2011. The Journal of Arachnology 39:506–510

SHORT COMMUNICATION

Bringing spiders to the multilocus era: novel anonymous nuclear markers for *Harpactocrates* ground-dwelling spiders (Araneae: Dysderidae) with application to related genera

Leticia Bidegaray-Batista[1], **Rosemary G. Gillespie**[2] and **Miquel A. Arnedo**[1]: [1]Institut de Recerca de la Biodiversitat & Departament de Biologia Animal, Universitat de Barcelona, Avenida Diagonal 643, 08020, Barcelona, Spain. E-mail: letigaray@yahoo.com; [2]Department of Environmental Science Policy and Management, University of California, 137 Mulford Hall, Berkeley, California 94720-3114

Abstract. Multilocus approaches are essential for accurately recovering the evolutionary processes underlying species and population history. However, historical inferences in non-model organisms are still almost exclusively based on mitochondrial DNA due to the difficulty of obtaining multiple informative loci. Here, we use a genomic library based approach, to generate 15 novel anonymous nuclear markers (ANMs) from the ground spider *Harpactocrates globifer* Ferrández 1986. The ANMs cross-amplify and sequence well in the target species and its two closest relatives, and some of them also in the more distantly related *H. ravastellus* Simon 1914. Levels of nucleotide diversity of the ANMs within *H. globifer* ranged from 0.05% to 1.4% and average sequence divergence between close congeneric relatives from 0.02% to 13.9%, supporting the utility of these loci in population and species-level analyses. Moreover, a cross-species amplification screened in other spider taxa showed that some of the loci could also potentially be useful in more distantly related genera.

Keywords: Genomic library, nuclear loci, multilocus approaches, non-model organisms

Multilocus approaches based on unlinked markers, including both mitochondrial (mtDNA) and nuclear markers, are essential for an accurate reconstruction of the evolutionary processes underlying species and population history. Inferences based exclusively on mtDNA markers are hampered by processes such as sex-biased behaviors (e.g., sex-biased dispersal), low dispersal ability and small population sizes, incomplete lineage sorting, introgression or selective sweeps (Avise 2000; Hare 2001; Irwin 2002; Ballard & Whitlock 2004; Wilkins 2004; Kuo & Avise 2005). In recent years the realization that gene trees and species trees can differ markedly in closely related species has sparked the development of a new generation of methods for species tree inference, which rely strongly on the information provided by multiple markers (Maddison 1997; Edwards 2009). Moreover, many studies have shown that the statistical power of parameters such as divergence times, population size and rates of gene flow, rely on the number of loci used (see Brumfield et al. 2003; Brito & Edwards 2009 and references therein). Recent advances, both technological (see reviews of Hudson 2007; Thomson et al. 2010; Ekblom & Galindo 2011) and methodological (e.g., Hey & Nielsen 2007; Heled & Drummond 2010; Hey 2010; Yang & Rannala 2010), have greatly facilitated the implementation of multilocus analyses. Unfortunately, the scarcity of available genomic information makes finding multiple informative loci in non-model organisms a daunting task (Thomson et al. 2010). This is especially true for spiders, in which just a handful of mitochondrial and nuclear markers that are variable at the population and species level can be reliably amplified and sequenced (see e.g., Hedin 1997 for nad1; Maddison & Hedin 2003 for nad1, 16S and EF-1 alpha; Ayoub et al. 2007 for EF-1 gamma; Bidegaray-Batista et al. 2007 for cox1 and 16S-nad1 fragment; Bond & Stockman 2008 for 12S-16S fragment and ITS-1 and ITS-2; Vink et al. 2008 for Actin 5C; Garb & Gillespie 2009 for cox1, 16S-nad1 fragment and EF-1 alpha; Muster et al. 2009 for ITS-1, 5.8S rDNA and ITS-2; De Busschere et al. 2010 for cox1 and 28S).

Here, we report on a method to circumvent this limitation by isolating novel anonymous nuclear markers (ANMs) from a spider genomic library. Our target organisms are species belonging to the ground-dwelling genus *Harpactocrates* Simon 1914 (family Dysderidae) which inhabit the mountain ranges of the Sistema Central in the Iberian Peninsula. From west to east, the species *Harpactocrates gredensis* Ferrández 1986, *H. globifer* Ferrández 1986 and *H. gurdus* Simon 1914 are found along the range, showing narrow, almost non-overlapping distributions. As with other species (about 13) in the genus, they are generally found at high elevation (above 1000 m) in temperate and moist forests, suggesting a preference for cool and humid environments. Preliminary phylogenetic and phylogeographic studies have shown that Pleistocene glaciations had a major role in structuring populations in *Harpactocrates.* These observations have led us to hypothesize that *Harpactocrates* species in the Sistema Central underwent population range expansion toward lower elevations during cooler periods, facilitating gene flow, whereas at interglacial periods they retreated to high elevation refuges, which then led to population fragmentation (e.g., Masta 2000; Knowles 2001; DeChaine & Martin 2005; Carstens & Knowles 2007b; Mardulyn et al. 2009; Schmitt 2009). With the aim of testing this hypothesis using a multilocus species/population approach, we have developed 15 novel ANMs from a genomic library of *H. globifer*, which cross-amplify and sequence well in the closely related species *H. gurdus* and *H. gredensis* and some of them in a more distant species *H. ravastellus* Simon 1914. Additionally, we have screened cross-species amplification in other spider taxa in order to evaluate the range and potential application of the novel markers (see below).

We constructed a genomic library from the pooled DNA of two *H. globifer* specimens. Total genomic DNA was extracted using SpeedTools Tissue DNA extraction kit (Biotools) and concentrated via ethanol precipitations. Genomic DNA (~10 µg) was digested with EcoRI restriction enzyme (50 µL total volume). The enzyme was selected following the recommendations of Carstens & Knowles (2006). The goal was to ensure that the enzyme did not cut the mitochondrial DNA into fragments smaller than 1.6 kilobase pairs (kb), thereby reducing the likelihood of cloning mtDNA fragments. EcoRI was selected based on the information provided by a complete mitochondrial genome of a new species of *Parachtes* Alicata 1964 (pending formal description, Pons, Bidegaray-Batista & Arnedo unpublished data), which is the sister genus to *Harpactocrates.* Digested DNA was visualized on a 1% agarose gel stained with ethidium bromide, and fragments between 0.8 and 1.5 kb in length

Table 1.—Primer sequences for fifteen anonymous nuclear loci developed from a genomic library of *Harpatocrates globifer*. Names indicate the loci, forward and reverse primers, PCR annealing temperature T (°C), size of amplicon and GenBank Accession No. of the original cloned fragment. Performance codes indicate successful amplification in the following species: *H. globifer* (1), *H. gurdus* (2), *H. gredensis* (3) and *H. ravastellus* (4), *Parachtes romandiolae* (a), *Dysdera erythrina* (b), *Harpactea corticalis* (c), *Loxosceles rufescens* (d), *Troglohyphantes lucifuga* and *T. pedemontanus* (e), *Nemesia randa* and *Iberesia brauni* (f). PCR products resulting in double bands (db) or multiple bands (mb) are indicated as superscripts.

Locus	Primer sequences (5′-3′)	T (° C)	Amplicon size (bp)	Accession no.	Performance
qgL1	F: AGACAGCATTCAGAGTCCAAGCG R2: GCCGAAATAGTTTGAGCTCGTTTGCG	56	416	JN654497	1,2,3,4, a^{mb}
qgL5	F: TGCCCACGCCCCACTAAAATAGG R: GCCAGGTTGCCAGTTAAAATCACG	58	424	JN654498	1,2,3,4, a, b
qgL10	F: AGCGACACATCCTTACCTGCGT R: GCGCATCTGGAGAGCCTTTGA	58	621	JN654506	1,2,3,4, a^{mb}, e
qgL12	F: TGGCACAGCAGTGGCCAGAA R: CATGTCAACCGAATAGAATC	55	617	JN654509	1,2,3
qgL21	F: ACGCCCGAACCGACCTTTGC R: ACGAGGGAGGTGCTAGAAGCG	58	580	JN654499	1,2,3, a^{db}, f^{mb}
qgL22	F: ACGATGCACCCTCGAAATGGTCG R: TTGGCGCGGAACCTCTCAGC	58	508	JN654511	1,2,3,4, a^{db}
qgL25	F: TGCCCACGCCACCCCTATCC R: AGGCCAACGCGAAAAGTCAGC	59	309	JN654510	1,2,3,4, a, b, d
qgL26	F: TCCCCGGGTCACGTGGGAAG R: TCCCCAACGTGAACGAACCGC	57	586	JN654500	1,2,3,4, a^{mb}
qgL28	F: GTCCCGTCGTCCGGGGTTTG R: GCCACCCATGCTTTTTGTGCTCC	59	401	JN654508	1,2,3,4, a
qgL29	F: TGGACTCCCGTTTCACAAGGCG R: CCACGCTATAATTGGCCCACAAGC	53	459	JN654502	1,2,3, a^{mb}
qgL32	F: AGCCCCAACATCCGTTTGACC R: CGTCGGCAAAAGGGACCACCC	58	567	JN654503	1,2,3,4, a^{mb}
qgL33	F: ACGTGCCACACTCTCTCTCTTTCG R: TGCCTGCTCGAATCAAACCCAGC	58	343	JN654504	1,2,3
qgL34	F: CGCGTCACCATAGGCTCTTCGC R: AGTTCGGTGTGTGGCCGAGC	58	408	JN654507	1,2,3, a^{mb}, b^{db}
qgL36	F: AGCGTAACGTAGGGCGTGCAG R: CGGCCGCTTGGAAGGTGGTC	58	627	JN654501	1,2,3,4, a^{mb}, b^{mb}, f^{db}
qgL38	F: TCACAGGCATACGAAAGCGCC R: GCCGAGCGTAGGCCTAGCATAAC	58	454	JN654505	1,2,3,4, a

were excised and purified from the gel using the kit QIAquick Gel Extraction kit (QIAGEN). Excised fragments were concentrated via ethanol precipitations to 8 ng/μL and cloned using the CloneJET™ PCR Cloning kit (Fermentas) and One Shot TOP10 competent cells (Invitrogen). Transformed cells were plated on agar plates containing ampicillin. Five hundred colonies were individually picked with a pipette tip and added directly to a 25 μL PCR mix containing the primers included in the cloning kit. Amplified inserts were visualized on a 1% agarose gel stained with ethidium bromide. Of the 500 amplified inserts, 206 that ranged in size from 300 to 1200 bp were selected for sequencing. Forty-one sequences were discarded because they were either identical to other sequences or did not sequence well. The remaining 165 sequences were locally blasted against the *Parachtes* sp. mitochondrial genome to ensure that there were no mtDNA sequences among the selected inserts, and in all cases they turned out to be part of the nuclear genome. Subsequently, we performed nucleotide BLAST searches against the GenBank database to characterize sequences. Most sequences (160 out of 165) reported non-significant BLAST hits. Thirty-eight of these sequences were subsequently selected for primer design based on features that ensured that they were non-coding regions, such as the presence of multiple stop codons in each of the six reading frames, and avoiding poly-A or -G runs, which are difficult to sequence. The primer pairs designed were initially screened in a set of individuals consisting of one from each of the three target species (*H. globifer*, *H. gurdus* and *H. gredensis*) and one belonging to a more distantly related species (*H. ravastellus* from the Pyrenees). Each primer pair was tested in a standard 25 μL PCR mix with the following conditions: 3 min at 94° C followed by 30 cycles of denaturation at 94° C for 30 s, annealing at three different temperatures of 52° C, 55° C and 58° C for 35 s, and extension at 72° C for 1 min, with a final single extension step of 72° C for 10 min. Of the 38 previously selected loci, 15 produced a single band product for at least one of the annealing temperatures tested and sequenced well in the three target species (Table 1). The annealing temperature of the PCR reaction was subsequently optimized (see Table 1), and the extension time was adjusted according to the length of the fragment.

Eight to 10 individuals of *H. globifer* from different populations were sequenced for each locus (Table 2) to investigate how informative the markers might be for population and phylogeographic studies. In addition, diversity indices and population statistics of the anonymous makers were compared with those of known mitochondrial genes and nuclear introns. With this aim, a mitochondrial fragment (*16S-nad1*) spanning the 3′ half of the 16S rRNA ribosomal subunit (*rrnL*), the complete tRNA leu (*trnL1*), the 5′ half of the NADH dehydrogenase subunit I (*nad1*) and the nuclear intron of the signal recognition particle 54-kDA subunit (*srp54*) were amplified and sequenced for 10 individuals of *H. globifer* and a single individual of *H. gurdus*, *H. gredensis* and *H. ravastellus*, respectivelly. The *16S-nad1* fragment was amplified with primer pairs LR-N-13398 (Simon et al. 1994) and N1-J-12350 (5′-CCTARTTGRCTAR-ARTTRGCRSATCARCCAATTG -3′) and the *srp54* with SRP54f1

Table 2.—Overall diversity measures and statistics estimated across 15 anonymous loci, *srp54* intron and *16s-nad1* mitochondrial fragment for *Harpactocrates globifer*. Sample size (SS) indicates the number of individuals sequenced for each locus, in brackets the number of individuals used in the estimations considering the 0.9 probability threshold in PHASE. The length (L) in bp for each locus after sequences end-trimming and excluding sites with gaps. The lengths of indels are indicated as they occurred in each locus (N/A: not applicable, if indels were not observed). The number of segregating sites (S) and haplotypes (H), nucleotide diversity (π), minimum number of recombination events (R_M) of Hudson (1985), linkage disequilibrium statistic (ZZ) of Rozas et al. (2001), Tajima's D test (D) of Tajima (1989), and the HKA test of Hudson (1987). Not significant (ns) and significant (*) values at $P < 0.05$ of statistics after coalescence simulations.

	H. globifer									HKA Test (*H. globifer*/*H. gredensis*) *srp54* vs. other locus
Locus	SS	L	Indel length	S	H	π	R_M	ZZ	D	
qgL1	10 (10)	313	N/A	8	3	0.0031	0	0.0000	−1.9072 *	$P = 0.4764$ ns
qgL5	10 (10)	342	N/A	5	4	0.0039	2 ns	0.0477 ns	−0.1917 ns	$P = 0.5292$ ns
qgL10	9 (6)	540	1, 8	12	7	0.0057	0	0.1707 ns	−0.9653 ns	$P = 0.7549$ ns
qgL12	8 (8)	542	1, 2	16	7	0.0071	0	0.2002 ns	−0.8083 ns	$P = 0.2500$ ns
qgL21	10 (9)	496	1, 2	15	10	0.0088	1 ns	0.1057 ns	−0.0070 ns	$P = 0.7649$ ns
qgL22	10(10)	429	3, 1, 2	16	5	0.0140	0	0.0360 ns	1.2410 ns	$P = 0.4838$ ns
qgL25	10 (9)	223	6, 1	1	2	0.0009	0	0.1170 ns	−0.5290 ns	$P = 0.2839$ ns
qgL26	10 (8)	512	N/A	2	3	0.0005	0	0.0000	−1.4979 *	$P = 0.3564$ ns
qgL28	10 (10)	317	N/A	8	6	0.0068	0	− 0.0570 ns	−0.1529 ns	$P = 0.4341$ ns
qgL29	10 (9)	373	N/A	12	6	0.0070	1 ns	0.1465 ns	−0.9463 ns	$P = 0.9255$ ns
qgL32	10 (9)	490	5, 11, 3, 10	21	5	0.0119	0	0.0665 ns	−0.1766 ns	$P = 0.5201$ ns
qgL33	10 (10)	266	N/A	12	7	0.0142	1 ns	−0.0074 ns	0.4320 ns	$P = 0.5290$ ns
qgL34	10 (10)	319	1	13	8	0.0108	0	0.0678 ns	−0.4578 ns	$P = 0.8684$ ns
qgL36	10 (8)	552	N/A	11	6	0.0057	0	−0.0444 ns	−0.1786 ns	$P = 0.5850$ ns
qgL38	10 (9)	366	N/A	5	3	0.0067	0	0.2154 ns	2.1431 ns	$P = 0.3583$ ns
srp54	10 (10)	119	N/A	7	8	0.0165	1 ns	−0.0154 ns	−0.0165 ns	-
16S-nad1	10 (10)	851	1	76	9	0.0237	7 ns	−0.0103 ns	−1.5465 ns	$P = 0.1021$ ns

and SRP52r1 (Jarman et al. 2002). PCR conditions were as follows: 94° C for 2 min; 35× (94° C for 35 s; 45° C for 45 s for *16S-nad1* and from 45° C to 50° C for 35 s for *srp54*; 72° C for 45 s for *16S-nad1* and 35 s for *srp54*; 72° C for 5 min).

The allelic phase of heterozygotic individuals was resolved using the algorithms provided by PHASE (Stephens et al. 2001; Stephens & Donnelly 2003) and implemented in DnaSP v5 (Librado & Rozas 2009). In the case where direct sequencing detected multiple copies of different lengths, either as a result of heterozygote individuals or paralogy, the PCR products were cloned and four to eight colonies sequenced. Cloning results were compatible with alleles of different length (i.e., high sequence similarity) rather than paralogous copies, assuming that polymorphisms due to singleton mutations among sequenced colonies represented errors of the *Taq* polymerase enzyme or cloning artifacts (see Villablanca et al. 1998; Calderón et al. 2009; Cummings et al. 2010; Dawson et al. 2010).

The taxonomic range of applicability of the novel markers was evaluated by screening a sample of specimens belonging to groups at different hierarchical levels: *Parachtes romandiolae* Caporiacco 1949 and *Dysdera erythrina* Walckenaer 1802 (Araneomorphae, Haplogynae, Dysderidae, Dysderinae), *Harpactea corticalis* Simon 1882 (Araneomorphae, Haplogynae, Dysderidae, Harpacteinae), *Loxosceles rufescens* Dufour 1820 (Araneomorphae, Haplogynae, Sicariidae), *Troglohyphantes lucifuga* Simon 1884 and *T. pedemontanus* Gozo 1908 (Araneomorphae, Entelegynae, Linyphiidae), and *Nemesia randa* Decae 2005 and *Iberesia brauni* L. Koch 1882 (Mygalomorphae, Nemesiidae). Two independent PCR amplifications were performed for each locus using the following touchdown cycle: 94° C 2 min; 10× [94° C for 35 s, 63° C for 35 s (−0.5° C/cycle), 72° C for 1 min]; 10× [94° C for 35 s, 58° C for 35 s, 72° C for 1 min]; 15× [94° C for 35 s, 52° C for 35 s, 72° C for 1 min]; 72° C for 10 min. Results of the amplification success are summarized in Table 1.

The average interspecific sequence divergence (*p* distance) of the anonymous loci calculated with DnaSP v5 ranged from 0.02% (qgL26) to 8.1% (qgL28) between *H. globifer* and *H. gurdus*, 0.41% (qgL26) to 6.9% (qgL33) between *H. globifer* and *H. gredensis*, 1.8% (qgL25) to 11.1% (qgL1) between *H. globifer* and *H. ravastellus*, 0.39% (qgL26) to 8.5% (qgL28) between *H. gurdus* and *H. gredensis*, 1.3% (qgL25) to 13.9% (qgL28) between *H. gurdus* and *H. ravastellus*, and 2.4% (qgL25) to 9.4% (qgL1) between *H. gredensis* and *H. ravastellus*. Percent nucleotide diversity within *H. globifer* ranged from 0.05% (qgL26) to 1.4% (qgL33). Genetic diversity indices, recombination and linkage disequilibrium measures and neutrality tests were calculated for each locus in *H. globifer* using DnaSP v5. Results are summarized in Table 2. We applied the HKA test of Hudson (1987) to assess whether levels of polymorphism and divergence were correlated, as predicted by neutral theory. The test was performed by comparing polymorphism in *H. globifer* and divergence between *H. globifer* and *H. gredensis* in the intron *srp54* versus each of the 15 anonymous loci and versus the mitochondrial fragment *16s-nad1*. Neither recombination nor linkage disequilibrium were found at any locus. None of the HKA tests showed deviation from neutral predictions, although Tajima's D test showed significant negative values for the loci qgL1 and qgL26. The mitochondrial fragment *16S-nad1* was 1.4 times more variable than the intron *srp54*, and from 1.7 to 47 times more variable than the anonymous loci qgL33 and qgL26, which are the most and least variable ANM's, respectively.

This study reports the first ANMs ever designed specifically for spiders. These kinds of markers, however, have been isolated and extensively applied in other organisms; e.g., grasshoppers (Carstens & Knowles 2006), halfbeaks and killifishes (De Bruyn et al. 2010a, b), oysters (Hare et al. 1996), birds (Lee & Edwards 2008) and lizards (Rosenblum et al. 2007; Noonan & Yoder 2009). ANMs are promising markers for multilocus approaches at the population and species level of closely related and recently diverged species, as has been shown in recent studies (Carstens & Knowles 2007a; Carstens & Richards 2007; Knowles et al. 2007; Lee & Edwards 2008). The novel ANMs designed here are potentially useful not only for the target species, but also for more distantly related species in the genus (e.g., *H. ravastellus*), as well as for species in related genera (e.g. *Parachtes*), or even subfamilies (e.g., *Dysdera*, see Table 1). However, the

applicability of these novel markers is compromised at higher taxonomic levels. A significant and negative relationship between performance and evolutionary distance from the target species is also observed among the popular microsatellite markers (Primmer et al. 1996). Although the strategy implemented in our study greatly facilitates and speeds up the isolation of variable markers for populations and species, the advent of the so-called next-generation sequencing (NGS) methods promise to revolutionize the discovery of novel markers in non-model organism even further. In fact, the protocols used here for ANM isolation can be easily coupled with NGS methods, simply by replacing the cloning step, to generate literally hundreds or thousands of novel markers for spiders. Moreover, NGS allows sequencing of lots of markers from multiple individuals and populations simultaneously. No doubt NGS methods will spawn a whole new era for phylogenetic, phylogeographic and population genetic studies in spiders and their allies.

ACKNOWLEDGMENTS

We are grateful to J. Pons for his invaluable technical advice. We also thank P. Croucher and A. Lam for their assistance in the lab. The project was funded by the Spanish Ministry of Science and Innovation (MICINN) grant CGL2006-08617 (MA) and the William and Esther Schlinger Chair in Systematic Entomology (RGG). LBB was supported by a graduate grant (FI-DGR 2009) and a short stay grant (BE 2009) from the Generalitat de Catalunya. Further funding support was provided by an ICREA Academia award for excellence in research from the Generalitat de Catalunya to MA.

LITERATURE CITED

Avise, J.C. 2000. Phylogeography: the History and Formation of Species. Harvard University Press, Cambridge, Massachusetts.

Ayoub, N.A., J.E. Garb, M. Hedin & C.Y. Hayashi. 2007. Utility of the nuclear protein-coding gene, elongation factor-1 gamma (EF-1γ), for spider systematics, emphasizing family level relationships of tarantulas and their kin (Araneae: Mygalomorphae). Molecular Phylogenetics and Evolution 42:394–409.

Ballard, J.W.O. & M.C. Whitlock. 2004. The incomplete natural history of mitochondria. Molecular Ecology 13:729–744.

Bidegaray-Batista, L., N. Macías-Hernández, P. Oromí & M.A. Arnedo. 2007. Living on the edge: demographic and phylogeographical patterns in the woodlouse-hunter spider *Dysdera lancerotensis* Simon, 1907 on the eastern volcanic ridge of the Canary Islands. Molecular Ecology 16:3198–3214.

Bond, J.E. & A.K. Stockman. 2008. An integrative method for delimiting cohesion species: finding the population-species interface in a group of Californian trapdoor spiders with extreme genetic divergence and geographic structuring. Systematic Biology 57:628–646.

Brito, P.H. & S.V. Edwards. 2009. Multilocus phylogeography and phylogenetics using sequence-based markers. Genetica 135:439–455.

Brumfield, R.T., P. Beerli, D.A. Nickerson & S.V. Edwards. 2003. The utility of single nucleotide polymorphisms in inferences of population history. Trends in Ecology & Evolution 18:249–256.

Calderón, I., X. Turon & H.A. Lessios. 2009. Characterization of the sperm molecule bindin in the sea urchin genus *Paracentrotus*. Journal of Molecular Evolution 68:366–376.

Carstens, B.C. & L.L. Knowles. 2006. Variable nuclear markers for *Melanoplus oregonensis* identified from the screening of a genomic library. Molecular Ecology Notes 6:683–685.

Carstens, B.C. & L.L. Knowles. 2007a. Estimating species phylogeny from gene-tree probabilities despite incomplete lineage sorting: an example from *Melanoplus* grasshoppers. Systematic Biology 56:400–411.

Carstens, B.C. & L.L. Knowles. 2007b. Shifting distributions and speciation: species divergence during rapid climate change. Molecular Ecology 16:619–628.

Carstens, B.C. & C.L. Richards. 2007. Integrating coalescent and ecological niche modeling in comparative phylogeography. Evolution 61:1439–1454.

Cummings, S.M., M. McMullan, D.A. Joyce & C. Van Oosterhout. 2010. Solutions for PCR, cloning and sequencing errors in population genetic analysis. Conservation Genetics 11:1095–1097.

Dawson, K., R.S. Thorpe, A. Malhotra & T. Mailund. 2010. Estimating genetic variability in non-model taxa: a general procedure for discriminating sequence errors from actual variation. PLoS One 5:551–554.

De Bruyn, M., W. Grail, A. Barlow & G.R. Carvalho. 2010a. Anonymous nuclear markers for SouthEast Asian halfbeak fishes (*Dermogenys*). Conservation Genetics Resources 2:325–327.

De Bruyn, M., W. Grail & G.R. Carvalho. 2010b. Anonymous nuclear markers for the Blue Panchax killifish (*Aplocheilus panchax*). Conservation Genetics Resources 3:53–55.

De Busschere, C., F. Hendrickx, S.M. Van Belleghem, T. Backeljau, L. Lens & L. Baert. 2010. Parallel habitat specialization within the wolf spider genus *Hogna* from the Galápagos. Molecular Ecology 19:4029–4045.

DeChaine, E.G. & A.P. Martin. 2005. Marked genetic divergence among sky island populations of *Sedum lanceolatum* (Crassulaceae) in the Rocky Mountains. American Journal of Botany 92:477–486.

Edwards, S.V. 2009. Is a new and general theory of molecular systematics emerging? Evolution 63:1–19.

Ekblom, R. & J. Galindo. 2011. Applications of next generation sequencing in molecular ecology of non-model organisms. Heredity 107:1–15.

Garb, J.E. & R.G. Gillespie. 2009. Diversity despite dispersal: colonization history and phylogeography of Hawaiian crab spiders inferred from multilocus genetic data. Molecular Ecology 18:1746–1764.

Hare, M.P., S.A. Karl & J.C. Avise. 1996. Anonymous nuclear DNA markers in the American oyster and their implications for the heterozygote deficiency phenomenon in marine bivalves. Molecular Biology and Evolution 13:334.

Hare, M.P. 2001. Prospects for nuclear gene phylogeography. Trends in Ecology and Evolution 16:700–706.

Hedin, M.C. 1997. Molecular phylogenetics at the population/species interface in cave spiders of the Southern Appalachians (Araneae: Nesticidae: *Nesticus*). Molecular Biology and Evolution 14: 309–324.

Heled, J. & A.J. Drummond. 2010. Bayesian inference of species trees from multilocus data. Molecular Biology and Evolution 27:570–580.

Hey, J. & R. Nielsen. 2007. Integration within the Felsenstein equation for improved Markov chain Monte Carlo methods in population genetics. Proceedings of the National Academy of Sciences USA 104:2785–2790.

Hey, J. 2010. Isolation with migration models for more than two populations. Molecular Biology and Evolution 27:905–920.

Hudson, M.E. 2007. Sequencing breakthroughs for genomic ecology and evolutionary biology. Molecular Ecology Resources 8:3–17.

Hudson, R.R. & N.L. Kaplan. 1985. Statistical properties of the number of recombination events in the history of a sample of DNA sequences. Genetics 111:147–164.

Hudson, R.R., M. Kreitman & M. Aguadé. 1987. A test of neutral molecular evolution based on nucleotide data. Genetics 116:153–159.

Irwin, D.E. 2002. Phylogeographic breaks without geographic barriers to gene flow. Evolution 56:2383–2394.

Jarman, S.N., R.D. Ward & N.G. Elliott. 2002. Oligonucleotide primers for PCR amplification of coelomate introns. Marine Biotechnology 4:347–355.

Knowles, L.L. 2001. Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshoppers. Molecular Ecology 10:691–702.

Knowles, L.L., B.C. Carstens & P. Sunnucks. 2007. Estimating a geographically explicit model of population divergence. Evolution 61:477–493.

Kuo, C.H. & J.C. Avise. 2005. Phylogeographic breaks in low-dispersal species: the emergence of concordance across gene trees. Genetica 124:179–186.

Lee, J.Y. & S.V. Edwards. 2008. Divergence across Australia's Carpentarian barrier: statistical phylogeography of the red-backed fairy wren (*Malurus melanocephalus*). Evolution 62:3117–3134.

Librado, P. & J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Maddison, W. & M. Hedin. 2003. Phylogeny of *Habronattus* jumping spiders (Araneae: Salticidae), with consideration of genital and courtship evolution. Systematic Entomology 28:1–22.

Maddison, W.P. 1997. Gene trees in species trees. Systematic Biology 46:523–536.

Mardulyn, P., Y.E. Mikhailov & J.M. Pasteels. 2009. Testing phylogeographic hypotheses in a Euro-Siberian cold-adapted leaf beetle with coalescent simulations. Evolution 63:2717–2729.

Masta, S.E. 2000. Phylogeography of the jumping spider *Habronattus pugillis* (Araneae: Salticidae): recent vicariance of sky island populations? Evolution 54:1699–1711.

Muster, C., W.P. Maddison, S. Uhlmann, T.U. Berendonk & A.P. Vogler. 2009. Arctic-alpine distributions-metapopulations on a continental scale? American Naturalist 173:313–326.

Noonan, B.P. & A.D. Yoder. 2009. Anonymous nuclear markers for Malagasy plated lizards (*Zonosaurus*). Molecular Ecology Resources 9:402–404.

Primmer, C.R., A.P. Møller & H. Ellegren. 1996. A wide range survey of cross species microsatellite amplification in birds. Molecular Ecology 5:365–378.

Rosenblum, E.B., N.M. Belfiore & C. Moritz. 2007. Anonymous nuclear markers for the eastern fence lizard, *Sceloporus undulatus*. Molecular Ecology Notes 7:113–116.

Rozas, J., M. Gullaud, G. Blandin & M. Aguade. 2001. DNA variation at the rp49 gene region of *Drosophila simulans*: evolutionary inferences from an unusual haplotype structure. Genetics 158:1147–1155.

Schmitt, T. 2009. Biogeographical and evolutionary importance of the European high mountain systems. Frontiers in Zoology 6:1–10.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu & P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87:651–701.

Stephens, M., N.J. Smith & P. Donnelly. 2001. A new statistical method for haplotype reconstruction from population data. American Journal of Human Genetics 68:978–989.

Stephens, M. & P. Donnelly. 2003. A comparison of bayesian methods for haplotype reconstruction from population genotype data. American Journal of Human Genetics 73:1162–1169.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Thomson, R.C., I.J. Wang & J.R. Johnson. 2010. Genome enabled development of DNA markers for ecology, evolution and conservation. Molecular Ecology 19:2184–2195.

Villablanca, F.X., G.K. Roderick & S.R. Palumbi. 1998. Invasion genetics of the Mediterranean fruit fly: variation in multiple nuclear introns. Molecular Ecology 7:547–560.

Vink, C.J., M. Hedin, M.R. Bodner, W.P. Maddison, C.Y. Hayashi & J.E. Garb. 2008. Actin 5C, a promising nuclear gene for spider phylogenetics. Molecular Phylogenetics and Evolution 48:377–382.

Wilkins, J.F. 2004. A separation-of-timescales approach to the coalescent in a continuous population. Genetics 168:2227–2244.

Yang, Z. & B. Rannala. 2010. Bayesian species delimitation using multilocus sequence data. Proceedings of the National Academy of Sciences USA 107:9264–9269.

Manuscript received 25 June 2011, revised 27 September 2011.