MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2011) doi: 10.1111/j.1755-0998.2011.03088.x

PERMANENT GENETIC RESOURCES NOTE

Permanent Genetic Resources added to Molecular Ecology Resources Database 1 August 2011–30 September 2011

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Abstract

This article documents the addition of 299 microsatellite marker loci and nine pairs of single-nucleotide polymorphism (SNP) EPIC primers to the Molecular Ecology Resources (MER) Database. Loci were developed for the following species: *Alosa pseudoharengus, Alosa aestivalis, Aphis spiraecola, Argopecten purpuratus, Coreoleuciscus splendidus, Garra gotyla, Hippodamia convergens, Linnaea borealis, Menippe mercenaria, Menippe adina, Parus major, Pinus densiflora, Portunus trituberculatus, Procontarinia mangiferae, Rhynchophorus ferrugineus, Schizothorax richardsonii, Scophthalmus rhombus, Tetraponera aethiops, Thaumetopoea pityocampa, Tuta absoluta* and *Ugni molinae*. These loci were cross-tested on the following species: *Barilius bendelisis, Chiromantes haematocheir, Eriocheir sinensis, Eucalyptus camaldulensis, Eucalyptus cladocalix, Eucalyptus globulus, Garra litaninsis vishwanath, Garra para lissorhynchus, Guindilla trinervis, Hemigrapsus sanguineus, Luma chequen. Guayaba, Myrceugenia colchagüensis, Myrceugenia correifolia, Myrceugenia exsucca, Parasesarma plicatum, Parus major, Portunus pelagicus, Psidium guayaba, Schizothorax richardsonii, Scophthalmus maximus, Tetraponera latifrons, Thaumetopoea bonjeani, Thaumetopoea ispartensis, Thaumetopoea libanotica, Thaumetopoea pinivora, Thaumetopoea pityocampa ena clade, Thaumetopoea solitaria, Thaumetopoea wilkinsoni* and *Tor putitora*. This article also documents the addition of nine EPIC primer pairs for *Euphaea decorata, Euphaea formosa, Euphaea ornata* and *Euphaea yayeyamana*.

This article documents the addition of 299 microsatellite marker loci and nine pairs of single-nucleotide polymorphism (SNP) genotyping primers to the Molecular Ecology Resources Database. Table 1 contains information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column. Table 2 presents information on SNP genotyping resources added to the MER database and presents data on the focal species, the number of sequencing primer pairs, the observed number of SNPs, other species the loci were tested in, and the number of allele specific primers or probes. The MER database and GenBank accession numbers and the authors responsible are also listed. Table 3 outlines additional permanent genetic resources that have been uploaded to the MER program wiki (http://tomato.biol.trinity.edu/programs/). A full description of the development protocol for the loci presented in Tables 1 & 2 can be found on the Molecular Ecology Resources Database (http://tomato.biol.trinity.edu/).

Table 1 Information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources (MER) Database and GenBank. The authors responsible for each set of loci are listed in the final column

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Alosa pseudoharengus, Alosa aestivalis	18	n/a	47166–47201	JN383992–JN384009	Labbe, Ellen M.; Argo, Emily E.; Schultz, Thomas F.; Palkovacs, Eric P.;Willis, Theodore V.

Correspondence: Molecular Ecology Resources Primer Development Consortium, E-mail: editorial.office@molecol.com

Table 1 (Continued)

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Aphis spiraecola	9	n/a	47081–47089	HM854169–HM854171, JN214382–JN214384, JN214386–JN214388	Mezghani-Khemakhem, M.; Kharrat, I.; Casse, N.; Bouktila, D.; Makni, M.; Makni H.
Argopecten purpuratus	8	n/a	47373–47380	JN674552–JN674559	Haye, P. A.; Segovia, N. I.; Gallardo-Escárate, C.
Coreoleuciscus splendidus	13	n/a	47143–47155	JF972368–JF972380	Kwan, Ye-Seul; Lee, Wan-Ok; Won, Yong-Jin
Garra gotyla	28	*G. para lissorhynchus*, *G. litaninsis vishwanath*, *Barilius bendelisis*, *Schizothorax richardsonii*, *Tor putitora*	47345–47372	HQ288484, HQ288485, HQ288489–HQ288499, HQ288501, HQ288502, HQ288504, HQ288506, HQ288507, HQ288510, HQ288511, HQ288517, HQ288526, HQ288661, JF268657, JF268662, JF268664, JF268665	Matura, Rakesh; Chandra, Suresh; Barat, Ashoktaru; Pande, Veena; Mahanta, Prabin Chandra
Hippodamia convergens	12	n/a	47397–47408	JN565049–JN565060	Michel, Andy P.; Zhang, W.; Gardiner, Mary M.
Linnaea borealis	10	n/a	47156–47165	JN674504–JN674512	A'Hara, S. W.; Scobie, A. R.; Broome, A.; Long, D.; Cottrell, J. E.
Menippe mercenaria, *M. adina*	22	n/a	46925–46968	GU970048–GU970069	Seyoum, Seifu; Bert, Theresa M.; Puchulutegui, Cecilia; Davis, Michelle C.; Muriel-Cunha, Janice; Crawford, Charles R.; Mcmillen-Jackson, Anne L.; Barbieri, Luiz
Parus major	15	n/a	47128–47142	HQ263118–HQ263132	Saladin, Verena; Richner, Heinz
Pinus densiflora	16	n/a	47381–47396	JN634766–JN634781	Lee, Kyung Mi; Kim, Yong Yul; Kim, Ki Hwan; Jeon, Ji Hyun; Cho, Kyung Jin
Portunus trituberculatus	11	*P. pelagicus*, *Eriocheir sinensis*, *Hemigrapsus sanguineus*, *Chiromantes haematocheir*, *Parasesarma plicatum*	46914–46924	JF505633–JF505643	Li, H.; Ye, N. H.; Liu, Y. G.; Zhang, Y. X.; Liu, S. S.

Table 1 (Continued)

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Procontarinia mangiferae	11	n/a	47057–47067	JF746879–JF746889	Amouroux, P.; Normand, F.; Nibouche, S.; Delatte H.
Rhynchophorus ferrugineus	15	n/a	47113–47127	JN374673–JN374687	Capdevielle-Dulac, C.; El-Mergawy, R. A. A. M.; Avand-Faghih, A.; Rochat, D.; Silvain, J.-F.
Schizothorax richardsonii	34	n/a	47292–47325	HM591233–HM591236, HM591238, HM591240–HM591242, HM591244, HM591246–HM591256, HM591258, HM591260, HM591264–HM591266, HM591270–HM591272, HM591276, HM591278, HM591279, HM591281, HM591283	Barat, Ashoktaru; Chandra, Suresh; Matura, Rakesh
Scophthalmus rhombus	15	*S. maximus*	47090–47104	JF900344–JF900358	Vandamme, S. G.; Maes, G. E.; Van Houdt, J. K. J.; Hellemans, B.; Robbens, J.; Parmentier, K.; Volckaert, F. A. M.
Tetraponera aethiops	14	*T. latifrons*	46982–47009	JN190035–JN190048	Piatscheck, F.; Djieto-Lordon, C.; Garcia, M.; Sauve, M.; Peccoud, J.; Dubois, M. P.; McKey, D.; Blatrix, R.
Thaumetopoea pityocampa	13	*T. p. ena clade, T. wilkinsoni, T. pinivora, T. libanotica, T. bonjeani, T. ispartensis, T. solitaria*	46969–46981	JN400258–JN400270	Burban, C.; Magnoux, E.; Rousselet, J.; Kerdelhué, C.
Tuta absoluta	19	n/a	47326–47344	JN680765–JN680783	Guillemaud, Thomas; Legoff, Isabelle; Blin, Aurélie; Tabone, Elisabeth; Desneux, Nicolas; Malausa, Thibaut

Table 1 (Continued)

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Ugni molinae	16	*Myrceugenia correifolia*, *M. colchagüensis*, *M. exsucca*, *Guindilla trinervis*, *Luma chequen*. *Guayaba*, *Psidium guayaba*, *Eucalyptus cladocalix*, *E. camaldulensis*, *E. globulus*	46809–46824	HQ917086–HQ917101	Ramos, R.; Ravest, G.; Méndez, M.A.; Hinrichsen, P.

Table 2 Information on the focal species, the sequencing primer pairs developed, the number of single-nucleotide polymorphisms (SNPs) observed and any other species the loci were tested in. The next columns contain the number of allele specific primers and probes developed and the Molecular Ecology Resources (MER) database and GenBank accession numbers, respectively. The authors responsible for each set of loci are listed in the final column

Species	No. primer pairs	No. SNPs in sequence	Other species tested	No. Allele specific primers/ probe	Target gene(s)	MER database numbers	Genbank Accession no.	Authors
Euphaea formosa, *E. yayeyamana*, *E. ornata*, *E. decorata*	9	See Table 2 in text for details.	n/a	n/a	See Table 1 in text for details.	47048–47056	JN246927–JN247002, JN389796–JN390424	Lee, Yat-Hung; Lin, Chung-Ping

Table 3 Information on other resources recently uploaded to the Molecular Ecology Resources program wiki (http://tomato.biol.trinity.edu/programs/). The authors are listed in the final column

Species	Category	Type of resource	Authors
Oncorhynchus tshawytscha	Technique	Microsatellite allele ladder-based standardization	LaHood, Eric; Schlei, Ora; Wenburg, John; Moran, Paul

Primers for amplification of nuclear introns in four East Asian black-banded gossamer-wing *Euphaea* damselflies

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Running title: EPIC primers for *Euphaea* damselflies

Abstract

This study describes the development and testing of exon-primed, intron-crossing (EPIC) degenerate primers for amplifying nuclear introns in four closely related *Euphaea* damselflies. Primers were developed by comparing sequence variations from a few model insects and the orthologous sequences of *E. formosa* and *E. yayeyamana* obtained from 454 pyrosequencing. Most of the amplified regions were highly variable and are being used to study the gene flow, divergence time and effective population sizes in sibling *Euphaea* species.

The gossamer-wing damselfly genus, *Euphaea* Selys 1840 (Odonata, Euphaeidae), is comprised of approximately 30 species occurring predominantly in lower to middle elevation forest streams of tropical and subtropical Asia (Orr & Hämäläinen 2003; Schorr & Paulson 2009). *Euphaea* damselflies are territorial because males exhibit aggressive behavior towards intruding con-specifics and females appear periodically inside these territories and mate with territory owners (Thompson 1998; Huang & Lin 2011). The four closely related East Asian *Euphaea* species, *E. formosa*, *E. yayeyamana*, *E. ornata* and *E. decorata* are characterized by distinctive black bands towards the tip of male hind wings, and are each endemic to Taiwan, Ishigaki and Iriomote of Japan, Hainan island of China, and continental Southeast Asia, respectively (Schorr & Paulson 2009). These damselflies provide an excellent "non-model" system to investigate the geographic modes of speciation (for example allopatric versus parapatric models) in natural populations residing in the Asian tropics, because they are each endemic to currently discontinuous geographic regions. For this reason, the evolutionary diversification of these damselflies is considered to be allopatric in origin (Hayashi 1990; Huang & Lin 2011). However, testing the validity of a strict allopatric speciation model in natural populations often requires utilization of a sufficient number of informative and unlinked loci under a theoretical framework of coalescence (Pinho & Hey 2010). For *Euphaea* damselflies, the available primers for population genetic analysis are limited to linked mitochondrial genes (Turgeon & McPeek 2002; Hayashi *et al*. 2005; Lin *et al*. 2010) and a few nuclear genes such as *ITS* and *EF-1α* (Jordan *et al*. 2003; Hayashi *et al*. 2004; Dumont *et al*. 2005). These nuclear genes generally show minimal sequence variation at the population level and among closely related species.

In this study, we developed EPIC (exon-primed, intron-crossing) loci for phylogeographic and population genetic inference in the four *Euphaea* damselflies. The

cDNA libraries were reconstructed for *E. formosa* and *E. yayeyamana* using the heads of damselflies preserved in RNA*later* stabilization solution (Ambion, Canada). The head tissue was frozen with liquid nitrogen, and ground into a fine power with the MagNa Lyser Instrument (Roche Diagnostic, Germany). The RNA was extracted with TRIzol reagent (Invitrogen, USA). The first strand of cDNA was synthesized using the Creator SMART cDNA library construction kit (ClonTech, USA) according to the manufacture's instructions. Double-stranded cDNA was prepared using the first-strand reaction by ligation-dependent PCR (LD-PCR) with provided primers. The normalized double-stranded cDNA of each species was used for a quarter run of 454 pyrosequencing on the Genome Sequencer GS FLX System (Roche Diagnostic, Germany) at the Mission Biotech, Taiwan. Totally 47,075 and 111,068 reads (average length = 248 and 161 nucleotides) were generated from the pyrosequencing runs for *E. yayeyamana* and *E. formosa*, respectively. The number of 454 reads was small for *E. yayeyamana* as a result of sub-optimal RNA isolation and cDNA construction. The original reads were assembled using GS *De Novo* Assembler software (Roche Diagnostic, Germany) without a reference genome. After assembling the 454 reads, we obtained between 49 and 163 contig sequences with lengths exceeding 500 base pairs (bp) for *E. yayeyamana* and *E. formosa*, respectively. These contig sequences were blasted in GenBank database to search for matches with known insect genes. The blast hits were used to assign annotations to the corresponding sequences. Approximately 40 annotated nuclear protein-coding genes were shared between the two species. We downloaded the full mRNA of these genes from various insect genomes, and aligned them with the orthologous contig sequences of the two *Euphaea* to map the locations of introns and exons. The degenerate primers were designed from areas of conserved amino acids flanking regions of exons that contain introns. We developed two to four forward and

two to three reverse primers for each locus, resulting in four to eight primer combinations per locus. For *EF1α*, we used primers developed for *Megalagrion* damselflies (Jordan *et al*. 2003).

Whole genomic DNA was extracted from thoracic muscle of 18 to 23 specimens of *E. decorata* (Tai Po Kau, Shing Mun Country Park and Wu Kau Tang of Hong Kong; Tam Dao and Me Linh of Vietnam), *E. formosa* (Tsengwen, Lienhuachih, Touchien, Shimenkeng of Taiwan), *E. ornata* (Jianfengling and Mt. Diaoluo of Hainan) and *E. yayeyamana* (Hoshino and Fuanasogawa of Ishigaki; Shiiragawa of Iriomote) preserved in 95 % ethanol using MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE, Wisconsin, USA). The primary combinations were used in polymerase chain reaction (PCR) to screen for successful amplification of target DNA fragments. The PCR reaction contained 1μl of genomic DNA (100 to 300 ng/μl), 1μl of ProTaq polymerase (2u/μl, Protech Technology, Taiwan), 2μl of forward and reverse primer (10mM), 4μl of dNTPs (1mM), 5μl of ProTaq buffer and 35μl of ddH_2O. The PCR cycling profile was as follows: one minute of denaturation at 94ºC, followed by 45 seconds of annealing at optimal annealing temperature (Tm), and then one minute of extension at 72ºC (repeated for 35 cycles) and finally 10 minutes of extension at 72ºC. The target DNA fragments were gel-purified and extracted using a Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan). Purified PCR products were sequenced directly from both directions (*act*, *awd2*, *EF1α*, *sdhB* and *anon*) or cloned into pCR® 2.l-TOPO vector (Invitrogen) and then sequenced (*arr2*, *fer*, *mlc* and *lop1*). Table 1 lists the nine most successful primer combinations for amplifying the appropriate products in four *Euphaea* species for each locus. The intron number and position for each locus were conserved across the four species. The DNA sequences used in this study

were deposited in GenBank (accession numbers JN246927-247002, JN389796-JN390424).

All degenerate primer pairs produced specific amplification except for the primers of an anonymous locus (*anon*) (Table 2). Most of the amplified regions were highly variable and had haplotype diversity (*Hd*) and nucleotide diversity (π) greater than 0.637 and 0.01, respectively, except for the less variable *act* and *sdhB*. Elevated levels of recombination were detected for a few loci (*arr*, *fer* and *anon*) suggesting possible intra-locus recombination events in these regions. The Fay and Wu's H (*H*) and Tajima's D (*D*) tests indicated that the majority of loci had not significantly deviated from the expectation of neutrality, except for *anon* in *E. ornata*, *fer* in *E. yayeyamana*, *mlc* in *E. formosa*, *E. yayeyamana* and *E. decorata*, and *lop1* in *E. yayeyamana*. The EPIC loci reported here provide useful and high-resolution markers for efficient population genetic and phylogeographic studies in damselflies giving us the ability to infer phylogenetic relationships and population structure, and estimate the extent and direction of gene flow, divergence time and effective population sizes between sibling species in these four *Euphaea* species.

Acknowledgements

This study was supported by research grants from the National Science Council of Taiwan (NSC 93WFD0105096, 97-2621-B-029-001) and a Genetic Core Facility grant from Tunghai University.

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Table 1 Locus information and primer sequences

Locus	Reference sequences	Region[1]	Identity[1]	Primers	Tm (°C)	Primer sequences (5'-3')
Actin (*act*)	*Drosophila melanogaster* NM169525 *Apis mellifera* NM001185146	767-1170	portion of exon1	Act-115F Act-539R	56-58	TGCTATGTSGCYCTKGACTTC ACTCGTCGTAYTCCTGYTTSGA
Arrestin 2 (*arr2*)	*Apis mellifera* XM623181 *Acyrthosiphon pisum* XM001952468 *Drosophila melanogaster* NM079252	757-1061	portion of exon4, intron4, exon5, intron5, portion of exon6	Arr-805F Arr-1087R	54-56	GARATCTACTACCAYGGYGAGAA ACCARRGTGGARGABGCMARGTT
Abnormal wing discs (*awd2*)	*Acyrthosiphon pisum* NM001126184 *Drosophila melanogaster* NM057413 *Tribolium castaneum* XM962410 *Apis mellifera* XM393351	434-587	portion of exon1, intron1, portion of exon2	Awd-195F Awd-329R	53-55	GGAGGGACTCAATTCTGTCAAA AATTTCCTTCTGAGCTGACTCAA
Elongation factor 1alpha (*EF1α*)	*Drosophila melanogaster* NM20659 *Apis mellifera* NM001011628 *Tribolium castaneum* NM001114363	1374-2148	portion of exon1, intron1, exon2, intron2, portion of exon3 (one additional intron within exon1 present in *Euphaea*)	EF-1-F-2361[2] EF1-R-3093[2]	54	YGGMCACAGRGATTTCATCAA[2] CCAGGRTGGTTRAGCACRATGA[2]

Ferritin (*fer*)	*Oncometopia nigricans* AY725783 *Homalodisca coagulata* AY588064	19-227	NA	Fer-29F Fer-344R	53-55	CAATSGCAWACTAYTTYGAYCAAG TCYTTRATGGCCTCRACYTGYT
Myosin light chain (*mlc*)	*Acyrthosiphon pisum* NM001162731 *Nasonia vitripennis* XM001608197 *Tribolium castaneum* XM969595 *Aedes aegypti* XM001655528	491-643	portion of exon3, intron3, exon4, intron4, portion of exon5	mlc-62F mlc-171R	56	CTGCTCACMCTTTTYGCCWACCG CAGGTCATAAGGGCGTGCCTGA
Long wavelength opsin (*lop1*)	*Nasonia vitripennis* NM001170908 *Anopheles gambiae* XM001238569	704-974	portion of exon3, intron4, portion of exon4	E-2409-opsin-F-417 E-2409-opsin-R-710	54-56	CCTTTGGCACGGAATCTTAG CATTGTATCTGTCCATGGCG
Succinate dehydrogenase B (*sdhB*)	*Acyrthosiphon pisum* NM001162436 *Tribolium castaneum* NM001170889 *Nasonia vitripennis* NM001172331 *Apis mellifera* NW003378042	295-547	portion of exon2, intron2, exon3, intron3, portion of exon4	SdhB-16F SdhB-268R	54-56	TAYCGATGGAAYCCRGAHAAGSC AYNACRTACATRTGBGGYARHGG
Anonymous (*anon*)	NA	NA	NA	mlc-44F mlc-182R	53	CCAATCAACTTCACCCAACTGCT GAACTTGTCTCCCCAGGTCATAAG

[1]Genomic regions refer to the mRNA sequences of the first species listed in reference sequences; numbers indicate to the 3' position of the primers; [2]Primers developed by Jordan *et al.* 2003.

Table 2 Polymorphism and summary statistics in four *Euphaea* species

Locus	Species	Coding	Non-coding	N	*S*	S_e	S_i	*Hd*	π	*Rm*	*H*	*D*
act	*formosa*	445	0	19	3	3	0	0.591	0.0015	0	0.444	-0.607
	yayeyamana	445	0	18	1	1	0	0.503	0.0011	0	0.183	1.378
	decorata	445	0	18	1	1	0	0.503	0.0011	0	0.183	1.378
	ornata	445	0	24	1	1	0	0.489	0.0011	0	-0.326	1.391
arr2	*formosa*	305	1029	15	25	5	20	0.838	0.0056	0	-2.590	-0.151
	yayeyamana	305	1029	19	51	5	46	0.883	0.0108	7	6.368	-0.154
	decorata	305	1016	21	64	16	48	0.952	0.0125	13	1.752	-0.363
	ornata	305	1016	20	49	10	39	0.947	0.0098	5	6.358	-0.329
awd2	*formosa*	154	212	19	5	1	4	0.789	0.0039	0	0.561	-0.084
	yayeyamana	154	212	17	16	2	14	0.809	0.0115	0	2.919	-0.695
	decorata	154	212	21	14	1	13	0.805	0.0095	1	1.214	-0.421
	ornata	154	212	19	12	2	10	0.813	0.0083	0	2.041	-0.705
EF1α	*formosa*	785	243	18	11	9	2	0.784	0.0018	0	0.601	-1.561
	yayeyamana	785	243	19	8	8	0	0.889	0.0020	2	1.076	-0.292
	decorata	782	243	21	10	8	2	0.814	0.0019	1	1.505	-1.027
	ornata	782	243	20	9	6	3	0.637	0.0014	0	-0.632	-1.441
fer	*formosa*	337	876	24	43	10	33	0.884	0.0059	3	-2.377	-1.576
	yayeyamana	337	876	25	18	5	13	0.900	0.0032	3	-9.930**	-0.705
	decorata	337	878	20	84	11	73	0.974	0.0120	7	-8.389	-1.624
	ornata	337	878	14	38	8	30	0.945	0.0139	2	2.022	1.699
mlc	*formosa*	193	696	22	53	4	49	0.952	0.0163	6	-9.333*	-0.313
	yayeyamana	193	696	20	40	1	39	0.953	0.0133	0	-12.294*	-0.412
	decorata	193	700	19	26	2	24	0.883	0.0085	1	-9.994*	-0.337
	ornata	193	700	24	53	3	50	0.975	0.0176	1	0.964	0.115
lop1	*formosa*	311	1070	18	85	4	81	0.922	0.0248	5	4.837	1.375
	yayeyamana	311	1070	16	38	0	38	0.875	0.0131	0	0.717	2.392*
	decorata	311	1057	23	47	5	42	0.960	0.0072	0	6.917	-0.920
	ornata	311	1057	21	52	5	47	0.967	0.0083	5	7.957	-0.890
sdhB	*formosa*	275	200	21	14	13	1	0.914	0.0080	1	-0.771	-0.344
	yayeyamana	275	200	17	6	5	1	0.750	0.0056	1	1.581	0.962
	decorata	258	200	14	3	2	1	0.495	0.0032	0	-1.187	1.753
	ornata	258	200	21	1	1	0	0.429	0.0009	0	0.257	0.959
anon	*formosa*	0	450	17	33	NA	NA	0.919	0.0228	6	0.816	-0.090
	yayeyamana	0	450	17	7	NA	NA	0.875	0.0048	1	-2.265	0.079
	decorata	0	459	20	35	NA	NA	0.953	0.0250	5	1.842	0.383
	ornata	0	459	24	32	NA	NA	0.902	0.0131	3	-10.601*	-1.121

N: number of sequences; *S*: number of polymorphic sites; S_e: number of polymorphic sites in exons; S_i: number of polymorphic sites in introns; *Hd*: haplotype diversity; π: nucleotide diversity; *Rm*: minimum number of recombination events; *H*: Fay and Wu's H; *D*: Tajima's D; NA: not available; * $p<0.05$, ** $p<0.01$; summary statistics were calculated in DnaSP v.5.0 (Rozas *et al*. 2003).