Blackwell Publishing

Cladistics 21 (2005) 390–395

Cladistics

www.blackwell-synergy.com

Targeting optimal introns for phylogenetic analyses in non-model taxa: experimental results in Asian pitvipers

Simon Creer*, Anita Malhotra, Roger S. Thorpe and Catharine E. Pook

School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, UK

Accepted 19 July 2005

Abstract

Nuclear introns are increasingly used as phylogenetic markers. Here, we present a multidisciplinary approach towards optimal locus selection and amplification using Asian pitvipers as an example of a non-model taxon, and raise the profile of length variant heterozygotes (LVHs) in intron loci. Taxon-specific primers were identified using a bioinformatic approach, and also designed from existing exon primed, intron crossing (EPIC) primer amplifications. Eleven further universal EPIC primer pairs were assayed using a range of PCR optimization strategies. Taxon-specific primers yielded the most consistent amplifications, but assaying a large number of universal EPIC primers yielded another appropriate locus for phylogenetic purposes. Modified *Taq* DNA polymerases such as JumpStart^{TM} *Taq* either significantly improved the specificity and yield of EPIC PCR amplifications (of low copy number nuclear targets), or resulted in amplifications that were not significantly worse than those derived from a generic *Taq* DNA polymerase. Finally, LVHs were detected in all loci that were sequenced suggesting that they are relatively common in introns. This study provides an efficient and cost effective template for the successful identification of intron markers for molecular systematics which is universally applicable to other non-model taxon groups.

The choice of molecular marker to be used in comparative evolutionary studies plays a crucial role in the successful acquisition of orthologous and phylogenetically informative gene fragments across a taxonomically diverse range of target taxa. Using markers which have worked well in closely related taxa, or because they are freely available, may be inappropriate strategies. Such choices can potentially result in the amplification and analysis of gene regions which may yield insufficient phylogenetic signal for a subset of the desired taxonomic range. Instead, if a small amount of time and money is invested in surveying the performance of different markers derived from a number of different sources, a subset of optimal markers can be identified for the necessary study (Slade et al., 1993). Although all project requirements will differ, the main criteria for optimal phylogenetic markers are successful and reliable PCR amplifications (and subsequent DNA sequencing) for all target taxa, and the attainment of the appropriate amount of phylogenetic signal.

Non-coding introns (Friesen, 2000) are emerging as reliable markers for independent phylogenetic studies (Oakley and Phillips, 1999; van Oppen et al., 2000), or for the nuclear corroboration of mitochondrial DNA (mtDNA) gene trees (Flynn and Nedbal, 1998; Pitra et al., 2000; Rockman et al., 2001). Notably, introns have also been identified as the primary candidate markers for single copy nuclear DNA population genetic analyses (Zhang and Hewitt, 2003). It is estimated that introns mutate at approximately one-quarter the rate of mtDNA (Prychitko and Moore, 1997, 2000; Creer et al., 2003). However, unlike mtDNA, introns are less subject to structural constraints which potentially facilitate the acquisition of phylogenetic signal from all base positions equally, accompanied by lower levels of homoplasy (Slade et al., 1993; Palumbi and Baker, 1994). It must be acknowledged that, given that

*Corresponding author.
E-mail address: s.creer@bangor.ac.uk

mtDNA has an effective population size four times smaller than nuclear DNA, mtDNA haplotypes are expected to coalesce (i.e., become monophyletic) and track speciation events more rapidly than intron loci (Moore, 1995; Wiens and Penkrot, 2002). Thus, introns may be of limited phylogenetic utility in tracking relatively recent speciation events. Nevertheless, studies have indicated that intron partitions of approximately 1000 base pairs (bp) afford interspecific taxonomic discrimination in a range of taxa (Palumbi and Baker, 1994; Weibel and Moore, 2002; Creer et al., 2003). Furthermore, gene partitions of this size can additionally (in most instances) be sequenced completely from both directions, minimizing the number of sequence reactions per OTUs that need to be visualized on an automated DNA sequencer.

Despite the potential utility of introns as phylogenetic markers, information on universally applicable primers is currently uncommon (Palumbi and Baker, 1994; Friesen et al., 1999; Jarman et al., 2002), and not well tested across a range of taxa. Furthermore, irrespective of the challenges associated with the alignment (Giribet and Wheeler, 1999; Sanchis et al., 2001; Belshaw and Quicke, 2002), and treatment, of diverse insertion/deletion (indel) events (see Giribet and Wheeler, 1999; Lutzoni et al., 2000; Simmons and Ochoterena, 2000 for different opinions), the amplification of low-copy number nuclear genes is not as straightforward as mtDNA markers. It is also rarely reported that introns can exist as intra-individual length variant heterozygotes (LVHs). Direct sequencing LVHs results in the apparent termination of sequencing reactions (Palumbi and Baker, 1994) due to the superimposition of two products of unequal length in chromatogram traces. Cloning and separate sequencing of the alleles is an easy solution for resolving the LVHs, but see also Zhang and Hewitt (2003) for alternative strategies. Therefore, in this study we wanted to identify a range of methodologies (which could be applied to any taxonomic group) to locate and characterize optimal nuclear introns for phylogenetic analysis in a diverse range of Asian pitvipers. Unique exon primed, intron crossing (EPIC) primers were designed and tested from single copy gene sequences available from GenBank, and also from sequences generated by using an existing EPIC primer across a representative taxonomic range. Finally, the utility of 11 universally designed EPIC primers were tested across the sample taxa in order to distinguish which markers would be most appropriate for application in a larger taxonomic study.

Materials and methods

Operational taxonomic units (OTUs) were selected from a broad taxonomic range of Asian pitvipers to ensure that any resulting selected primers would be more likely to cross-amplify throughout the desired target range. These consisted of representatives from throughout the *Trimeresurus* radiation of pitvipers (*Cryptelytrops albolabris*, *Viridovipera vogeli*, *Popeia popeiorum* and *Trimeresurus borneensis*), *Tropidolaemus wagleri*, *Garthius chaseni* (Malhotra and Thorpe, 2004) and *Calloselasma rhodostoma*. Intron primers were selected via three different approaches:

(a) Designing taxonomic group-specific EPIC primers from cDNA mRNA and genomic sequences from GenBank

At the time of analysis, the only compatible, single copy cDNA mRNA—genomic DNA sequence data comparisons that were available on GenBank were derived from the characterization of the *TATA box-binding protein* gene from *Viridovipera stejnegeri* (Malhotra and Thorpe, 2004) and *Protobothrops flavoviridis* (referred to as *Trimeresurus gramineus* and *Trimeresurus flavoviridis*, respectively, in Nakashima et al., 1995). Comparisons of the size and pair-wise sequence divergences of all seven introns of the *TATA box-binding protein* gene (*TBP*) in Nakashima et al. (1995), suggested that intron 3 (718 bp in length and exhibiting approximately 7.5% uncorrected sequence divergence) would be the most appropriate marker for phylogenetic purposes. Thus, the EPIC primer *TRIMTBPI3F* – 5′-CCTTTACCAGGAACCACACC-3′ (107 bp upstream from the 3rd exon–3rd intron boundary), *TRIMTBPI3R* – 5′-CGAAGGGCAATGGTTTTTAG-3′ (60 bp downstream from the 3rd intron–4th exon boundary) was designed for the amplification of the third intron of the *TATA box-binding* gene. All primer designs were perfomed with the assistance of Primer3 (Rosen and Skaletsky, 1998).

(b) Designing taxonomic group-specific primers from sequences generated by existing universal EPIC primers

Previous work has shown that the universal EPIC primers designed to amplify the 7th intron of the β-fibrinogen gene (*7IFGB*) in vertebrates cross amplify comparatively successfully in Asian pitvipers (Creer et al., 2003). Thus, the Asian pitviper specific EPIC primers *7IFGB TRIMFGB-I7U* – 5′-AGAGACAATGATGGATG*GTAAG-3′ (* designates the 7th exon–7th intron boundary) and *TRIMFGB-I7L* – 5′-CCTTTTGGGATCTGGGTGTA-3′ (22 bp downstream from the 7th intron–8th exon boundary) were designed from sequences amplified from the same OTUs above (Creer et al., 2003) generated using the *7I*βFIB primers listed in (Prychitko and Moore, 1997).

(c) Surveying and testing candidate universal EPIC primers

Putative EPIC universal primers were selected that were designed to amplify introns in *fructose 1,6-biphosphate aldolase B* (*ALDOB*), α-enolase (*ENO1*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPD*), *lamin A* (*LMNA*) (Friesen et al., 1997), *lactate dehydrogenase B* (*LDHB*), *myelin proteolipid protein* (*MBP*), *ornithine decarboxylase* (*ODC1*), *ribosomal protein 40* (*MRPL40*), *tropomyosin* (*TPM1*) (Friesen et al., 1999), *actin* (Palumbi and Baker, 1994) and *histone* (*H2AF* – Slade et al., 1993) genes. The exon locations of all primers are shown in Table 1.

Whole genomic DNA extractions from liver and blood samples were as in Creer et al. (2003). Primers were initially tested on the candidate OTUs according to the PCR thermal cycling parameters outlined in the source literature. If unsuccessful, further PCR reactions were carried out with combinations of the following generic PCR program. Initial denaturation of 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C (1 min), annealing at 47–60 °C (1 min), extension at 72 °C (1 min), and a final extension of 72 °C (15 min). If necessary, all 25 μL PCR reactions (1×PCR buffer, 0.8 mM total dNTPs, 0.4 μM primers) were further optimized by assaying different concentrations of magnesium (1–4 mM $MgCl_2$) and using either a standard generic *Taq*, or Sigma JumpStart™ *Taq* DNA polymerase. All PCRs were performed on an Applied Biosystems GeneAmp® PCR System 3700 alongside a negative control (upH_2O) to exclude the possibility of contamination. Double stranded PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining (Dowling et al., 1996). If the PCR assays yielded discrete bands of the appropriate size, PCR products were then excised, pooled and cleaned using Qiaquick columns (QIAGen). Single stranded sequencing was then perfomed using dye-labeled terminators (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit), and subsequently run on an ABI Prism 377 DNA sequencer.

If unexpected perturbations were experienced in the sequencing reactions of any OTU of a locus displaying significant phylogenetic signal, the PCR product was cloned using an Invitrogen™ life technologies TOPO TA Cloning® kit (incorporating pCR®2.1-TOPO® and TOP10F′ One Shot® Chemically Competent cells) according to the manufacturer's instructions. If a heterozygote was suspected, five individual transformed One Shot® colonies were sequenced using combinations of standard M13 forward and reverse primers to ensure that both alleles of the intron would be detected.

Table 1
Summary data for the optimal PCR reactions resulting from the assayed EPIC primer pairs. Align. size shows the total size (bp) of resulting sequence alignments with the exception of the third intron of the *TATA-box binding protein* and the 7th intron of the *β-fibrinogen* gene, that correspond to the characterized actual intron size. The species abbreviations apply to *Cryptelytrops albolabris* (*C. a*), *Viridovipera vogeli* (*V. v*), *Popeia popeiorum* (*P. p*), *Trimeresurus borneensis* (*T. b*), *Tropidolaemus wagleri* (*T. w*), *Protobothrops mucrosquamatus* (*P. m*), *Garthius chaseni* (*G. c*) and *Calloselasma rhodostoma* (*C. r*). The intron loci abbreviations are as in the Materials and methods. Var. and PI show the number of variable sites and the number of parsimony informative sites, respectively. YES corresponds to PCR reactions that yielded a discrete band of the appropriate size according to the relevant primer source. NO and a horizontal dashed line corresponds to PCR reactions that resulted in failure and multiple, smeared products, respectively. Underlined YESs represent PCR fragments that were successfully sequenced and [h] denotes that a LVH was recovered via cloning

Locus	Exon primer sites	*C. a*	*V.v*	*P. p*	*T. b*	*T. w*	*P. m*	*G. c*	*C. r*	Align. size	Var.	PI
TBP	3, 4	YES	YES	YES	YES	NO	YES	YES	YES	772	117	24
FGB	7, 8	YES	YES[h]	YES	YES	YES	YES	YES[h]	YES	1387	195	54
ALDOB	3, 5	–	–	–	–	–	–	–	–	–	–	–
ENO1	8, 9	YES	YES	YES	YES	YES	YES	YES	YES	178	8	0
GAPD	11, 12	YES	YES	YES	YES		YES	YES	YES	296	24	3
LMNA	3, 4	YES	YES	YES	YES	YES	YES	YES	YES	731	121	17
LDHB	3, 4	NO	NO	NO	NO	NO	NO	NO	NO	–	–	–
MBP	4, 5	–	–	–	–	–	–	–	–	–	–	–
ODC1	6, 8	NO	YES	NO	YES	YES	YES	NO	YES	516	33	3
MRPL40	5, 6	YES	YES	YES	YES	YES	YES	YES	YES	436	46	7
TPM1	5, 6	YES	YES	YES	YES	YES	YES	YES	YES	875	86	5
Actin	1, 2	–	–	–	–	–	–	–	–	–	–	–
H2AF	2, 4	NO	NO	NO	NO	NO	NO	NO	NO	–	–	–

GenBank accession numbers are: *TBP—C.a.* DQ117520, *V.v.* DQ117511, *P.p.* DQ117496, *T.b.* DQ117495, *P.m.* DQ131649, *O.c.* DQ131650, *C.r.* DQ131651; *FGB—C.a.* DQ131656, *V.v.* DQ116989-90, *P.p.* DQ116977, *T.b.* DQ116973, *T.w.* DQ131655, *P.m.* DQ131657, *O.c.* DQ131652-53, *C.r.* DQ131654; *ENO1–T.w.* DQ131660, *P.m.* DQ131659, *C.r.* DQ131658; *GAPD—V.v.* DQ131662, *T.b.* DQ131663, *P.m.* DQ131664, *O.c.* DQ131661, *C.r.* DQ131665; *LMNA—C.a.* DQ131666, *V.v.* DQ131667, *P.p.* DQ131668, *T.b.* DQ131669, *T.w.* DQ131670, *P.m.* DQ131671, *O.c.* DQ131672, *C.r.* DQ131673; *ODC1–V.v.* DQ131676, *T.w.* DQ131675, *P.m.* DQ131674, *C.r.* DQ131677, *MRPL40–C.a.* DQ131684, *V.v.* DQ131685, *P.p.* DQ131683, *T.b.* DQ131681, *T.w.* DQ131680, *P.m.* DQ131679, *O.c.* DQ131682, *C.r.* DQ131678; *TPM1–P.p.* DQ131689, *T.b.* DQ131690, *T.w.* DQ131686, *G.c.* DQ131688 and *C.r.* DQ131687.

Resulting sequence chromatograms were read using Chromas (Technelysium Pty. Ltd, 1988–2000) and all mutations/indels were individually checked by eye at all sites across all taxa. In order to confirm, or refute intron identity, sequences were subjected to the National Center for Biotechnology Information (NCBI), nucleotide BLAST search engine (Altschul et al., 1997). Finally, the sequences were aligned using ClustalX (Thompson et al., 1997) and MEGA2 (Kumar et al., 1993) was used to record the number of variable sites and parsimony informative sites (PI – i.e., present or absent in just one OTU).

Results and discussion

Referring to Table 1, the third intron of the *TATA box-binding protein* gene (*3ITBP*) was amplified and could be sequenced in seven out of eight of the taxa. Thus, although the primers designed to amplify the *3ITBP* were derived from just two genetically disparate taxa (*Viridovipera stejnegeri* and *Protobothrops flavoviridis*), the theory underpinning EPIC primer design (Lessa, 1992; Slade et al., 1993) and the conserved nature of the exons at this taxonomic level facilitate cross amplification in a wide range of target taxa. Likewise, the *7IFGB* Asian pitviper specific primers proved successful in all of the test taxa. Therefore, both taxon-specific primer design approaches (cDNA mRNA—genomic comparisons and redesigning internal primers from existing successful EPIC amplifications (Slade et al., 1993)) were highly effective protocols for effective acquisition of target data. Furthermore, the introns were of sufficient length (alignments of 772–1387 bp) and exhibited enough phylogenetic signal (between 117 and 195 variable positions yielding 24–54 PI sites) to be considered as suitable intron markers for Asian pitviper phylogenetics (Creer et al., 2003).

Conversely, the universal primers derived from Slade et al. (1993), Palumbi and Baker (1994) and Friesen et al. (1997, 1999) revealed less consistent results. The *ALDOB*, *LDHB*, *MBP*, actin and *H2AF* loci could not be amplified at all, or resulted in smeared or multiple products. More success resulted from amplifications with the *GAPD* and *ODC1* primers and discrete PCR bands were observed from all amplifications with the *ENO1*, *LMNA*, *MRPL40* and *TPM1* loci. Therefore, although EPIC primers are constructed adhering to universal primer design strategies (Slade et al., 1993; Palumbi, 1996; Jarman et al., 2002), exon evolution in genetically disparate taxa can result in the failure of EPIC primers to anneal to putative priming sites (Zhang and Hewitt, 2003). Notably, the JumpStart™ *Taq* DNA polymerase either significantly improved the specificity and yield of PCR amplifications, or resulted in amplifications that were not significantly worse than those derived from a generic *Taq* DNA polymerase. The latter observation strongly suggests that a specialized *Taq* DNA polymerase should be routinely used when using universal EPIC primers on low copy number (Friesen, 2000) nuclear gene regions.

The resulting alignments ranged from 178 to 1387 bp in length, and the number of variable and PI positions ranged from 8–195 and 0–54, respectively. For phylogenetic purposes, the number of PI positions yielded by a marker is of vital importance. Thus, although the *TPM1* locus resulted in an 875 bp alignment, the data only yielded five parsimony informative positions, which would not fulfil the requirements of an optimal intron marker. Similarly, the *ENO1*, *GAPD*, *ODC1* and *MRPL40* amplifications were either too small, or yielded too little phylogenetic signal to warrant further investment of time and money. The screen of the generic EPIC primers did however, reveal that the third intron of the *lamin A* gene (*3ILMNA*) was of an appropriate size (731 bp) and yielded sufficient signal (17 PI sites from 121 variable positions) to be considered as a suitable intron marker for the target genetic range.

Two cases of LVHs were detected from the *7IFGB* locus and further screening (unpublished data) has revealed that LVHs occur in the *3ITBP* and the *3ILMNA* gene regions. A considerable amount of studies have used introns as phylogenetic markers, but many have either not detected, or not reported the occurrence of LVHs (Johnson and Clayton, 2000; Pitra et al., 2000; Jenkins et al., 2001; Rockman et al., 2001; Braband et al., 2002; Ericson et al., 2002; Rowe and Honeycutt, 2002). Recently however, studies have detected and incorporated LVH data into phylogenetic appraisals (Beltrán et al., 2002; Sota and Vogler, 2003; Pons et al., 2004). The discrepancy between such studies is paradoxical, but the present data suggest that LVHs are relatively common when working with EPIC primers.

Conclusion

To summarize, a taxon-specific approach to EPIC primer design (of gene regions of up to approximately 1000 bp) appears to be the best and most cost-effective strategy towards identifying optimal intron markers for phylogenetic reconstruction. The latter can take the form of a bioinformatic approach, or redesigning internal primers from existing EPIC amplified products that are of sufficient length (Slade et al., 1993). If these strategies are not possible, screening large numbers of universal EPIC primers (using diverse strategies of PCR optimization) on a genetically disparate target range of OTUs is likely to reveal suitable loci, or at least provide further sequences that taxon-specific EPIC primers can be designed from. Moreover, LVHs appear to be

common in intron loci, and are the predominant reason for apparent terminations in direct sequencing reactions. This study has shown that a small investment in time, consumables and primers at the outset of a project can successfully identify markers that are appropriate for intron-based molecular systematics. We predict that similar approaches will be universally applicable to other non-model taxon groups.

Acknowledgements

This work was primarily funded by The Wellcome Trust (057257/Z/99/Z; 060384/Z/00/Z) and The Leverhulme Trust (F174/O) grants to R.S.T. and A.M. We would like to thank Ross McEwing for valuable advice on PCR chemistry and Bryan Stuart and Alec Knight for providing comments that improved an earlier version of this manuscript.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25, 3389–3402.

Belshaw, R., Quicke, D.L.J., 2002. Robustness of ancestral state estimates: evolution of life history strategy in ichneumonoid parasitoids. Syst. Biol. 5, 450–477.

Beltrán, M., Jiggins, C.D., Bull., V., Linares, M., Mallet, J., McMillan, W.O., Bermingham, E., 2002. Phylogenetic discordance at the species boundary: comparative gene genealogies among rapidly radiating *Heliconius* butterflies. Mol. Biol. Evol. 19, 2176–2190.

Braband, A., Richter, S., Hiesel, R., Schlotte, G., 2002. Phylogenetic relationships within the Phyllopoda (Crustacea, Branchiopoda) based on mitochondrial and nuclear markers. Mol. Phyl. Evol. 25, 229–244.

Creer, S., Malhotra, A., Thorpe, R.S., 2003. Assessing the phylogenetic utility of four mitochondrial genes and a nuclear intron in the Asian pit viper genus *Trimeresurus*: Separate, simultaneous, and conditional data combination analyses. Mol. Biol. Evol. 20, 1240–1251.

Dowling, T.E., Moritz, C., Palmer, J.D., Rieseberg, L.M., 1996. Nucleic acids III: Analysis of fragments and restriction sites. In: Hillis, D.M., Mable, B.K., Moritz, C. (Eds.), Molecular Systematics, 2nd edn. Sinauer Associates, Sunderland, MA, pp. 249–320.

Ericson, P.G.P., Christidis, L., Irestedt, M., Norman, J.A., 2002. Systematic affinities of the lyrebirds (Passeriformes: Menura), with a novel classification of the major groups of passerine birds. Mol. Phyl. Evol. 25, 53–62.

Flynn, J.J., Nedbal, M.A., 1998. Phylogeny of the Carnivora (Mammalia): Congruence vs. incompatability among multiple data sets. Mol. Phyl. Evol. 9, 414–426.

Friesen, V.L., 2000. Introns. In: Baker, A.J. (Ed.), Molecular Methods in Ecology. Blackwell Science, pp. 274–294.

Friesen, V.L., Congdon, B.C., Kidd, M.G., Birt, T.P., 1999. Polymerase chain reaction (PCR) primers for the amplification of five nuclear introns in vertebrates. Mol. Ecol. 8, 2141–2152.

Friesen, V.L., Congdon, B.C., Walsh, H.E., Birt, T.P., 1997. Intron variation in marbled murrelets detected using analyses of single-stranded conformational polymorphisms. Mol. Ecol. 6, 1047–1058.

Giribet, G., Wheeler, W.C., 1999. On gaps. Mol. Phyl. Evol. 13, 132–143.

Jarman, S.N., Ward, R.D., Elliot, N.G., 2002. Oligonucleotide primers for PCR amplification of coelomate introns. Mar. Biotechnol. 4, 347–355.

Jenkins, T.M., Dean, R.E., Verkerk, R., Forschler, B.T., 2001. Phylogenetic analyses of two mitochondrial genes and one nuclear intron region illuminate European subterranean termite (Isoptera: Rhinotermitidae) gene flow, taxonomy, and introduction dynamics. Mol. Phyl. Evol. 20, 286–293.

Johnson, K.P., Clayton, D.H., 2000. Nuclear and mitochondrial genes contain similar phylogenetic signal for pigeons and doves (Aves: Columbiformes). Mol. Phyl. Evol. 14, 141–151.

Kumar, S., Tamura, K., Nei, M., 1993.MEGA: Molecular Evolutionary Genetics Analysis 1.01. Penn State University, USA.

Lessa, E.P., 1992. Rapid surveying of DNA sequence variation in natural populations. Mol. Biol. Evol. 9, 323–330.

Lutzoni, F., Wagner, P., Reeb, V., Zoller, S., 2000. Integrating ambiguously aligned regions of DNA sequences in phylogenetic analyses without violating positional homology. Syst. Biol. 49, 628–651.

Malhotra, A., Thorpe, R.S., 2004. A phylogeny of four mitochondrial gene regions suggests a revised taxonomy for Asian pitvipers (*Trimeresurus and Ovophis*). Mol. Phyl. Evol. 32, 83–100.

Moore, W.S., 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. Evolution. 49, 718–726.

Nakashima, K., Nobuhisa, I., Deshimaru, M., Ogawa, T., Shimohigashi, Y., Fukumaki, Y., Hattori, M., Sakaki, Y., Hattori, S., Ohno, M., 1995. Structures of genes encoding TATA box-binding proteins from *Trimeresurus gramineus and T. flavoviridis* snakes. Gene. 152, 209–213.

Oakley, T.H., Phillips, R.B., 1999. Phylogeny of salmonine fishes based on growth hormone introns: Atlantic (*Salmo*) and Pacific (*Oncorhynchus*) salmon are not sister taxa. Mol. Phyl. Evol. 11, 381–393.

van Oppen, M.J.H., Willis, B.L., van Vugt, H.W.J.A., Miller, D.J., 2000. Examination of species boundaries in the *Acropora cervicornis* group (Scleractina, Cnidaria) using nuclear DNA sequence analyses. Mol. Ecol. 9, 1363–1373.

Palumbi, S.R., 1996. Nucleic acids III: The polymerase chain reaction. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics, 2nd edn. Sinauer Associates, Sunderland, MA, pp. 205–248.

Palumbi, S.R., Baker, C.S., 1994. Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. Mol. Biol. Evol. 11, 426–435.

Pitra, C., Lieckfeldt, D., Alonso, J.C., 2000. Population subdivision in Europe's great bustard inferred from mitochondrial and nuclear DNA sequence variation. Mol. Ecol. 9, 1165–1170.

Pons, J., Barraclough, T.G., Theodorides, K., Cardoso, A., Vogler, A.P., 2004. Using exon and intron sequences of the gene Mp20 to resolve basal relationships in *Cicindela* (Coleoptera: Cicindelidae). Syst. Biol. 53, 554–570.

Prychitko, T.M., Moore, W.S., 1997. The utility of DNA sequences of an intron from the β-fibrinogen gene in phylogenetic analysis of woodpeckers (Aves: Picidae). Mol. Phyl. Evol. 8, 193–204.

Prychitko, T.M., Moore, W.S., 2000. Comparative evolution of the mitochondrial cytochrome b gene and nuclear β-fibrinogen intron 7 in woodpeckers. Mol. Biol. Evol. 17, 1101–1111.

Rockman, M.V., Rowell, D.M., Tait, N.N., 2001. Phylogenetics of *Planipapillus*, lawn-headed onychophorans of the Australian Alps, based on nuclear and mitochondrial gene sequences. Mol. Phyl. Evol. 21, 103–116.

Rosen, S., Skaletsky, H.J., 1998. Primer3. Code available at http://www-genome.wi.mit.edu/genomesoftware/other/primer3.html.

Rowe, D.L., Honeycutt, R.L., 2002. Phylogenetic relationships, ecological correlates, and molecular evolution within the Cavioidea (Mammalia, Rodentia). Mol. Biol. Evol. 19, 263–277.

Sanchis, A., Michelena, J.M., Latorre, A., Quicke, D.L.J., Gärdenfors, U., Belshaw, R., 2001. The phylogenetic analysis of variable-length sequence data: elongation factor-1α introns in European populations of the parasitoid wasp genus *Pauesia* (Hymenoptera: Braconidae: Aphidiinae). Mol. Biol. Evol. 18, 1117–1131.

Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49, 369–381.

Slade, R.W., Moritz, C., Heidemann, A., Hale, P.T., 1993. Rapid assessment of single-copy nuclear DNA variation in diverse species. Mol. Ecol. 2, 359–373.

Sota, T., Vogler, A.P., 2003. Reconstructing species phylogeny of the carabid beetles *Ohomopterus* using multiple nuclear DNA sequences: heterogenous information content and the performance of simultaneous analyses. Mol. Phyl. Evol. 26, 139–154.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24, 4876–4882.

Weibel, A.C., Moore, W.S., 2002. A test of a mitochondrial gene-based phylogeny of woodpeckers (genus *Picoides*) using an independent nuclear gene β-Fibrinogen intron 7. Mol. Phyl. Evol. 22, 247–257.

Wiens, J.J., Penkrot, T.A., 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). Sys. Biol. 51, 69–91.

Zhang, D.-X., Hewitt, G.M., 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Mol. Ecol. 12, 563–584.