nucleotides had fewer informative sites than any other positions. Homogeneity of nucleotide composition was not rejected by the χ^2 test for each gene (Table 3). Obvious saturation was found in ND2 gene transitions.

Overall average sequence divergences among ingroup taxa for three genetic markers were also evaluated. The largest inter-group genetic distance (uncorrected divergence) for Adh gene occurred between the *nannoptera* species group and the *quadrisetata* species group (20.8%), and the smallest divergence (12.8%) between the *robusta* species group and the *melanica* species group. The inter-group divergence for ND2 gene varied between 11.5% (*quadrisetata* group vs. *robusta* group) and 20.3% (*polychaeta* group vs. *angor* group), sequence divergence for COI gene ranged between 10.3% (*quadrisetata* group vs. *melanica* group) and 13.5% (*clefta* group vs. *nannoptera* and *annulimana* group).

3.2. Phylogenetic relationships

3.2.1. Mitochondrial genes

We analyzed two mitochondrial genes (COI and ND2) together in this study, since these two genes share a single geneology and they have close localities in mitochondria and separated by only three tRNA genes (Clary and Wolstenholme, 1985). Our unweighted MP analysis for concatenated mt gene produced 36 equally most parsimonious trees of length 9462 steps (CI 0.227, RI 0.670, and RC 0.152). Transversion analysis for same data sets resulted in 6 MP trees of 4293 steps with CI = 0.196, RI = 0.793, and RC = 0.145. Transversional MP, ML, and Bayesian analysis yielded essentially the similar topology, with many common branches particularly in shallow divergences. Topology of Bayesian tree is presented in Fig. 1A and transversion MP tree is showed in Fig. 1B. As seen from the figure, the monophyly of most species groups, including the *angor* group, the *clefta* group and the *virilis* group, was identified. All analyses provided strong support for the monophyly of the *polychaeta* group, pairing the *polychaeta* group with *D. fluvialis* Toda et Peng (BP from 75 to 100%, PP 100%), an ungrouped tropical species. Although the Bayesian topology rendered the *quadrisetata* species group monophyletic, the support for this arrangement was tenuous (PP = 76%, not shown). Both MP analyses recovered the non-monophyly of the *quadrisetata* species group, which was split into two branches with one branch sister to undescribed *clefta* species group, which inhabit in the forest of highland of subtropical regions.

Table 3
Description of genes used in this study, with the exclusion of outgroups

Gene	TS	VS	PIS	χ^2	*P*	Variable sites by codon position			Base frequencies			
						1st (%)	2nd (%)	3rd (%)	%A	%C	%G	%T
Adh	711	436	375 (52.7%)	185.50 (df, 303)	1.0	27.8	20.6	51.6	24.3	27.6	25.8	22.3
ND2[a]	1023	652	545 (53.3%)	116.38 (df, 315)	1.0	31.4	19.9	48.6	34.8	11.0	8.6	45.7
COI	1500	616	538 (35.9%)	147.58 (df, 318)	1.0	18.5	5.0	76.5	29.8	15.6	15.5	39.1

TS, total sites; VS, variable sites, PIS, parsimony informative sites.
[a] Aligned length, excluding indels.

The resulted topology produced by Bayesian approach featured three clades consisting of: (1) the monophyletic *virilis* species group; (2) the *polychaeta* species group, *D. fluvialis*, *angor* group, *repleta* group, *annulimana* group and the *nannoptera* groups and (3) the *melanica*, *robusta*, *quadrisetata* and *clefta* species groups. Bayesian tree topology revealed the sister relationship of clades 1 and 3 with robust support (PP = 98%). While unweighted MP supported that clades 2 and 3 were mutual sister to each other, albeit the confidence of this relationship was very weak (<50%, not shown). Comparing with the two results mentioned, transversion MP analysis offers ambiguous association of these species group at deep branches. Additionally, substantial differences regarding the relationships within some groups were found. For example, the sister relationship of *D. hirtipes* and *D. polychaeta* X revealed by unweighted MP analysis was not observed in transversion MP and Bayesian topology.

The *melanica* group and the *robusta* group, as well as each of three subgroups within the *robusta* group were revealed to be non-monophyletic taxonomic units. The paraphyletic *melanica* species group was uncovered, and all topologies identified *D. moriwakii* Okada et Kurokawa as the sister taxon to the *robusta* subgroup represented by *D. sordidula* Kikkawa et Peng, *D. pseudosordidula* Kaneko, Tokumitsu et Tdkada and *D. robusta* Sturtevant. The

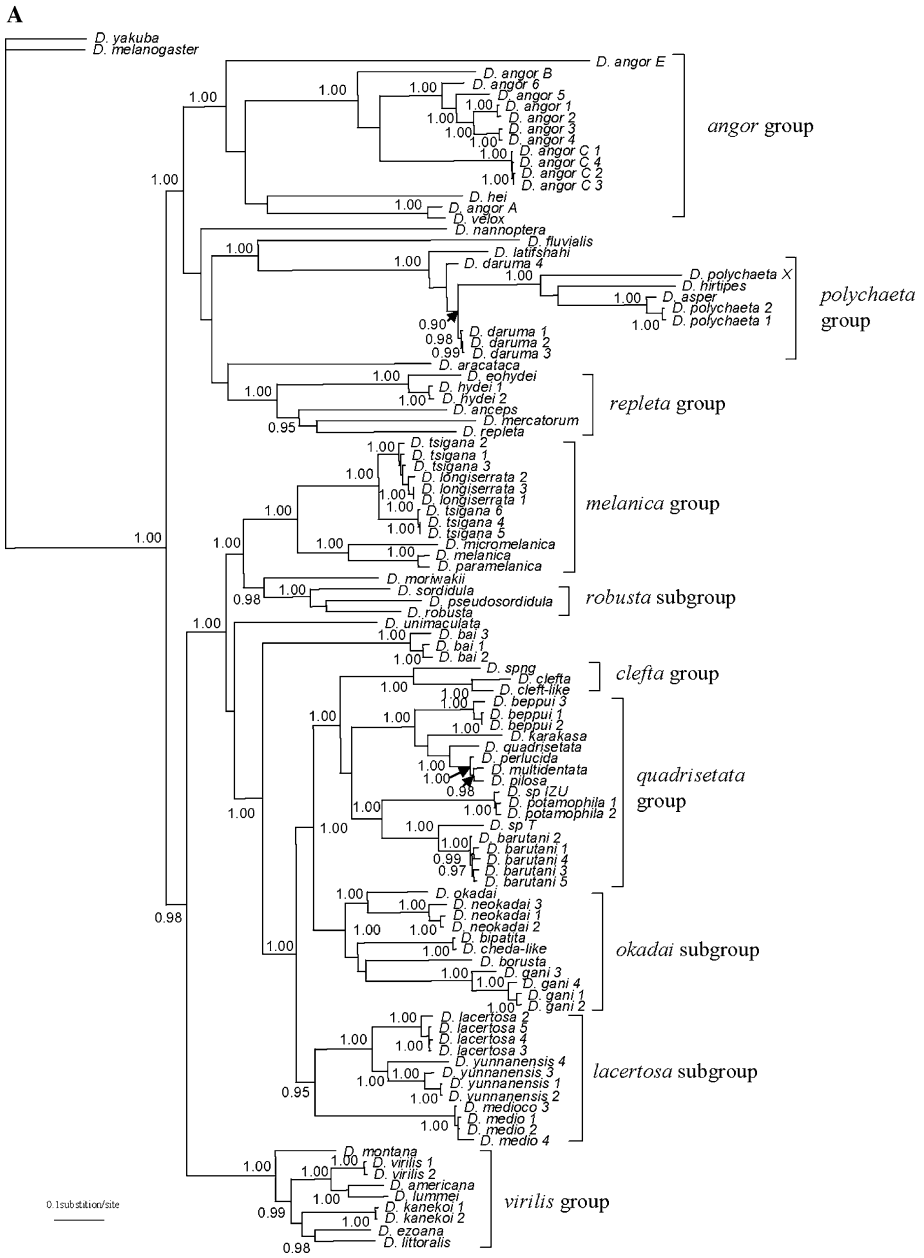

Fig. 1. (A) Consensus tree derived from the Bayesian inference for mt genes. Numbers indicate the posterior probability of the associated clade. Posterior probabilities >0.95 are shown in the tree. (B) Tree topology based on the MP analysis for transversion of mt genes. Numbers indicate the support from 1000 bootstrap calculation to the associated clade. Bootstrap values >50% are shown in the tree.

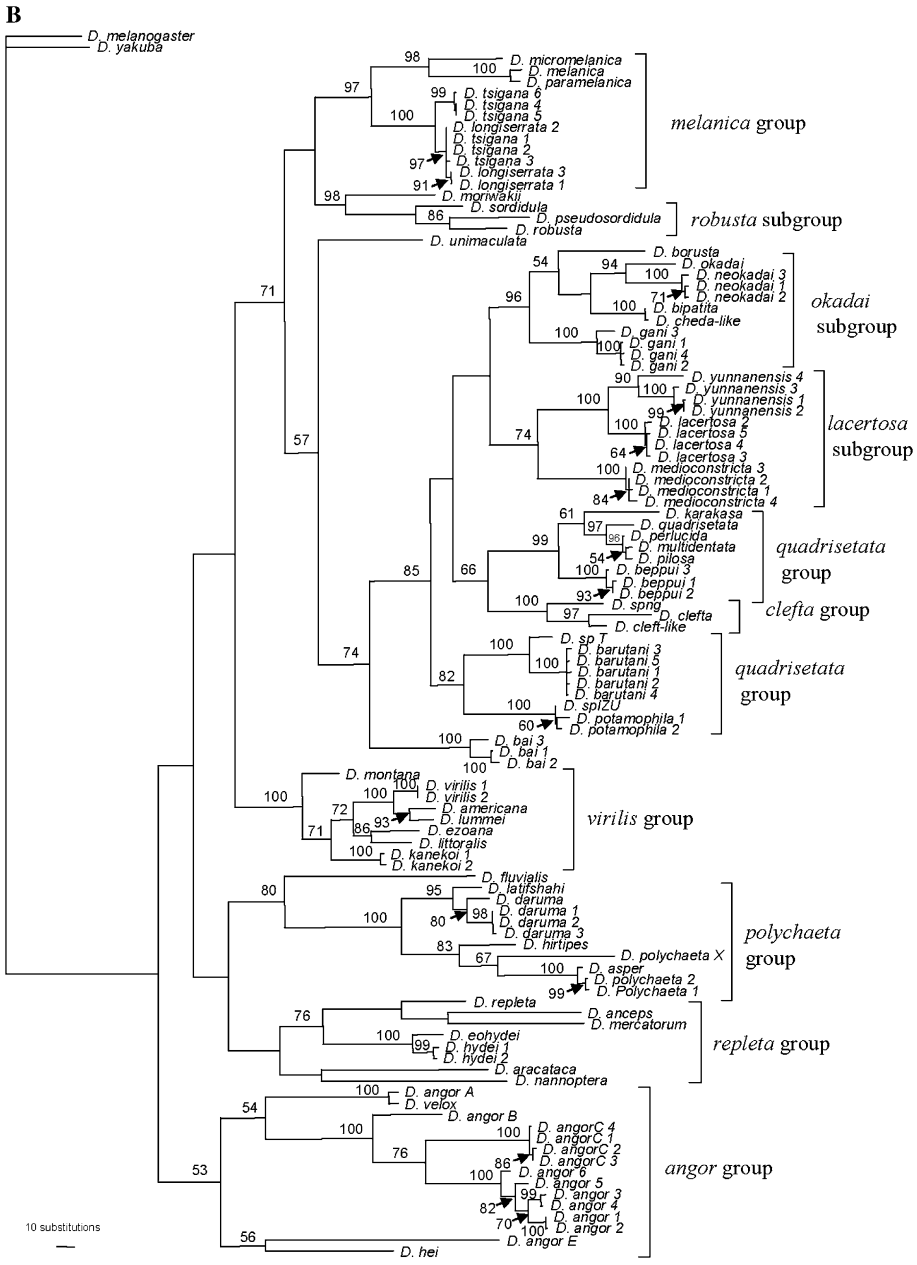

Fig. 1 (*continued*)

largest incongruence among these methodologies concerned the placement of *D. unimaculata* Strobl. In transversion MP analysis, this species is sister to the clade composed by the *quadrisetata* group, the *clefta* group, the *lacertosa* and *okadai* subgroup species. But unweighted MP grouped *D. unimaculata* with *D. moriwakii* and the *robusta* subgroup with the exclusion of *D. borusta* and *D. cheda*-like. Bayesian inference based on mt genes provided different picture from both MPs. Under this method, *D. unimaculata* was arranged to be a close relative to the clade formed by the *clefta*, the *quadrisetata* group, the *okadai* and the *lacertosa* group as well as *D. borusta* and *D. cheda*-like. Another worthy noting is that all analyses suggested a distinct division of *D. tsigana* Burla et Gloor along essentially geographic lines. The Chinese *D. tsigana* united *D. longiserrata* Toda, a sympatric species to the former. However, the conspecific strain of *D. tsigana* inhabiting in Japan seems to be distantly related to its Chinese counterpart.

3.2.2. Nuclear gene

The nucleotide dataset contained 314 (out of 711) parsimony informative sites. The unweighted parsimony analysis based on Adh coding region identified over 100 MP trees of 2067 steps with a CI of 0.350, RI of 0.782, and RC of 0.273. We presented MP tree in Fig. 2A. In comparison, the resolve ability of Adh gene is generally similar or a little higher than the mt genes in deep divergence of the tree. Many relationships appeared in mt gene tree were also supported based on analyses for Adh gene. MP analysis put *D. nannoptera* Wheeler in the basal position of the tree and

identified the sister relationship between the *annulimana* species group (here represented by *D. aracataca* Vilela et Val) and the *repleta* species group. However, in ML tree topology (not shown), *D. aracataca* was nested between *D. nannoptera* and all *repleta* species group. Tree topology derived from Bayesian approach did not support the basal position the *nannoptera* species group (Fig. 2B). In Bayesian tree, it was imbedded into the group formed by *D. fluvialis*, the *annulimana* species group and the *repleta* species group. However, the support for this arrangement was unreliable (PP < 95%). With regard to the placement of *D. unimaculata*, MP approach supported the sister relationship between it and the cluster consisting of *D. bai*, *D. moriwakii* and the *robusta* subgroup, whereas ML topology identified the sister relationship between *D. unimaculata* and the clade composed by the *quadrisetata* group, the *okadai* and the *lacertosa* subgroup. In all Adh gene trees, a strange result concerned the placement of *D. angor* B and *D. angor* C. They are confirmed as sibling species, but all analyses grouped them to one sample of *D. lacertosa* Okada, one species from the *robusta* species group with relatively high supports.

Once again, analyses for Adh gene strongly suggested the division of *D. tsigana* based on geographic distribu-

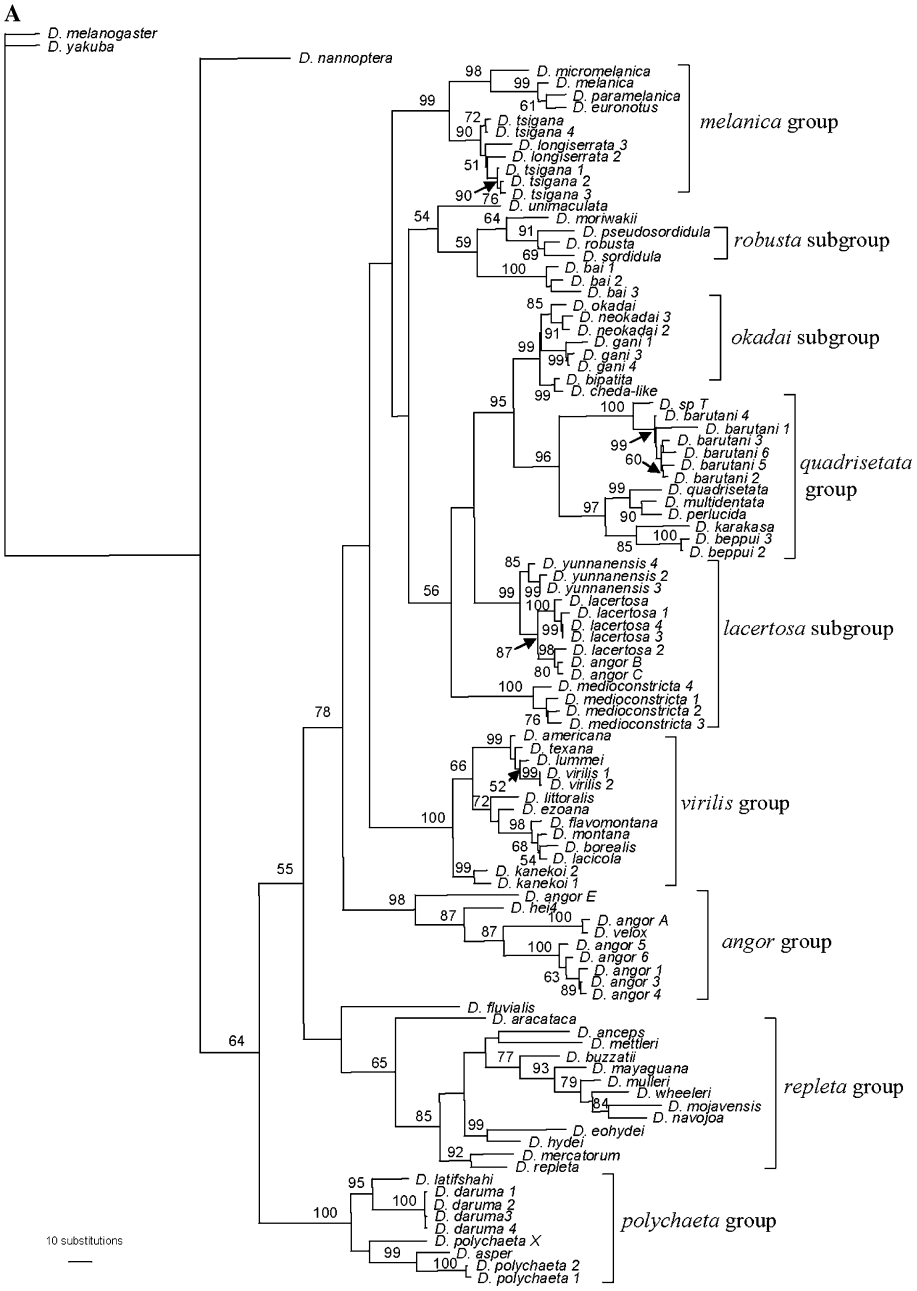

Fig. 2. (A) Tree topology based on the MP analysis for Adh gene. Numbers indicate the support from 1000 bootstrap calculation to the associated clade. Bootstrap values >50% are shown in the tree. (B) Consensus tree derived from the Bayesian inference for Adh genes. Numbers indicate the posterior probability of the associated clade. Posterior probabilities >0.95 are shown in the tree.

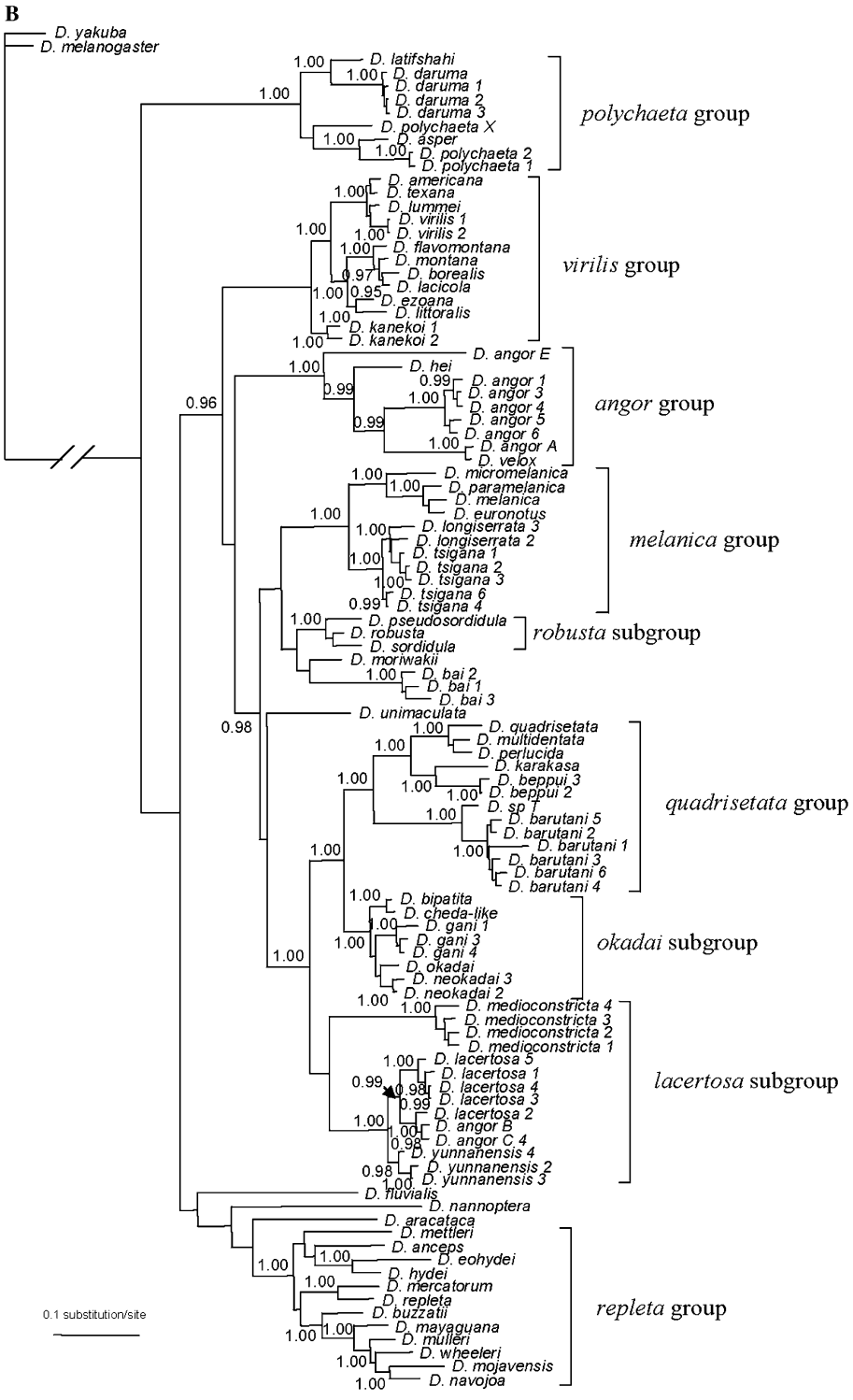

Fig. 2 (*continued*)

tions, and there is a tendency of the Chinese *D. tsigana* to form a sister taxon of *D. longiserrata*.

3.2.3. Combined mitochondrial and nuclear genes

Partition Homogeneity Test (PHT) for mt and nuclear genes rejected the combination of these two genetic lineages. However, when only transversion of mt gene was considered, homogeneity of the two data partitions can be accepted at 99% confidence level ($P = 0.02$), hence we combined these two data partitions for MP, ML, and Bayesian analyses. The tree topology produced by Bayesian analysis is shown in Fig. 3. The general accordance across different methodologies concerned the cluster of species that comprises members of the *robusta* species group, the *melanica* group, the *quadrisetata* group, which were clearly revealed by mt genes (Fig. 1). For example, the sister relationship

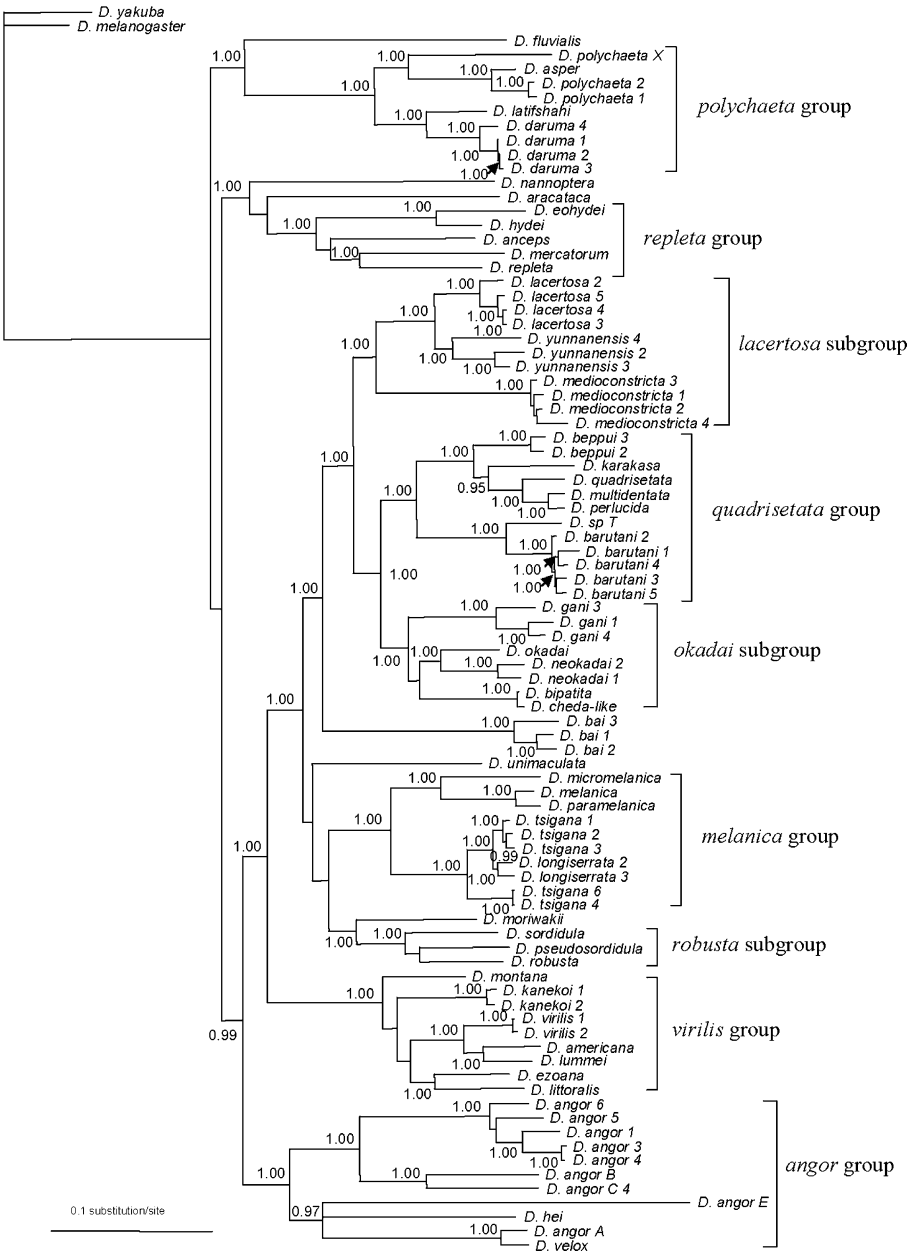

Fig. 3. Consensus tree derived from the Bayesian inference for concatenated mt and nuclear genes. Numbers indicate the posterior probability of the associated clade. Posterior probabilities >0.95 are shown in the tree.

between *D. lacertosa* and *D. yunnanensis* Watabe et Liang, additionally, they were grouped together with *D. mediоconstricta* Watabe, Zhang et Gan with robust posterior probability (100%). However, a few disagreements were observed between individual loci and the concatenated data sets. For instance, the sister relationship between *D. nannoptera* and *D. aracataca* revealed by MP analysis for mt gene was not supported by ML analysis for combined data sets. Under ML framework, the *annulimana* group (*D. aracataca*) sistered to the *repleta* species group, and the *nannoptera* species group was sister to them.

Set aside many well-supported clusters, some phylogenetic relationships indicated by different methods remained ambiguous. For example, the sister relationship between *D. angor* A and *D. velox* Watabe et Peng were recognized by ML methods. Nevertheless, Bayesian analysis rendered *D. angor* E, *D. hei*, *D. angor* A and *D. velox* as a compact cluster, and nested *D. hei* within *D. angor* E and *D. angor* A–*D. velox* (Fig. 3. PP = 97%).

4. Discussion

The high species diversity and greater ecological breadth (from tropical to cold regions) of the *Drosophila virilis* section invite an interesting question regarding the phylogeny and evolutionary history of the lineage, particularly in regard to their origin and historical biogeography. For the first time, the present study represents the most extensive

taxon sampling of the *virilis* section, especially including many new species which are confined to the Southern China, the potential place of origin of the *virilis–repleta* radiation suggested by Throckmorton (1975). Moreover, our study provides first significant contribution in clarifying the phylogenetic relationships among and within some groups at molecular level.

4.1. Phylogeny

4.1.1. The polychaeta species group

DNA sequence comparisons strongly suggest the monophyly of the *polychaeta* group, one of the earlier tropical assemblages in the *virilis–repleta* radiation (Throckmorton, 1975). Moreover, all analyses based on combined datasets confirm with strong support (Fig. 1. PP 100%) the sister relationship between the *polychaeta* group and the ungrouped species, *D. fluvialis*. Based on our analyses, the *polychaeta* group is clearly divided into two distinct sublineages composed of: (1) *D. latifshahi* Gupta et Ray-Chaudhuri and *D. daruma* Okada and (2) A new species found in Southwest China, *D. polychaeta X* and two closely related species, *D. asper* Lin et Tseng and *D. polychaeta* Petterson et Wheeler. *D. latifshahi* was originally assigned as one member of the subgenus *Scaptodrosophila* (Gupta and Ray-Chaudhuri, 1970), and later was shifted to the *polychaeta* species group (Toda and Peng, 1989). In this study, unambiguously *D. latifshahi* is one member of the *polychaeta* group and sister taxon of *D. daruma*. An important finding encompasses the position of an Afro-tropical member, *D. hirtipes*. Although we are not aware of its position in Adh gene tree since its Adh sequence is unavailable, analysis for mt loci support its close relationship to the second clade mentioned above. Bayesian inference based on mt data set recognized this species as close relative of the other members of the *polychaeta* group. Present result is consistent with the observation based on the analysis for ribosomal RNA data (Pelandakis and Solignac, 1993), in which the sister relationship between two representatives of the *polychaeta* group, *D. hirtipes* (Pelandakis and Solignac's *D. iri*, conspecific to *D. hirtipes*, Tsacas, 1994) and *D. polychaeta*, was identified. Regardless of the allopatric distribution of *D. hirtipes* and other three species within this species group, the close relationships among them revealed by our molecular data suggest their common origin, and their current distribution most likely reflect the dispersal event between Asia and Africa.

The high level of morphological resemblance in external genitalia of *D. polychaeta and D. asper* as well as asymmetric mating preference (87% between *D. asper* ♀ and *D. polychaeta* ♂, while 4% in reciprocal cross, Watabe and Toribe, 1996) suggest that they are conspecific species, representing two ecotype of *D. polychaeta*. The reason exists in that *D. asper* can be found only in natural environments, and *D. polychaeta* itself may be collected from artificial environments, such as brewery or timberyard (Watabe and Peng, 1991). In present result, the sister relationship between the two species is recognized by both mt and nuclear data under varied analyses, which supports Watabe and Toribe's (1996) conclusion to a great extent. However, whether these two are conspecific need future confirmation with more evidences, such as more gene fragments under background of population genetics.

4.1.2. The angor species group

Including 4 new species, a total of 7 species are subjected to molecular phylogenetic assessment in present study. Both combined datasets and mitochondrial genes strongly support the monophyly of this group, and *D. angor* A is sister to *D. velox* with high level of confidence. However, Adh gene data matrix overcome the monophyly of the *angor* group. Although the sister relationship between *D. angor* B and *D. angor* C is recognized by Adh data, they seems to be nearer to *D. lacertosa* than to their own group with robust support (PP = 100%). The conflict placement of *D. angor* B and *D. angor* C between mt and nuclear gene sequence may reflect the different evolutionary history of two gene lineages. In our study, only one species of *D. angor* B was used, and only one Adh gene among several samples of *D. angor* C was obtained. It is clear that multiple individuals are required in the future to determine the position of *D. angor* B and *D. angor* C with certainty.

The cluster formed by *D. angor* itself was divided into four strains essentially according to geographic origin (Table 1). Average genetic distance among them varied from 0.9% (GX vs. YN) to 2.4% (GX vs. JP) for Adh gene, from 5.0% (GX vs. TW) to 6.0% (GX vs. JP) for COI gene and from 3.9% (GX vs. YN) to 5.5% (GX or YN vs. TW) for ND2, respectively. Although morphological differences particularly in genitalia between these strains may indicate their divergence at the species level, our molecular data analyses show that their genetic differentiations represent only intraspecific variation, and their differences in morphology have nothing to do with the genetic background.

4.1.3. The quadrisetata group and the clefta group

All tree construction methods based on mt data set distinguish within the *quadrisetata* species group two clades: (1) the sibling species of *D. sp* T and *D. barutani* Watabe et Liang as well as *D. potamophila* Toda et Peng and *D.* sp IZU and (2) the remaining taxa with *D. beppui* Toda et Peng and *D. karakasa* Watabe et Liang at the basal position. However, the monophyly of the *quadrisetata* species group is problematic in this study. The sister relationship between the *clefta* group and either entire *quadrisetata* group or only the second clade are identified. In this study, we are inclined to suggest the non-monophyly of the *quadrisetata* species group, since the posterior probability for this phylogenetic relationship is quite low (Fig. 1. PP = 76%). Owing to the absence of Adh sequence of *clefta* group, presently it is ambiguous of contributions of nuclear gene to the *clefta* group species in the tree. Nevertheless, morphological evidence corroborates the close relationship of the *quadrisetata* and *clefta* group, and members of both

groups share identical environments (Watabe, unpubl. data). In other words, the result obtained from molecular analyses is consistent with morphology. In conclusion, our present study revealed the close association between the *quadrisetata* and the *clefta* species group. Further research with complete Adh gene data will tell the detail relationships with confidence.

Based on mt data set, the sister-group relationship between *D. potamophila–D.* sp IZU and *D. barutani–D.* sp T was recovered in the first clade mentioned above, suggesting monophyly of this clade. The remaining *quadrisetata* taxa form a compact cluster (clade 2), and morphologically distant *D. beppui* was found to be basal to the clade based on all analyses except Bayesian inference for combined datasets. In the tip of this clade, *D. pilosa* Watabe et Peng, *D. perlucida* Zhang et Liang and *D. multidentata* Watabe et Zhang are relatives, but the hierarchical ranks were resolved with uncertainty. Bayesian inference and transversion MP analysis for mt gene support the sibling species of *D. pilosa* and *D. multidentata*, while equally weighted MP identified that *D. pilosa* was a sister to *D. perlucida*. Their exact relationships are still kept as an ambiguity and need further research.

4.1.4. The melanica and robusta species group

These two species groups have been well studied from the angle of speciation and evolution (Levitan, 1982; Narayanan, 1973; Stalker, 1966, 1972). However, the taxon sampling was extremely limited in previous investigations, and thus the phylogenetic relationships derived from inadequate number of species were far less complete. In regard to the *melanica* species group, our results confirm the non-monophyly of this group, given the taxonomic revision of *D. moriwakii* by Beppu (1988). Without any case in our analyses was *D. moriwakii* supported as one close relative to other members of the *melanica* species group. Although the position of *D. moriwakii* altered across different analytical methods and different datasets, the sister relationship of this species to the *robusta* subgroup was distinguished in most cases and strongly supported by assessment based on separate and combined datasets. In view of the close association of *D. moriwakii* and the *robusta* subgroup, it seems likely that the revision of *D. moriwakii* (Beppu, 1988) indicate the incorrect taxonomic arrangement of this species.

With the exception of *D. moriwakii*, the remaining *melanica* group species formed two clades, one consisting of the Old World members and the other, the New World ones. Phylogeny deduced from cytotaxonomic data suggested the ancestral of *D. micromelanica* Petterson, from which four species evolved in the order of *D. melanura* Miller, *D. euronotus*, *D. paramelanica* Petterson and *D. melanica* Sturtvant (Levitan, 1982; Stalker, 1966). In our result, *D. micromelanica* is placed at the most basal position in the New World *melanica* clade in all trees, which is in accord with the results based on cytological researches (Stalker, 1966). In contrast to the phylogenetic relationships revealed by cytotaxonomic data, among three species with identical karyotypes, *D. melanica* was confirmed by our molecular study as a species diverged earlier than the sister species of *D. euronotus–D. paramelanica* (Fig. 2).

Of the five species of the Old World *melanica* species group, we compared three. The remaining two members, *D. afer* Tan, Hsu et Sheng and *D. bisetata* Toda, are considered as synonyms of *D. tsigana* (Watabe and Toda, unpubl. data). Regarding species involved in this study, *D. moriwakii* has been discussed above. We sampled in present paper several individuals in each of two geographic strains of *D. tsigana*, one of which is from Southwestern China and sympatric to *D. longiserrata* (Table 1), and the other is from Japan. Interestingly, the geographic separation of *D. tsigana* was found repeatedly regardless of analytical methods and genetic loci. The sympatric *D. tsigana* and *D. longiserrata* are undoubtedly sister taxa, while JP *D. tsigana* is found distantly related to CN *D. tsigana–D. longiserrata*. When *D. tsigana* monophyly was constrained in unweighted MP tree, no significant difference was observed between constrained and unconstrained trees ($P = 0.2$). Nevertheless, constrained topology of monophyletic *D. tsigana* required extra 25 steps (TL = 11711, CI = 0.245, RI = 0.690), suggesting the reliability of present hypothesis with regard to paraphyletic *D. tsigana*. In addition, genetic distances for three genes imply that both strains may differentiate to a large extent (data not shown). This genetic differentiation is consistent with the external morphology variations, for instance, males of Chinese strain are almost entirely black in the second to fifth tergites, while those of Japanese *D. tsigana* have small black caudal bands interrupted in the middle (Watabe et al., 1990a). Besides, although there is no difference in the genitalia structure of the two strains of *D. tsigana*, results from cross experiments indicate asymmetric mating preference between these two strains (unpubl. data), suggesting their strong genetic differentiation, may represent allopatric speciation.

Previous study based on morphology split the *robusta* species group into three species subgroups, the *robusta* subgroup, the *lacertosa* subgroup and the *okadai* subgroup (Watabe and Nakata, 1989). However, the monophyly of the *robusta* species group and three subgroups have not been checked by any investigation. Present molecular studies show that the *robusta* species group is non-monophyletic lineage, nor are the monophyly of at least two subgroups supported, although most morphologically identified members within each same subgroup constitute strong cluster, representing respective taxonomic unit at subgroup level. The most unexpected result is that the *robusta* species group was confirmed as an incompact taxonomic unit, three subgroups within this group are not particularly close to one another. By comparison, the *lacertosa* subgroup is closer to the *okadai* subgroup than to the *robusta* subgroup. Three members of the *robusta* subgroup, *D. robusta*, *D. sordidula* and *D. pseudosordidula* always appear as relatives. Two assumed members of the *robusta* subgroup, *D. borusta*

and *D. cheda*-like were recognized to have close affinity to the *okadai* subgroup (Fig. 1). Hence, the monophyly/paraphyly of the *robusta* subgroup relied greatly on the taxonomic arrangement of the *D. borusta* and *D. cheda*-like based on morphology. Within the compact lineage comprising of *D. pseudosordidula*, *D. sordidula* and *D. robusta*, basal position of either *D. sordidula* or *D. pseudosordidula* was identified, depending on methods used. The phylogenetic placement of *D. robusta* in the tree indicated at any rate the derived status of this New World member of the *robusta* group, agreeing with the result based on chromosomal research and supporting the conclusion that the New World is the second habitat of the *robusta* group (Levitan, 1982; Narayanan, 1973).

As for the paraphyletic *lacertosa* subgroup, *D. bai* was recovered to be distantly associated to the clade constituted by closely linked*D. lacertosa*, *D. yunnanensis* and *D. mediconstricta* (Figs. 1 and 2), with the divergence of the last species predating the divergence of *D. lacertosa* and *D. yunnanensis*. The present molecular result with regard to the *lacertosa* subgroup is accordant to observation of morphological characteristics. For example, the *lacertosa* subgroup species are morphologically closely related by novasternum of male genitalia with submedian spines, wherein *D. mediconstricta* differed in a relatively smaller value of *C*-index of wing than those of the other two species (Watabe et al., 1990b). Despite of the same morphological characteristic to other *lacertosa* subgroup members (Watabe et al., 1990a), *D. bai* inhabit in the place which is much higher than that of those three species. In this regard, the habitat height of the *lacertosa* subgroup may be indicative of their phylogenetic relationships.

The non-monophyly of the *okadai* subgroup was strongly suggested at two points: (1) In MP tree topology derived from transversion mt gene, *D. unimaculata* was distantly related to other members of the same subgroup. Furthermore, in concatenated data it was closer to the clade formed by the *melanica* species group and the *robusta* subgroup than to other *okadai* subgroup members, and (2) two assumed *robusta* subgroup species, *D. borusta* and *D. cheda*-like, were nested within the clade consisting of the *okadai* subgroup members. Sister relationship of *D. okadai* Takada and *D. neokadai* Kaneko et Takada always received high bootstrap support or high posterior probability. *D. bipatita* and *D. cheda*-like formed a sister taxa with high level support in our analyses. However, very low genetic distances for three genes between these two species may suggest their conspecific status. Within this clade, *D. gani* Liang et Zhang was indicated to diverge earlier than the divergence of other species, a consistent result to morphological observation, since this taxon differs from other clade members substantially in external genitalia (Watabe et al., 1990a).

Previous work suggested that the *okadai* subgroup might occupy an ancestral position in the *robusta* group (Watabe et al., 1997), since its members possessed a "ancestral-like karyotype" of the genus *Drosophila* ($2n = 12$ and rod-like X chromosome), and the other two subgroups might be derived due to their large V-shaped X chromosomes. However, our present study do not agree with the results derived from the viewpoint of karyotypic evolution (Watabe et al., 1997) in that firstly, the monophyly of the *okadai* subgroup is not supported, and secondly, the lineage composing of *D. okadai*, *D. neokadai* and *D. gani* is not at the basal in all tree topologies.

The phylogenetic relationships regarding the clade containing the *melanica* group, the *robusta* group, the *quadrisetata* group and the *clefta* group might be related to the similarity of ecological environments. For example, although most species of the *virilis* section are associated with riparian communities (Throckmorton, 1975; Watabe et al., 1990a), the degree of water-dependence is discrepant among different lineages. The *robusta* subgroup and the *melanica* group are often collected from sites in the temperate forests distant from streams, while the *okadai* and the *lacertosa* subgroup, the *quadrisetata* group and the *clefta* group are usually found along watersides (Beppu et al., 1989; Watabe and Peng, 1991). Therefore, our phylogeny reflects a well adaptation of *Drosophila* species to the different ecological environments, and this adaptation might have played an important role in the speciation processes of those species.

4.1.5. The virilis species group

Until recently, the phylogenetic relationships within this species group based on complete taxon sampling were investigated using molecular study (Orsini et al., 2004; Spicer and Bell, 2002). Although the taxa involved in this group are not complete in present study (*D. canadiana* is absent), some agreements are observed between our results and that derived from analyses for mitochondrial 12S/16S rRNA datasets (Spicer and Bell, 2002). Under the background of the *virilis* section, monophyly of the *virilis* phylad was strongly supported by our studies. Moreover, monophyly of the *montana* phylad as well as the sister relationship between the *littoralis* subphylad and the *kanekoi* subphylad were identified by MP and ML methods for Adh data, while ML approach based on concatenated sequences revealed the sister relationship between the *vrilis* phylad and the *littoralis* subphylad, with *D. kanekoi* predating the divergence of them. It was noteworthy that our result with regard to the phylogeny of the *montana* subphylad is not completely consistent with Nurminsky et al. (1996), although we used several identical Adh sequences. Our analyses for Adh gene support the basal position of *D. flavomontana* within the *montana* subphylad, which is coincident with Nurminsky et al., whereas the sister relationship between *D. borealis* and *D. lacicola* presented by our analysis was not supported by Nurminsky et al. (1996). The uncertain phylogenetic relationships within the *montana* subphylad also confirmed recently by Orsini et al. (2004). It is necessary to contain more genetic markers to determine the phylogeny with certainty.

4.1.6. The repleta species group, nannoptera group, and annulimana group

Although the most basal position of the *nannoptera* species group in the ingroup taxa was revealed by MP analysis for Adh gene, both ML and Bayesian approaches based on mt sequence data identified the close relationships between *D. nannoptera* and the *annulimana* and the *repleta* species group (Fig. 3). Since only one representative of each of the *nannoptera* and *annulimana* species groups was employed in this study, further analysis of extensive taxa sampling is required.

It should be cautious to assume the monophyly of the *repleta* species group, since high number of species were put into this group and substantial molecular data did not suggest it a monophyletic lineage (Durando et al., 2000), albeit authors owed non-monophyly of the *repleta* group to the poor resolution at the base of the phylogenetic tree obtained. In this study, we only sample very limited representative taxa within this group for comparison, and we cannot assume that these species are necessarily typical of the *repleta* group as a whole. Therefore, although it seems that the phylogenetic relationships revealed by our molecular study are consistent to the result produced via cytological methods (Wasserman, 1982), certain tentative conclusion was drawn only at the subgroup level, and the valid conclusion about the interspecific relationships should be considered until more extensive studies have been made on this group.

4.2. Biological implications of molecular phylogeny

Among all species groups, phylogenetic approaches based on Adh and concatenated data sets suggest that the *polychaeta* group and the *repleta* group are closer to the basal position of the tree than other taxa (Figs. 2 and 3). Concerning the position of the *polychaeta* group, this result is partly consistent with that obtained from molecular phylogeny based on Amd and Ddc sequences (Tatarenkov and Ayala, 2001). Nevertheless, our result does not support the derived position of the *relpleta* group revealed by Tatarenkov and Ayala (2001). It is noteworthy that the relative basal placement of the *polychaeta* group suggested by our analyses provides support for Throckmorton's (1975) hypothesis that the *polychaeta* species group is one of a heterogeneous assemblage of groups representing the early tropical *virilis–repleta* radiation. However, all analytical methods based on mt data sets indicate the close affinity of the *polychaeta* group and the *repleta* group (Figs. 1A and B), which is conflict with that uncovered by Adh and combined data sets. We owed the disagreement of the tree topologies to the different evolution pattern between nuclear and mitochondrial genes. For instance, Modeltest analyses under Akaike Information Criterion (AIC) reveal higher alpha value of Adh (0.8413) than that of mt gene (0.5186), which means that higher level of homoplasy of mt gene than Adh is most likely, given the suggestion by Lin and Danforth (2004) that there exist a positive correlation between alpha value and consistency index (CI). This correlation is detected not only by us but also by Robe et al. (2005). Besides, base compositional bias between nuclear and mitochondrial genes, as well as distribution differences of variable sites among codon positions between two different genetic lineages (Table 3) are most likely the source of homoplasy. The performance of homoplasy can be seen especially in the base of the tree when mitochondrial genes are employed as molecular markers, and different phylogenetic approaches can improve the resolution little (Figs. 1A and B).

Owing to the absence of the bootstrap value for the ML tree topology, we primarily put our discussion of biological inference based on Bayesian method framework for combined datasets. As seen from the result (Fig. 3), with the exception of the cosmopolitan "domestic" species, such as *D. polychaeta*, *D. repleta* Duda, *D. hydei* Spencer and *D. virilis* Sturtvant, which may disperse through human activities (Dobzhansky, 1965; Throckmorton, 1975), the distribution of the *virilis* section species in the tree is generally corresponding to their colonization in different temperature bands. For instance, *D. fluvialis*, the *polychaeta*, and *angor* species groups are oriental tropical colonizers and the *repleta*, *nannoptera*, and *annulimana* species groups inhabit neotropical regions, and they are placed at the base of the tree as more ancestral clades than other taxa.

The remaining ingroup taxa are split into two clades (*virilis* clade and *quadrisetata-clefta-robusta-melanica* clade) representing cold and from subtropical to warm-temperate to cool-temperate species, respectively, despite the considerable overlap in their distribution. The latter clade further divide into two subclades: one is formed by the *quadrisetata* group, the *clefta* group, the *lacertosa* subgroup and most of the *okadai* subgroup species, they are reported to inhabit in the natural forests near waterside (Toda and Peng, 1989; Watabe et al., 1990a; Watabe and Peng, 1991). The other subclade is made up of *D. unimaculata*, the *melanica* species group and the *robusta* subgroup with exception of *D. borusta* and *D. cheda*-like. Except for *D. unimaculata* (Bachli, personal commun.), these species colonize temperate forests far from rivulet (Toda, 1988; Levitan, 1982). Hence, just like many other species of insects (Goto et al., 2000), it is most likely that the taxa of the *virilis* section originated in the Old World tropics and adapted to warm climate, represented by the common ancestors of the *polychaeta* and *angor* species group. Ancestor of the *repleta* species group might have evolved in the Old World tropics and been extinct since its descendants dispersed into neotropical region and diversified there. We assume that some taxa within the *virilis* section succeeded in colonizing temperate region during migration from low to high latitude in order to void warm temperature. Among these temperate species, some members of the *virilis* species group, such as *D. virilis*, *D. borealis* Petterson and *D. canadiana* Takada et Yoon, became winners in colonizing cold regions. Nevertheless, the primitive type of the remaining species were far less cold tolerant than the *virilis* group species. They evolved as two

lineages with the lineage of the *quadrisetata* group-*clefta* group-*okadai* subgroup-*lacertosa* subgroup adapting to subtropical and warm-temperate climates and possess waterside niche. The other lineage contains the *melanica* group and the *robusta* subgroup, and they could take advantage of the natural forests far from ghyll along with adaptations to cool-temperate climates. It was noteworthy that the distribution pattern of the *virilis*, *robusta* and *melanica* species groups are generally same in both Old and New World. Considering the phylogenetic placement of the Old and New World members of these three species groups, it is likely that at least five independent migrations might have occurred from the Old World to the New World via Beringia, such as (1) the *virilis* phylad and (2) *montana* phylad, (3) *D. robusta* within the *robusta* species group, (4) the *repleta* species group and (5) the *melanica* species group. Under this consideration, extant *repleta* group species and North American members of the *virilis* group, the *melanica* group and the *robusta* group might have evolved in new differentiate center there.

Acknowledgments

We are grateful to Dr. T. Katoh (Hokkaido University) for his invaluable comments on the manuscript and to Dr. G. Baechli (Zurich University) for providing us with samples of European members of the *virilis* section. The manuscript was significantly benefited from two anonymous reviewers. This study was funded by the State Key Basic Research and Development Plan (2003CB415105), Bureau of Science and Technology of Yunnan Province, NSFC (30021004), and JSPS (No. 12375002).

References

Atrian, S., Sanchez-Pulido, L., Gonzalez-Duarte, R., Valencia, A., 1998. Shaping of *Drosophila* alcohol dehydrogenase through evolution: relationship with enzyme functionality. J. Mol. Evol. 47, 211–221.

Baker, W.K., 1980. Evolution of the alpha-esterase duplication within the *montana* subphylad of the *virilis* species group of *Drosophila*. Genetics 94, 733–748.

Beppu, K., 1988. Systematic positions of three *Drosophila* species (Diptera: Drosophilidae) in the *virilis–repleta* radiation. Proc. Jpn. Soc. Zool. 37, 55–58.

Beppu, K., Peng, T.X., Xie, L., 1989. An ecological study on species (Diptera: Drosophilidae) living at watersides in southern China. Jpn. J. Ent. 57, 185–198.

Chen, H.Z., Watabe, H.A., 1993. The *Drosophila virilis* section (Diptera: Drosophilidae) from eastern China, with description of two new species. Jpn. J. Ent. 61, 313–322.

Clary, D.O., Wolstenholme, D.R., 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. J. Mol. Evol. 22, 252–271.

Crozier, R.H., Crozier, Y.C., 1993. The mitochondrial genome of the honeybee *Apis mellifera*: Complete sequence and genome organization. Genetics 133, 97–117.

Dobzhansky, T., 1965. Wild and Domestic species of *Drosophila*. In: Baker, H.G., Stebbins, G.L. (Eds.), The Genetics of Colonizing Species. Academic Press, New York, pp. 533–546.

Durando, C.M., Baker, R.H., Etges, W.J., Heed, W.B., Wasserman, M., DeSalle, R., 2000. Phylogenetic analysis of the *repleta* species group of the genus *Drosophila* using multiple sources or characters. Mol. Phylogenet. Evol. 16, 296–307.

Farris, J.S., Kallersjo, M., Kluge, A.G., Bult, C., 1995. Testing significance of incongruence. Cladistics 10, 315–319.

Flook, P.K., Rowell, C.H., Gellissen, G., 1995. The sequence, organization, and evolution of the *Locusta migratoria* mitochondrial genome. J. Mol. Evol. 41, 928–941.

Goto, S.G., Kitamura, H.W., Kimura, M.T., 2000. Phylogenetic relationships and climatic adaptations in the *Drosophila takahashii* and *montium* species groups. Mol. Phylogenet. Evol. 15, 147–156.

Gupta, J.P., Ray-Chaudhuri, S.P., 1970. Some new and unrecorded species of *Drosophila* (Diptera: Drosophilidae) from India. Proc. Roy. Ent. Soc. Lond. (B) 39, 57–72.

Hsu, T.C., 1949. The external genital apparatus of male Drosophilidae in relation to systematics. Univ. Texas Publ. 4920, 80–142.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Mayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5, 150–163.

Levitan, M., 1982. The *robusta* and *melanica* groups. In: Ashburner, M., Carson, H.L., Thompson, J.N. (Eds.), The Genetics and Biology of *Drosophila*, vol. 3b. Academic press, London, pp. 141–192.

Lin, C.P., Danforth, B.N., 2004. How do insect nuclear and mitochondrial gene substitution patterns differ? Insights from Bayesian analysis of combined datasets. Mol. Phylogenet. Evol. 30, 686–702.

Narayanan, Y., 1973. The phylogenetic relationships of the members of the *Drosophila robusta* group. Genetics 73, 319–350.

Nurminsky, D.I., Moriyama, E.N., Lozovskaya, E.R., Hartl, D.L., 1996. Molecular phylogeny and genome evolution in the *Drosophila virilis* species group: duplications of the alcohol dehydrogenase gene. Mol. Biol. Evol. 13, 132–149.

O' Grady, P.M., 1999. Reevaluation of phylogeny in the *Drosophila obscura* species group based on combined analysis of nucleotide sequences. Mol. Phylogenet. Evol. 12, 124–139.

Orsini, L., Huttunen, S., Schlotterer, C., 2004. A multilocus microsatellite phylogeny of the *Drosophila virilis* group. Heredity 93, 161–165.

Pelandakis, M., Solignac, M., 1993. Molecular phylogeny of *Drosophila* based on ribosomal RNA sequences. J. Mol. Evol. 37, 525–543.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Remsen, J., DeSalle, R., 1998. Character congruence of multiple data partitions and the origin of the Hawaiian Drosophilidae. Mol. Phylogenet. Evol. 9, 225–235.

Robe, R.J., Valente, V.L.S., Budnik, M., Loreto, E.L.S., 2005. Molecular phylogeny of the subgenus *Drosophila* (Diptera, Drosophilidae) with an emphasis on Neotropical species and groups: a nuclear versus mitochondrial gene approach. Mol. Phylogenet. Evol. 36, 623–640.

Rodriguez-Trelles, F., Tarrio, R., Ayala, F.J., 1999. Molecular evolution and phylogeny of the *Drosophila saltans* species group inferred from the *Xdh* gene. Mol. Phylogenet. Evol. 13, 110–121.

Russo, C.A.M., Takezaki, N., Nei, M., 1995. Molecular phylogeny and divergence times of Drosophilid species. Mol. Biol. Evol. 12, 391–404.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Spicer, G.S., 1992. Reevalution of the phylogeny of the *Drosophila virilis* species group (Diptera: Drosophilidae). Ann. Entomol. Soc. Am. 85, 11–25.

Spicer, G.S., Bell, C.D., 2002. Molecular phylogeny of the *Drosophila virilis* species group (Diptera: Drosophilidae) inferred from mitochondrial 12S and 16S ribosomal RNA genes. Ann. Entomol. Soc. Am. 95, 156–161.

Stalker, H.D., 1966. The phylogenetic relationships of the species in the *Drosophila melanica* group. Genetics 53, 327–342.

Stalker, H.D., 1972. Intergroup phylogenies in Drosophila as determined by comparisons of salivary banding patterns. Genetics 70, 457–474.

Stone, W.S., Guest, W.C., Wilson, F.D., 1960. The evolutionary implications of the cytological polymorphism and phylogeny of the *virilis* group of *Drosophila*. Proc. Natl. Acad. Sci. USA 46, 350–361.

Sturtevant, A.H., 1942. The classification of the genus *Drosophila*, with descriptions of nine new species Univ. Texas Publ. 4213, 5–51.

Swofford, D.L., 2000. PAUP*: Phylogenetic analysis using parsimony (∗and other methods), Ver. 4. Sinauer, Sunderland, MA.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.

Tatarenkov, A., Ayala, F.J., 2001. Phylogenetic relationships among species groups of the *virilis–repleta* radiation of *Drosophila*. Mol. Phylogenet. Evol. 21, 327–331.

Throckmorton, L.H., 1975. The phylogeny, ecology, and geography of *Drosophila*. In: King, R.C. (Ed.), Handbook of Genetics, vol. III. Plenum Publ., New York, pp. 421–469.

Throckmorton, L.H., 1982. The *virilis* species group. In: Ashburner, M., Novistky, E. (Eds.), The Genetics and Biology of *Drosophila*, vol. 3b. Academic press, London, pp. 227–297.

Toda, M.J., 1988. Drosophilidae (Diptera) in Burma. III. The subgenus *Drosophila*, excepting the *D. immigrans* species-group. Kontyu 56, 625–640.

Toda, M.J., Sidorenko, V.S., Watabe, H.A., Kholin, S.K., Vinokurov, N.N., 1996. A revision of the Drosophilidae (Diptera) in East Siberia and Russian Far East: Taxonomy and biogeography. Zool. Sci. 13, 455–477.

Toda, M.J., Peng, T.X., 1989. Eight species of the subgenus *Drosophila* (Diptera: Drosophilidae) from Guangdong Province, southern China. Zool. Sci. 6, 155–166.

Tsacas, L., 1994. *Drosophila* iri Bular , 1954, nouveau synonyme de *Drosophila hirtipes* Lamb, 1914 (Diptera). Ann. Soc. Entomol. Fr (N.S.) 30, 251–252.

Wasserman, M., 1982. Evolution of the *repleta* group. In: Ashburner, M., Carson, H.L., Thompson, J.N. (Eds.), The Genetics and Biology of *Drosophila*, vol. 3b. Academic press, London, pp. 61–139.

Watabe, H.A., Nakata, S., 1989. A comparative study of genitalia of the *Drosophila robusta* and *D. melanica* species-groups (Diptera: Drosophilidae). J. Hokkaido Univ. Edu. (Sec. II B). 40, 13–30.

Watabe, H.A., Peng, T.X., 1991. The *Drosophila virilis* section (Diptera: Drsophilidae) from Guangdong Province, southern China. Zool. Sci. 8, 147–156.

Watabe, H.A., Toribe, N., 1996. A new synonym of *Drosophila polychaeta*, with the results of cross-experiments. Zool. Sci. 13 (Suppl.), 35 (abstract only).

Watabe, H.A., Liang, X.C., Zhang, W.X., 1990a. The *Drosophila robusta* species-group (Diptera: Drosophilidae) from Yunnan Province, southern China, with the revision of its geographic distribution. Zool. Sci. 7, 133–140.

Watabe, H.A., Liang, X.C., Zhang, W.X., 1990b. The *Drosophila polychaeta* and the *D. quadrisetata* species groups (Diptera: Drosophilidae) from Yunnan Province, southern China. Zool. Sci. 7, 459–467.

Watabe, H.A., Park, J., Aotsuka, T., 1997. A karyotype study on the *Drosophila robusta* species-group (Diptera: Drosophilidae). Zool. Sci. 14, 855–858.

Xia, X., Xie, Z., 2001. DAMBE: Data analysis in molecular biology and evolution. J. Hered. 92, 371–373.

Zhang, W.X., Liang, X.C., 1994. Three new species of Drosophilid flies (Diptera: Drosophilidae) from Yunnan and Hubei. China Entomotaxonomia. 16, 213–219.