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Evolutionary Relationships in the *Drosophila ananassae* Species Cluster Based on Introns of Multiple Nuclear Loci

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The *Drosophila ananassae* species cluster includes *D. ananassae*, *D. pallidosa*, *D. parapallidosa*, and the cryptic species "pallidosa-like", "pallidosa-like Wau", and "papuensis-like". Some of the taxa are sympatric in the South Pacific, Papua New Guinea, and Southeast Asia, and gene flow between different taxa has been suspected for a handful of genes. In the present analysis, we examined DNA sequences of introns in four loci: *alpha actinin* (*Actn*) on XL, *white* (*w*) on XR, *CG7785* on 2L, and *zinc ion transmembrane transporter 63C* (*ZnT63C*) on 2R. Phylogenetic trees (neighbor-joining and haplotype network) were inconsistent among these loci. Some haplotypes shared between taxa were found for *w*, *CG7785*, and *ZnT63C*, suggesting recent gene flow. However, no haplotypes were shared, for example, between *D. ananassae* and *D. pallidosa* for *CG7785*, which is close to the proximal breakpoint of *In(2L)D*. This suggests that taxon-specific inversions prevent gene flow, as predicted by the chromosomal speciation hypothesis.

Key words: cryptic species, *Drosophila ananassae*, gene flow, introgression, inversion polymorphism, phylogeography

INTRODUCTION

The *Drosophila ananassae* species complex is one of three species complexes recognized in the ananassae group, recently elevated from a subgroup of the melanogaster group (Kaneshiro and Wheeler, 1970; Lemeunier et al., 1986; Da Lage et al., 2007). Twelve species have been described in the *D. ananassae* species complex (Bock and Wheeler, 1972; Tobari, 1993; Matsuda et al., 2009), but two of them were recently removed from this complex (Da Lage et al., 2007); the complex now includes *D. ananassae*, *D. atripex*, *D. cornixa*, *D. ironensis*, *D. monieri*, *D. nesoetes*, *D. ochrogaster*, *D. pallidosa*, *D. parapallidosa* (= "taxon-K"), and *D. phaeopleura*. Among them *D. ananassae*, *D. pallidosa*, and *D. parapallidosa* are difficult to discriminate by morphology and may include several cryptic species, namely, "pallidosa-like", "pallidosa-like Wau", and "papuensis-like" (Futch, 1966; Tobari, 1993; Sawamura et al., 2008a; Matsuda et al., 2009), which together we refer to as the "*D. ananassae* species cluster." Despite their morphological similarity, the cryptic species are reproductively isolated from each other by sexual behavior and hybrid sterility and are differentiated by certain chromosomal rearrangements and acoustic signals emitted by males (Futch, 1966; Tomimura et al., 1993; Yamada et al., 2002; Matsuda et al., 2009; H. Yamada, T. Sakai, and Y. Oguma, unpublished observations).

To understand geographic differentiation of populations of *D. ananassae* and the relationship of the latter to *D. pallidosa*, microsatellites and mitochondrial DNA have been investigated, which led to the realization that *D. ananassae* is a polytypic species even in the same locality (Schug et al., 2004, 2007, 2008). It is still unclear, however, whether any of the sub-populations recognized by Schug et al. (2007, 2008) correspond to the taxa mentioned above. On the other hand, Sawamura et al. (2008a) and Matsuda et al. (2009) analyzed genes from chromosome 4, chromosome Y, and the mitochondrial genome to understand relationships in the *D. ananassae* species cluster, but the phylogenetic resolution was too low to resolve the relationships, presumably because of introgression and/or lineage sorting. In the present study we chose four loci in different genomic regions to circumvent the potential problem that certain loci may have been affected by introgression. To enhance phylogenetic resolution, we sequenced introns and included these data in our analysis because most introns, owing to their relatively neutral evolutionary impact, generally evolve more rapidly than exons (Kimura, 1983).

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MATERIAL AND METHODS

Flies and sequencing

Test lines were identified to species by cross experiments. Genomic DNA deposited after the previous study of the *D. ananassae* species cluster (Sawamura et al., 2008a) was used for sequencing (Table 1). Briefly, DNA was isolated from a single male fly from each iso-female line (30 lines). *Drosophila atripex*, *D. monieri*, *D. ochrogaster*, and *D. phaeopleura* were used as outgroup taxa (Table 1). Sequences from four loci—*alpha actinin* (*Actn*), *white* (*w*), *CG7785*, and *zinc ion transmembrane transporter 63C* (*ZnT63C*)—were amplified by PCR. Forward (F) and reverse (R) primers were designed based on comparisons between *D. ananassae* and *D. melanogaster* genomic sequences available from UCSC Genome Bioinformatics (http://genome.ucsc.edu/), because annotation of the *D. ananassae* genome had not been completed when we started this study. The reference sequences for *D. ananassae* are from the AABBg1 strain (*Drosophila* 12 Genomes Consortium, 2007).

PCR was conducted at 95°C for 4 min followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 7 min. Intron 4 of the gene homologous to *Actn* of *D. melanogaster* was amplified by using primers Actn.4F (–103 to –84; 5'-GACTATGCCAAGCTGTCCAA-3') and Actn.4R (+104 to +85; 5'-ATGCGTCACATTTCCAACCT-3'). Intron 3 of the gene homologous to *w* of *D. melanogaster* was amplified by using primers w.3F (–128 to –109; 5'-ACGGTCATTCTGACGATCCA-3') and w.3R (+96 to +77; 5'-CTGTCCCGGGATTCGATCTC-3'). Intron

Table 1. Iso-female lines analyzed and DDBJ/EMBL/GenBank accession numbers for sequences. Donor or collector: 1, inbred line (the strain sequenced); 2, Y. Fuyama, E. Takanashi (Matsuura), Y. N. Tobari; 3, E. Takanashi (Matsuura), Y. N. Tobari; 4, University of Texas; 5, H. L. Carson, T. Okada; 6, O. Kitagawa, K. I. Wakahama, T. Watanabe; 7, Y. Fuyama, F. Hihara, T. K. Watanabe; 8, A. Fukatami, H. Ikeda, O. Kitagawa; 9, Centre National de la Recherche Scientifique, Gif-Sur-Yvette; 10, Bowling Green State University. Sequences of AABBg1 are from UCSC Genome Bioinformatics. NA, not amplified.

Taxon	Strain	Locality	Year	Donor or collector	Locus			
					Intron 4 of *Actn*	Intron 3 of *w*	Intron 1 of *CG7785*	Intron 2 of *ZnT63C*
D. ananassae								
	AABBg1	Hawaii, USA (reference for sequences)	1945	1	scaffold_12929(reverse): 1923472–1923796	scaffold_13248: 365734–366080	sacaffold_13340(reverese): 11866124–11866425	scaffold_13337: 8892002–8892446
	NAN84	Lautoka, Fiji	1981	2	AB474414	AB474447	1: AB474479, 2: AB474480	AB474519
	NOU78	Noumea, New Caledonia	1981	2	AB474415	AB474448	AB474481	AB474520
	WAU138	Wau, Papua New Guinea	1981	3	AB474416	AB474449	1: AB474482, 2: AB474483	AB474521
	POM431	Port Moresby, Papua New Guinea	1981	3	AB474417	AB474450	AB474484	AB474522
	WAU120	Wau, Papua New Guinea	1981	3	AB474418	AB474451	AB474485	1: AB474523, 2: AB474524
	LAE119	Lae, Papua New Guinea	1981	3	AB474419	AB474452	1: AB474486, 2: AB474487	AB474525
	PPG183	Pago Pago, Samoa	1981	2	AB474420	AB474453	AB474488	AB474526
	TBU136	Tongatapu, Tonga	1981	2	AB474421	AB474454	AB474489	AB474527
	TBU209	Tongatapu, Tonga	1981	2	AB474422	AB474455	AB474490	AB474528
	TNG	Tongatapu, Tonga	1966	4	AB474423	AB474456	AB474491	AB474529
	VAV161	Vava'u, Tonga	1981	2	AB474424	AB474457	AB474492	AB474530
D. pallidosa								
	NAN4	Lautoka, Fiji	1981	2	AB474425	AB474458	AB474493	AB474531
	NAN64	Lautoka, Fiji	1981	2	AB474426	AB474459	AB474494	AB474532
	NAN66	Lautoka, Fiji	1981	2	AB474427	AB474460	AB474495	AB474533
	TBU155	Tongatapu, Tonga	1981	2	AB474428	AB474461	AB474496	AB474534
	VAV92	Vava'u, Tonga	1981	2	AB474429	AB474462	AB474497	1: AB474535, 2: AB474536
	NAN57	Lautoka, Fiji	1981	2	AB474430	AB474463	AB474498	AB474537
pallidosa-like								
	LAE346	Lae, Papua New Guinea	1981	3	AB474431	AB474464	AB474499	1: AB474538, 2: AB474539
	POM446	Port Moresby, Papua New Guinea	1981	3	AB474432	AB474465	AB474500	AB474540
	POM458	Port Moresby, Papua New Guinea	1981	3	AB474433	AB474466	AB474501	AB474541
	POM473	Port Moresby, Papua New Guinea	1981	3	AB474434	AB474467	AB474502	AB474542
pallidosa-like-Wau								
	BLO79	Bulolo, Papua New Guinea	1979	5	AB474435	AB474468	1: AB474503, 2: AB474504	AB474543
	WAU61	Wau, Papua New Guinea	1981	3	AB474436	AB474469	AB474505	AB474544
	WAU92	Wau, Papua New Guinea	1981	3	AB474437	AB474470	AB474506	AB474545
papuensis-like								
	WAU142	Wau, Papua New Guinea	1981	3	AB474438	AB474471	1: AB474507, 2: AB474508	1: AB474546, 2: AB474547
	WAU145	Wau, Papua New Guinea	1981	3	AB474439	AB474472	1: AB474509, 2: AB474510	1: AB474548, 2: AB474549
	LAE360	Lae, Papua New Guinea	1981	3	AB474440	AB474473	AB474511	AB474550
	LAE376	Lae, Papua New Guinea	1981	3	AB474441	AB474474	AB474512	AB474551
D. parapallidosa								
	B43	Kota Kinabalu, Malaysia	1971	6	AB474442	AB474475	AB474513	AB474552
	T184	Kota Kinabalu, Malaysia	1979	7	AB474443	AB474476	AB474514	AB474553
D. atripex								
	B190	Chiang Mai, Thailand	1971	8	AB474444	NA	AB474515	AB474554
D. monieri								
	k-aac001	Moorea, the Society Islands	1987	9	AB474445	AB474477	AB474516	AB474555
D. ochrogaster								
	k-aad001	Noumea, New Caledonia	1992	9	NA	AB474478	AB474517	AB474556
D. phaeopleura								
	k-aaf001	Suva, Fiji	1966	10	AB474446	NA	AB474518	AB474557

1 of the gene homologous to *CG7785* of *D. melanogaster* was amplified by using primers CG7785.1F (–98 to –79; 5'-CTGCCTACGCACCTGTCTCA-3') and CG7785.1R (+214 to +195; 5'-GCGTTGTCGGAACACAAGCA-3). Intron 2 of the gene homologous to *ZnT63C* of *D. melanogaster* was amplified by using primers ZnT63C.2F (–65 to –46; 5'-GCTCTCGTCAACGCCGTCTT-3') and ZnT63C.2R (+61 to +42; 5'-ACCCAAGGCTCCGACGATCA-3'). PCR amplifications were successful for all samples of the *D. ananassae* species cluster, but not for all outgroup samples. In these cases, only species with successful amplification were included in the phylogenetic analyses.

Both strands of the PCR products were sequenced directly with the 3730xl DNA Analyzer (Applied Biosystems) using the amplification primers for PCR (and internal primers, if necessary) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Because each DNA sample was from a single male, we were not concerned about sequence polymorphism for X-linked loci. If inspection of the chromatograms indicated heterozygosity, as can occur in some autosomal sequences, PCR products were cloned into the M13 vector, and at least five clones were sequenced to obtain two sequences. We numbered these sequences (_1 and _2) after the name of the strain. The sequences were edited with Sequencher ver. 4.5 (Gene Codes Corporation).

In-situ hybridization

The cytological location of the genes was determined by in-situ hybridization of polytene chromosomes of *D. ananassae*, as previously described (Sawamura et al., 2006). PCR products labeled with digoxigenin-11-dUTP (PCR DIG Labeling Mix, Roche) were used as probes; the DIG Nucleic Acid Detection Kit (Roche) was used to detect hybridization. If in-situ hybridization was unsuccessful despite our having tried several PCR products covering different regions of the gene, we selected neighboring genes based on the *D. ananassae* genome annotation available from UCSC Genome Bioinformatics.

Sequence alignment and phylogenetic analysis

ClustalX 2.0 (Thompson et al., 1997) was used to align the sequences. We then carefully adjusted the alignments to reduce further the number of substitutions and insertions/deletions (indels) using Se-Al v2.0a11 (A. Rambaut; http://tree.bio.ed.ac.uk/software/seal/); indels were excluded from the analysis. A neighbor-joining (NJ) tree based on Kimura two-parameter distances was constructed for each locus by using MEGA4 (Tamura et al., 2007). Five hundred bootstrap replicates were analyzed to obtain support values for nodes. To incorporate the possibility of recombination in phylogenetic reconstruction, a haplotype network was also constructed by using the statistical parsimony method in TCS v1.21 (Clement et al., 2000; http://darwin.uvigo.es/software/tcs.html).

Population genetic analysis

The average number of pairwise differences between sequences within species (nucleotide diversity π) (Nei, 1987), the average number of pairwise differences between sequences between species (nucleotide divergence D_{XY}) (Nei, 1987), and the F_{ST} adapted for DNA sequence data (Hudson et al., 1992) were estimated by using the program SITES, developed by J. Hey (available at http://lifesci.rutgers.edu/~heylabSoftware.htm#SITES). The number of polymorphisms unique to *D. ananassae* or *D. pallidosa*, shared polymorphisms, and fixed differences between the species were calculated for each locus.

RESULTS

Localization of genes

Actn, *w*, and *CG7785* were localized by in-situ hybridization on cytological positions 10A (XL, the left arm of chromosome X), 17C (XR, the right arm of chromosome X), and 30B-C (2L, the left arm of chromosome 2), respectively (see also Schaeffer et al., 2008 for *w*). The location of *CG7785* was consistent with that of its neighboring gene, *DNaseII*, on 30B (Schaeffer et al., 2008). Although in-situ hybridization was unsuccessful for *ZnT63C*, neighboring genes *pst* (56A), *CG3634* (56A), and *CG12077* (55D-56A) were localized. Therefore, *ZnT63C* appears to be on 56A (2R, the right arm of chromosome 2).

Structure of introns

Intron 4 of *Actn*, intron 1 of *CG7785*, and intron 2 of *ZnT63C* had fewer indels, and thus their alignment was straightforward. In contrast, intron 3 of *w* varied in length among the strains (Fig. 1). First, four strains (NAN84, POM431, LAE119, and POM458) had a duplication, and two strains (NOU78 and T184) had a triplication, in the region corresponding to nucleotide positions 142–297 of AABBg1. It was difficult to determine a priori which segments of the repeats were homologous to each other, because a large number of sequence variations were accumulated among the segments. Therefore, we analyzed each repeated sequence separately and independently from the entire sequence outside the repeats; the repeated sequences were labeled by adding _R1 and _R2, or _R1A, _R1B and _R2 after the name of the strain (the R2 repeats in the duplication and the triplication had a high degree of sequence similarity; see below). Second, POM446 had an 837-bp insertion and WAU92 had an 863-bp insertion into the site between nucleotides 249 and 250 (249; 250) and 290; 291 of AABBg1, respectively. The insertions had a high degree of sequence similarity; many similar sequences exist in the AABBg1 genome, one of which is adjacent to *pseudo-COI* on chromosome 4 (Sawamura et al., 2008a). These transposon-like insertions were excluded from the analysis described below. Third, WAU61 had a 184-bp deletion and LAE360 had a 77-bp deletion in the regions corresponding to 75–258 and 17–93 of AABBg1, respectively.

Phylogeny of *Actn* introns

According to the NJ tree constructed for *Actn* intron 4 (Fig. 2A), no taxa except *D. parapallidosa* (shown in red) were monophyletic. Eleven *D. ananassae* sequences (black) formed a cluster, and this cluster also included a pallidosa-like Wau sequence (orange) other than the two *D. parapallidosa* sequences. Five *D. pallidosa* sequences (gray) formed a cluster, and three pallidosa-like sequences (green) also formed a cluster. Three pallidosa-like Wau sequences and four papuensis-like sequences (blue) were scattered on the tree, although two of the latter formed a local cluster.

The haplotype network constructed for *Actn* intron 4 (Fig. 2B) was slightly different from the NJ tree. *Drosophila ananassae* sequences were separated into three groups: (1) VAV161, TBU136, TNG, and AABBg1; (2) TBU209, WAU120, and NOU78; and (3) PPG183, WAU138, POM431, NAN84, and LAE119. Five *D. pallidosa* sequences formed a group, which was intermediate between groups 1 and 2 of *D. ananassae*. The separated *D. pallidosa* sequence (TBU155) was closer to group 3 of *D. ananassae*. Three pallidosa-like sequences formed a group, which was closer

to group 2 of *D. ananassae* and the major group of *D. pallidosa*. The separated pallidosa-like sequence (POM446) was closer to group 3 of *D. ananassae*. Three pallidosa-like Wau sequences were scattered on the network; among them, WAU92 and BLO79 were closer to groups 1 and 3 of *D. ananassae*, respectively. Three papuensis-like sequences formed a group, which was closer to group 1 of *D. ananassae* and the major group of *D. pallidosa*. The separated papuensis-like sequence (LAE360) was intermediate between groups 2 and 3 of *D. ananassae*. Two *D. parapallidosa* sequences were also intermediate between groups 2 and 3 of *D. ananassae*.

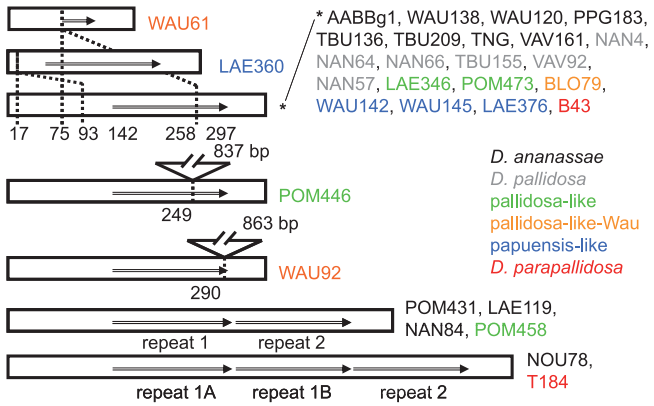

Fig. 1. Structure of intron 3 of the *w* gene. Nucleotide positions are based on the AABBg1 sequence reported in UCSC Genome Bioinformatics. Deletions are indicated by dotted lines, and insertions are indicated by open triangles over dotted lines (insertion sites). Arrows represent sequences repeated twice (repeats 1 and 2) or three times (repeats 1A, 1B, and 2).

Phylogeny of *w* introns (5' and 3' regions)

As was mentioned above, phylogenetic analyses were carried out separately for the 5' and 3' regions and the repeat region of *w* intron 3. According to the NJ tree of the 5' and 3' regions (Fig. 3A), most of the sequences belonged to a cluster, but the other cluster (WAU61 and LAE360) may have been an artifact caused by large deletions in the sequences. Within the major cluster, the tree was not very informative because of low branch resolution (bootstrap values < 50%). Among the recognized sub-clusters, two were pairs composed of different taxa: TNG of *D. ananassae* and NAN57 of *D. pallidosa*, and NOU78 of *D. ananassae* and BLO79 of pallidosa-like Wau. Only *D. parapallidosa* was monophyletic.

The haplotype network constructed for the 5' and 3' regions of *w* intron 3 (Fig. 3B) was also complicated, presumably because of recurrent recombination between haplotypes and/or multiple substitutions. Two sequences (WAU92 and LAE376) did not link to any other sequences, and two others (WAU61 and LAE360) were linked to each other but were isolated from the other sequences. *Drosophila ananassae* and *D. pallidosa* sequences were scattered on the network and intermingled with each other. Interestingly, TNG of *D. ananassae* and NAN57 of *D. pallidosa* shared a haplotype. Four pallidosa-like sequences were scattered on the network, although LAE346 and POM446 were close to each other. A pallidosa-like Wau sequence (BLO79) had only one substitution relative to NOU78 of *D. ananassae*. Two papuensis-like sequences were the same haplotype, and two *D. parapallidosa* sequences differed only by one substitution.

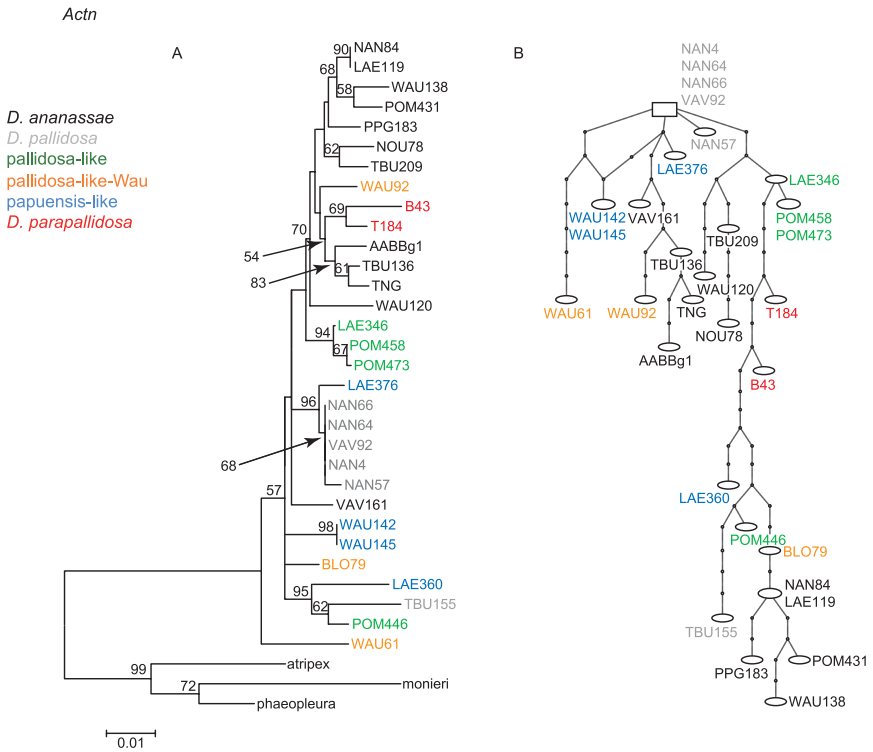

Fig. 2. Phylogeny of the *D. ananassae* species cluster based on intron 4 of the *Actn* gene. **(A)** NJ tree. Only bootstrap values > 50% are indicated. The scale bar at the lower left indicates branch length in substitutions per site. **(B)** Haplotype network. Ovals (or a rectangle) and small circles represent extant and hypothetical haplotypes, respectively. The haplotype with the highest outgroup probability is displayed as a rectangle.

Phylogeny of *w* introns (repeat region)

According to the NJ tree of the repeat region of *w* intron 3 (Fig. 4A), most of the sequences (and repeats) belonged to a cluster; the exception was WAU61, but this may have been an artifact caused by a large deletion in the sequence. Interestingly, the second repeat of sequences containing a duplication (POM431_R2, POM458_R2, LAE119_R2, and NAN84_R2) formed a cluster. Repeat 2 (the 3'-most repeat) of sequences with a triplication (NOU78_R2 and T184_R2) and sequence LAE360 formed a higher-order cluster that included the cluster mentioned above. This suggests that the 3'-most repeats have a common ancestry that differs from that of the sequences with a single repeat, of the first repeat of

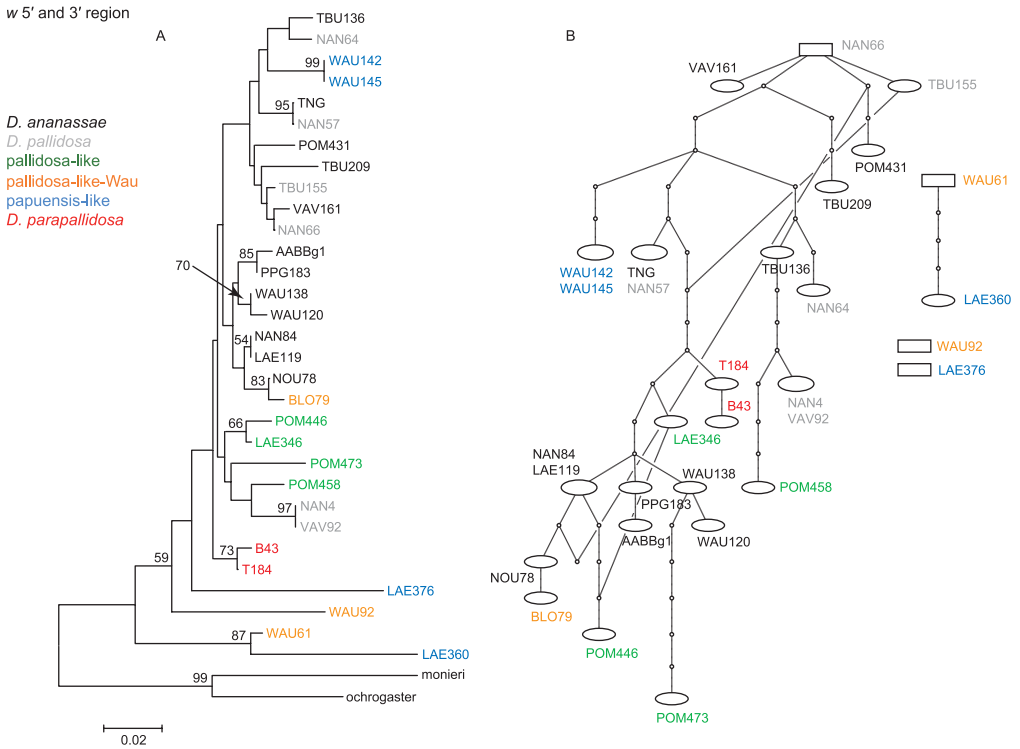

Fig. 3. Phylogeny of the *D. ananassae* species cluster based on intron 3 (5' and 3' regions) of the *w* gene. **(A)** NJ tree. **(B)** Haplotype network. See the legend to Fig. 2 for general explanations.

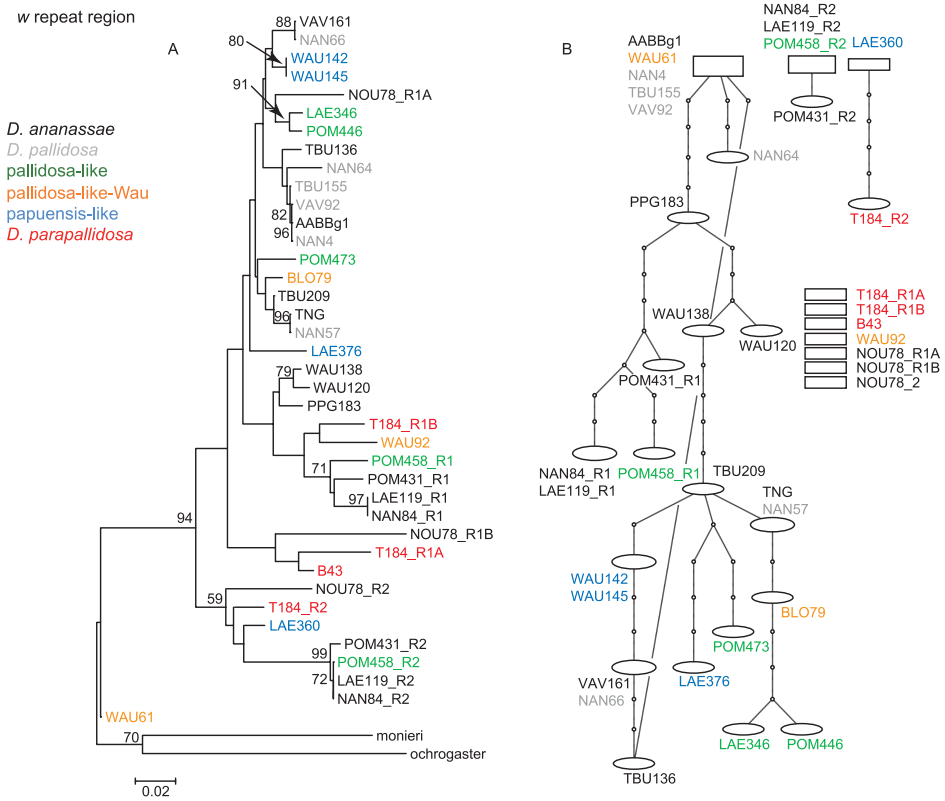

Fig. 4. Phylogeny of the *D. ananassae* species cluster based on intron 3 (repeat region) of the *w* gene. **(A)** NJ tree. **(B)** Haplotype network. See the legend to Fig. 2 for general explanations and the legend to Fig. 1 for repeat nomenclature.

sequences containing a duplication (four R1s), and of the first and second repeats of sequences containing a triplication (two R1A–R1Bs). It is also interesting that the first repeat of sequences containing a duplication (POM431_R1, LAE119_R1, and NAN84_R1 of *D. ananassae* and POM458_R1 of pallidosa-like) formed a cluster. This suggests that after the duplication event, gene flow took place recently between *D. ananassae* and pallidosa-like. The tree was not informative for the other sequences because of low branch resolution (< 50%). Among the recognized subclusters, some included sequences from different taxa: VAV161 of *D. ananassae* and NAN66 of *D. pallidosa*; AABBg1 of *D. ananassae* and TBU155, VAV92, and NAN4 of *D. pallidosa*; and TNG of *D. ananassae* and NAN57 of *D. pallidosa*.

The haplotype network constructed for the repeat region of *w* intron 3 (Fig. 4B) was also complicated, presumably because of recurrent recombination between haplotypes, multiple substitutions, and/or conversions between repeats. Several sequences (and repeats) did not link to the main network, although some had the same sequence or were linked to each other. *Drosophila ananassae* and *D. pallidosa* sequences were scattered on the network, although there were some local clusters. Further, the *D. ananassae* and *D. pallidosa* sequences were intermingled, and the two species shared some haplotypes: AABBg1 with NAN4 and VAV92 (TBU155 has a deletion not incorporated in the figure), VAV161 with NAN66, and TNG with NAN57. WAU61 of pallidosa-like Wau appeared to share a haplotype with *D. ananassae* and *D. pallidosa*, but this could have been an artifact caused by a large

deletion in WAU61. Pallidosa-like and papuensis-like sequences were also scattered on the network, although there were some local clusters.

Phylogeny of *CG7785* introns

According to the NJ tree constructed for *CG7785* intron 1 (Fig. 5A), *D. pallidosa* and *D. parapallidosa* were each monophyletic. All *D. ananassae* sequences were included in a cluster that also contained a pallidosa-like, two pallidosa-like Wau, and four papuensis-like sequences. Thus, *D. ananassae* sequences and *D. pallidosa* sequences were clearly separated in different clusters. Four pallidosa-like, four pallidosa-like Wau, and six papuensis-like sequences were scattered on the tree, although some of them formed local clusters. BLO79_2 of pallidosa-like Wau clustered with five *D. ananassae* sequences, WAU145_1 and WAU142_2 of papuensis-like clustered with four *D. ananassae* sequences, and BLO79_1 of pallidosa-like Wau clustered with three *D. ananassae* sequences.

The haplotype network constructed for *CG7785* intron 1 (Fig. 5B) was slightly different from the NJ tree. Two sequences (LAE360 and LAE376) did not link to any other sequences, and two (WAU61 and WAU92) were linked to each other but were isolated from the other sequences. All *D. ananassae* sequences formed a group, each separated by at most two substitutions. *Drosophila pallidosa* sequences also formed a group, each separated by two substitutions. In contrast, *D. ananassae* sequences and *D. pallidosa* sequences were separated by at least seven substitutions. Four pallidosa-like sequences were scattered on the network; among them LAE346 was closer to *D. ananassae*. Pallidosa-like Wau shared two haplotypes with *D. ananassae* sequences. Papuensis-like also shared one haplotype with a *D.*

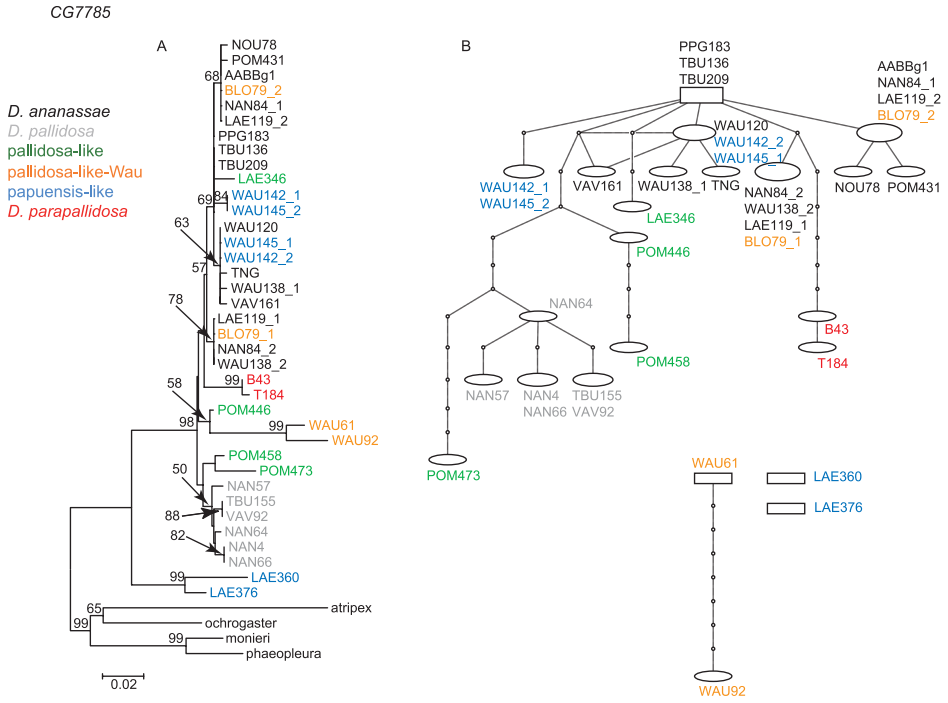

Fig. 5. Phylogeny of the *D. ananassae* species cluster based on intron 1 of the *CG7785* gene. **(A)** NJ tree. **(B)** Haplotype network. See the legend to Fig. 2 for general explanations.

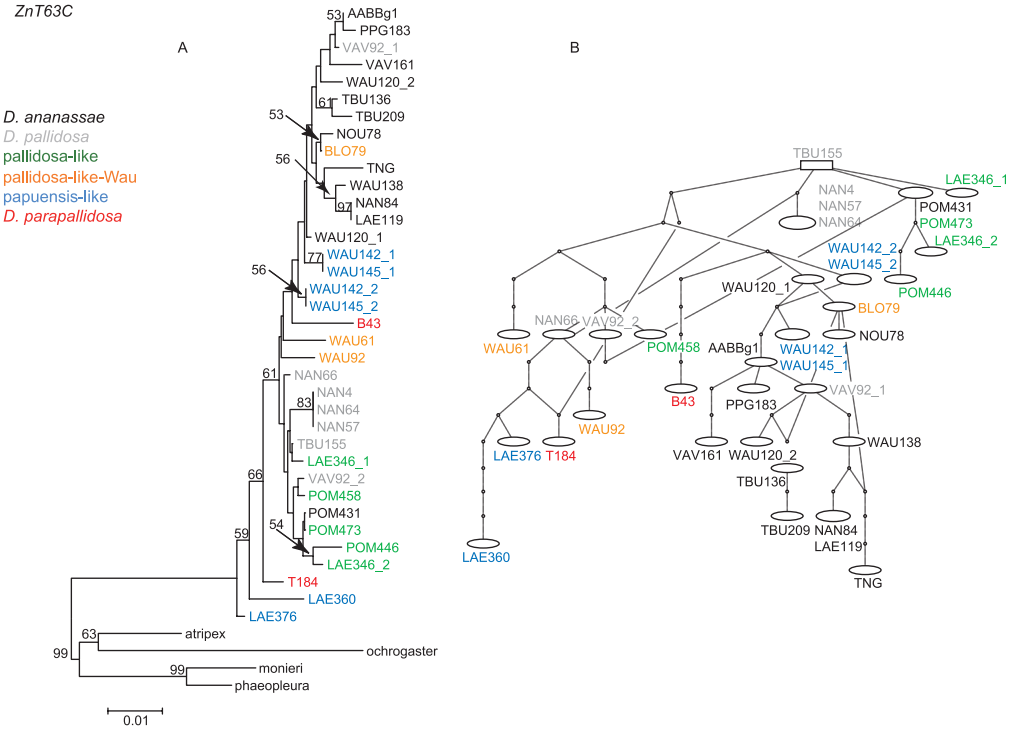

Fig. 6. Phylogeny of the *D. ananassae* species cluster based on intron 2 of the *ZnT63C* gene. **(A)** NJ tree. **(B)** Haplotype network. See the legend to Fig. 2 for general explanations.

ananassae sequence, and another haplotype (WAU142_1 and WAU145_2) differed from a *D. ananassae* sequence by two substitutions. Two *D. parapallidosa* sequences linked to each other by one substitution and were one branch point away from a *D. ananassae* haplotype.

Phylogeny of *ZnT63C* introns

According to the NJ tree constructed for *ZnT63C* intron 2 (Fig. 6A), most of the sequences belonged to a cluster; the exceptions were LAE376 and LAE360 of papuensis-like and T184 of *D. parapallidosa*, which were basal to the cluster. Within the cluster, however, the tree was not very informative because of low branch resolution (bootstrap value < 50%). Among the sub-clusters recognized, one was a pair of different taxa, NOU78 of *D. ananassae* and BLO79 of pallidosa-like Wau.

The haplotype network constructed for *ZnT63C* intron 2 (Fig. 6B) was also complicated, presumably because of recurrent recombination between haplotypes and/or multiple substitutions. Twelve *D. ananassae* sequences formed a group, but POM431 was separated from the other *D. ananassae* sequences. Six *D. pallidosa* sequences formed a group, but VAV92_1 was separated from the other *D. pallidosa* sequences. The exceptional *D. ananassae* sequence (POM431) was close to the major group of *D. pallidosa*; there was only one substitution between TBU155 and POM431. In contrast, the exceptional *D. pallidosa* sequence (VAV92_1) was in the center of the major *D. ananassae* group. Most of the pallidosa-like sequences formed a group, although POM458 was separated from the others by at least four substitutions. This group also included the exceptional *D. ananassae* sequence (POM431, the same haplotype as POM473) and the major *D. pallidosa* group. Three pallidosa-like Wau sequences were scattered on the network; among them BLO79 was included in the major *D. ananassae* group. Papuensis-like sequences were separated into two or three groups; the major group included four sequences (WAU142_1, WAU145_1, WAU142_2, and WAU145_2) belonging to two haplotypes that were in the major *D. ananassae* group or intermediate between the major *D. ananassae* and major *D. pallidosa* groups. The two *D. parapallidosa* sequences were separated; B43 was closer to the major *D. ananassae* group, whereas T184 was closer to the major *D. pallidosa* group.

Population genetic analyses of the four loci

Intronic sequences of *Actn*, *w*, *CG7785*, and *ZnT63C* were analyzed for nucleotide diversity (π) within each taxon and the average number of pairwise differences (D_{XY}) for each pair of taxa. At the *w* locus, because it was difficult to determine which segments of the repeats were homologous to one another, sequences with duplication (NAN84, POM431, LAE119, and POM458) or triplication (NOU78 and T184) were excluded from the analysis; sequences with large deletions (WAU61 and LAE360) were also excluded.

Nucleotide diversity (Table 2) was generally larger in pallidosa-like Wau and papuensis-like than in the other taxa,

Table 2. Nucleotide diversity (π/bp) within each taxon for each locus. At the *w* locus, NAN84, POM431, LAE119, POM458, NOU78, T184, WAU61, and LAE360 were excluded from the analysis (see text for details). The number of sequences included in each analysis is indicated in parentheses. ND, not determined.

Taxon	Locus			
	Actn	*w*	*CG7785*	*ZnT63C*
D. ananassae	0.01942 (12)	0.03831 (8)	0.00743 (15)	0.01210 (13)
D. pallidosa	0.01044 (6)	0.02753 (6)	0.00821 (6)	0.00800 (7)
pallidosa-like	0.01523 (4)	0.03090 (3)	0.02939 (4)	0.00739 (5)
pallidosa-like-Wau	0.02964 (3)	0.08333 (2)	0.04824 (4)	0.01421 (3)
papuensis-like	0.01795 (4)	0.05323 (3)	0.05008 (6)	0.01201 (6)
D. parapallidosa	0.01553 (2)	ND (1)	0.00329 (2)	0.02278 (2)

Table 3. Average number of pairwise differences (D_{XY}) for each pair of taxa for each locus. D_{XY} divided by the larger and smaller π is indicated in parentheses. π was not calculated for the *w* locus in *D. parapallidosa* (see Table 2).

Compared taxa	Locus			
	Actn	*w*	*CG7785*	*ZnT63C*
D. ananassae vs. *D. pallidosa*	0.0254	0.03584	0.0233	0.0146
	(1.3–2.4)	(0.9–1.3)	(2.8–3.1)	(1.2–1.8)
D. ananassae vs. pallidosa-like	0.02337	0.04322	0.02402	0.01738
	(1.2–1.5)	(1.1–1.4)	(0.8–3.2)	(1.4–2.4)
D. ananassae vs. pallidosa-like-Wau	0.02551	0.06068	0.03486	0.01496
	(0.9–1.3)	(0.7–1.6)	(0.7–4.7)	(1.1–1.2)
D. ananassae vs. papuensis-like	0.02899	0.05087	0.03243	0.01524
	(1.5–1.6)	(1.0–1.3)	(0.6–4.4)	(1.3–1.3)
D. ananassae vs. *D. parapallidosa*	0.02449	0.05022	0.02786	0.0193
	(1.3–1.6)	(1.3 ~)	(3.7–8.5)	(0.8–1.6)
D. pallidosa vs. pallidosa-like	0.01646	0.03956	0.02676	0.00883
	(1.1–1.6)	(1.3–1.4)	(0.9–3.3)	(1.1–1.2)
D. pallidosa vs. pallidosa-like-Wau	0.02237	0.06316	0.04745	0.0117
	(0.8–2.1)	(0.8–2.3)	(1.0–5.8)	(0.8–1.5)
D. pallidosa vs. papuensis-like	0.01853	0.04759	0.04577	0.0148
	(1.0–1.8)	(0.9–1.7)	(0.9–5.6)	(1.2–1.9)
D. pallidosa vs. *D. parapallidosa*	0.03135	0.0484	0.03032	0.01672
	(2.0–3.0)	(1.8 ~)	(3.7–9.2)	(0.7–2.1)
pallidosa-like vs. pallidosa-like-Wau	0.0249	0.06013	0.04489	0.01382
	(0.8–1.6)	(0.7–1.9)	(0.9–1.5)	(1.0–1.9)
pallidosa-like vs. papuensis-like	0.02483	0.05346	0.0446	0.01716
	(1.4–1.6)	(1.0–1.7)	(0.9–1.5)	(1.4–2.3)
pallidosa-like vs. *D. parapallidosa*	0.02481	0.05556	0.03833	0.01787
	(1.6–1.6)	(1.8 ~)	(1.3–12)	(0.8–2.4)
pallidosa-like-Wau vs. papuensis-like	0.02775	0.073	0.05888	0.01335
	(0.9–1.5)	(0.9–1.4)	(1.2–1.2)	(0.9–1.1)
pallidosa-like-Wau vs. *D. parapallidosa*	0.03108	0.06831	0.05155	0.01584
	(1.0–2.0)	(0.8 ~)	(1.0–16)	(0.7–1.3)
papuensis-like vs. *D. parapallidosa*	0.03226	0.06322	0.05051	0.01703
	(1.8–2.1)	(1.2 ~)	(1.0–15)	(0.7–1.4)

Table 4. Population genetic analysis between *D. ananassae* and *D. pallidosa*. F_{ST} represents the degree of genetic differentiation between species (Hudson et al., 1992).

	Locus			
	Actn	*w*	*CG7785*	*ZnT63C*
Average sequence length analyzed (bp)	312	343	296	441
Number of *D. ananassae* sequences	12	8	15	13
Number of *D. pallidosa* sequences	6	6	6	7
Unique polymorphisms in *D. ananassae*	18	15	9	11
Unique polymorphisms in *D. pallidosa*	6	3	6	2
Shared polymorphisms	4	17	0	8
Fixed differences	0	0	4	0
F_{ST}	0.402	0.083	0.663	0.310

although it was also large in *D. ananassae* at *Actn* and *ZnT63C*. (Nucleotide diversity was exceptionally large in *D. parapallidosa* at *ZnT63C*, and this result was omitted from the description below.) This suggests that pallidosa-like Wau and papuensis-like (and maybe also *D. ananassae*) are quite heterogeneous, presumably due to the large population size, gene flow from other taxa, or a higher mutation rate. On the other hand, nucleotide diversity was relatively small in *D. pallidosa*, indicating that this species is relatively homogeneous. In each taxon, nucleotide diversity was largest at *w* and generally smallest at *ZnT63C*, although in *D. ananassae* it was smallest at *CG7785*. This suggests that the evolutionary history differs among loci. The *w* locus has become heterogeneous, whereas the *ZnT63C* locus has become homogeneous within each taxon, although this phenomenon might reflect differences in mutation rate.

Pairwise divergence between taxa (Table 3) was generally comparable to or slightly larger than nucleotide diversity within each taxon; the ratio of divergence to diversity (D_{XY} divided by larger or smaller π) was ~1, as shown in parentheses in the table. This suggests that the *D. ananassae* species cluster has not had enough time for clear separation of taxa; alternatively, the results may reflect gene flow between taxa. There was a weak trend that the ratio was larger at *CG7785* than at the other loci. This may mean that only this locus has been unaffected by gene flow, but the small diversity of *D. parapallidosa* at this locus must also have affected the result. In any case, the present test does not seem sensitive enough to determine the presence of gene flow.

As the sample sizes of *D. ananassae* and *D. pallidosa* were relatively large, the actual numbers and kinds of polymorphic sites in these species are indicated in Table 4. There were 17 shared polymorphic sites at the *w* locus, which might be a consequence of gene flow or incomplete lineage sorting. *ZnT63C* and *Actn* also exhibited shared polymorphisms (eight and four sites, respectively), and thus the possibility of gene flow or incomplete lineage sorting cannot be excluded. There were no fixed differences at these three loci (*Actn*, *w*, and *ZnT63C*). In contrast, *CG7785* had no shared polymorphisms but did have four fixed differences. This strongly suggests that *CG7785* has not been exchanged between *D. ananassae* and *D. pallidosa*. The observed small F_{ST} appears to represent gene flow or lineage sorting. F_{ST} was smallest at *w*, suggesting that gene flow had occurred; F_{ST} was greatest for *CG7785*, suggesting that gene flow had been restricted.

DISCUSSION

Among the *D. ananassae* species cluster, *D. ananassae* and *D. pallidosa* are sympatric in the South Pacific; *D. ananassae*, pallidosa-like, pallidosa-like Wau, and papuensis-like are sympatric in Papua New Guinea (and partly in northern Australia); and *D. ananassae* and *D. parapallidosa* are sympatric in southeast Asia (Futch, 1966; Lemeunier et al., 1986; Tobari, 1993; Sawamura et al., 2008a; Matsuda et al., 2009). Our interest here is whether sympatric populations of different taxa share sequences at any loci. This will be discussed below for each combination of taxa.

Drosophila ananassae versus *D. pallidosa*

The evolutionary relationship between *D. ananassae* and *D. pallidosa* was incongruent among the four loci examined. *Drosophila ananassae* and *D. pallidosa* sequences for *CG7785* were clearly separated (Fig. 5; Table 4), suggesting that no gene flow had occurred at this locus. In contrast, *w* sequences were not clearly separated into two groups (Figs. 3, 4; Table 4). Furthermore, the species shared haplotypes: TNG of *D. ananassae* and NAN57 of *D. pallidosa* had the same sequences, and VAV161 of *D. ananassae* and NAN66 of *D. pallidosa* differed by only one substitution. These could be the result of recent gene flow. The results for *Actn* and *ZnT63C* were intermediate between those for *CG7785* and *w* (Figs. 2, 6; Table 4). *Drosophila ananassae* and *D. pallidosa Actn* sequences would have been clearly separated if TBU155 of *D. pallidosa* and VAV161 of *D. ananassae* were omitted. (Although *D. ananassae* sequences were separated into three groups in the haplotype network [Fig. 2B], this might simply reflect a large population size.) Furthermore, the two species' sequences for *ZnT63C* would have been clearly separated if POM431 of *D. ananassae* and VAV92_1 of *D. pallidosa* were omitted. Such exceptional sequences might be a consequence of incomplete lineage sorting or gene flow.

Cryptic species in Papua New Guinea

None of the three cryptic species (pallidosa-like, pallidosa-like Wau, and papuensis-like) was monophyletic at any of the loci examined here, although some of the sequences formed local clusters. Among them, pallidosa-like sequences exhibited the lowest diversity (Table 2). Furthermore, pallidosa-like sequences of *Actn* would have been monophyletic if POM446 was omitted (Fig. 2). POM446 might have resulted from ancient gene flow from different taxa or may represent incomplete lineage sorting. Interestingly, pallidosa-like shared a haplotype of *ZnT63C* with *D. ananassae* (POM473 and POM431) (Fig. 6), which strongly suggests that recent gene flow occurred from pallidosa-like to *D. ananassae* at Port Moresby, Papua New Guinea. It is also interesting that a pallidosa-like sequence for *w* (POM458) shared the same repeat structure with three *D. ananassae* sequences (Fig. 1), and that within the repeats themselves there was a high degree of similarity, although the degree of similarity among the sequences was low out-

side this region (Figs. 3, 4). This might have been the result of recombination after ancient gene flow from *D. ananassae* to pallidosa-like.

Among the pallidosa-like Wau strains, BLO79 was unique. At *CG7785*, BLO79_1 and BLO79_2 each shared the haplotype with three sequences of *D. ananassae* (Fig. 5). Also, at *ZnT63C*, BLO79 had only one substitution compared with NOU78 of *D. ananassae* (Fig. 6). These findings strongly suggest that BLO79 resulted from recent gene flow from *D. ananassae*. In fact, variations in BLO79 correlate with certain sexual behaviors (H. Yamada, Y. Oguma, M. Matsuda, Y. N. Tobari, unpublished observations). Among the papuensis-like strains, WAU142 and WAU145 were exceptional. At *CG7785*, WAU142_2 and WAU145_1 had the same haplotype as one of *D. ananassae*, and WAU142_1 and WAU145_2 had only two substitutions compared with a haplotype of *D. ananassae* (Fig. 5). At *ZnT63C*, WAU142_1, WAU142_2, WAU145_1, and WAU145_2 had only two substitutions compared with WAU120_1 of *D. ananassae* (Fig. 6). These results suggest that WAU142 and WAU145 resulted from recent gene flow from *D. ananassae*.

Drosophila ananassae versus *D. parapallidosa*

Our study suggests that there was no effective gene flow between *D. ananassae* and *D. parapallidosa*. In fact, hybrid males are generally sterile when *D. ananassae* females are crossed with *D. parapallidosa* males (Matsuda et al., 2009). Monophyly of *D. parapallidosa* was supported, although only two strains were examined, by the analyses of *Actn*, *w*, and *CG7785*, but not by *ZnT63C* (Figs. 2–6). The position of *D. parapallidosa*, with respect to whether it was derived from a local population of one taxon (e.g., *D. ananassae*) or is basal to the other taxa, could not be definitively determined.

Potential gene flow and evolutionary implications

Some shared or very closely related sequences between different taxa suggest the occurrence of gene flow. Thus, recent gene flow was suggested (1) at *w* between *D. ananassae* and *D. pallidosa*; (2) at *CG7785* from *D. ananassae* to pallidosa-like Wau and papuensis-like; and (3) at *ZnT63C* from *D. ananassae* to *D. pallidosa*, pallidosa-like Wau, and papuensis-like, and from pallidosa-like to *D. ananassae*. Gene flow was previously suggested to have occurred for chromosomes 4 and Y and between mitochondrial genomes (Sawamura et al., 2008a; Schug et al., 2008; Matsuda et al., 2009), although ruling out the possibility of incomplete lineage sorting is difficult. Certain coalescent-based models, such as the "isolation with migration" model (Hey and Nielsen, 2004), may be helpful in estimating gene flow between species, but this model was difficult to apply to our dataset because of the large number of apparent intra-locus recombinations, which the model does not assume. The conclusion we draw from our results is that gene flow may not have been restricted to a small portion of the genome in the *D. ananassae* species cluster.

Inversions are highly polymorphic in this species cluster (Futch, 1966; Tomimura et al., 1993; Matsuda et al., 2009), and this could have affected the evolutionary relationships for each locus if sympatric populations of different taxa had exchanged genes via hybridization; gene flow may have been efficiently restricted only in the inverted regions. Cytology indicates that *Actn* (10A) is within (and close to the proximal breakpoint of) *In(XL)A* [4A; 10D], which is specific to *D. pallidosa*, pallidosa-like, pallidosa-like Wau, and papuensis-like (the breakpoints of inversions hereafter are from Tobari et al. [1993]). Our data do not suggest that recent gene flow occurred at this locus, although ancient gene flow cannot be ruled out. XR, where *w* (17C) is located, is a region nearly free of inversions; only papuensis-like was polymorphic for an inversion in XR. Thus, it is not surprising that *w* sequences were exchanged between *D. ananassae* and *D. pallidosa*, which accounted for the observed small F_{ST}. *CG7785* (30B-C) is within (and close to the proximal breakpoint of) *In(2L)D* [26C; 30D], which is specific to *D. pallidosa* and pallidosa-like (*In(2L)D* is always on *In(2L)C* [22A; 28A]). Thus, we suspect that gene flow between *D. ananassae* and *D. pallidosa* was strongly inhibited at this locus. Because *D. ananassae* as well as pallidosa-like Wau, papuensis-like, and *D. parapallidosa* do not usually have *In(2L)D*, there is a chance that *CG7785* introgression occurred from *D. ananassae* to these taxa. *ZnT63C* (56A) is within (and close to the proximal breakpoint of) *In(2R)A* [55B; 62C], which is specific to taxa other than *D. ananassae*. Double cross-overs or gene conversion must have taken place between *D. ananassae* and the other taxa (*D. pallidosa*, pallidosa-like, and pallidosa-like Wau).

In conclusion, our findings partly support the chromosomal speciation hypothesis (Navarro and Barton, 2003; Butlin, 2005) at the DNA sequence level in the *D. ananassae* species cluster, as has been reported for the species pair *D. pseudoobscura* and *D. persimilis* (Machado et al., 2002, 2007; Noor et al., 2007; Kulathinal et al., 2009). This corroborates our previous conclusion at the level of gene function: genes related to postmating and premating isolation have been mapped in or near species-specific inversions in this species cluster (Sawamura et al., 2006, 2008b). Such a case has been documented as well in the species pair mentioned above (Noor et al., 2001). Loci closely linked to genes involved in reproductive isolation are needed to construct more reliable phylogenetic trees, as has been accomplished for the *D. simulans* clade (Ting et al., 2000).

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