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PRIMER NOTE

Primers for amplifying the hypervariable, male-transmitted *COII-COI* junction region in amblemine freshwater mussels (Bivalvia: Unionoidea: Ambleminae)

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Abstract

Freshwater bivalves in the superfamily Unionoidea possess distinct male (M)- and female (F)-transmitted mitochondrial DNA (mtDNA). The former evolves independently of and at a significantly faster rate than the latter. Thus, population genetic and phylogenetic analyses of M sequences facilitate the generation of independent estimates of genetic variation and evolutionary relationships which are often more robust than those provided by analyses of F sequences alone. However, M mtDNA's rapid substitution rate often renders polymerase chain reaction (PCR) amplification difficult with 'universal' primers. Herein, we report on three pairs of PCR primers that consistently amplify the hypervariable M *COII-COI* gene junction region in 25 bivalve genera (Unionoidea: Ambleminae).

Keywords: Ambleminae, *COI*, *COII*, DUI, hypervariable region, mtDNA

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Unionoidean bivalves exhibit a unique mode of mitochondrial DNA (mtDNA) inheritance, termed doubly uniparental inheritance (DUI) (e.g. Walker *et al.* 2006). In this system, progeny inherit distinct female (F)- and male (M)-transmitted mitochondrial genomes. Female progeny are usually homoplasmic for the F genome while male progeny are typically heteroplasmic, possessing F mtDNA in somatic tissues and M mtDNA concentrated within the testes tissues. Estimated intra-male divergence between M and F mtDNA lineages, based on p-distances of partial *COI* sequences, ranged from 28 to 33% in three species of freshwater mussels with the M lineage evolving more rapidly than F (Hoeh *et al.* 1996). The presence of DUI in unionoidean bivalves allows the utilization of independently evolving mtDNA genetic markers in this ancient and generally endangered group of animals (e.g. Curole 2004; Walker *et al.* 2006). However, consistent amplification of the M mtDNA is often difficult using the currently available polymerase chain reaction (PCR) primers due to the rapidly evolving nature of the M mtDNA and the presence of contaminating F mtDNA in DNA extracts from testes.

Curole & Kocher (2002) developed a forward primer, UNIOCOII.2 which, when used in conjunction with the HCO2198 reverse primer (Folmer *et al.* 1994), amplified the cytochrome *c* oxidase subunit II (*COII*)/cytochrome *c* oxidase subunit I (*COI*) junction region in F and M genomes from various unionoidean bivalve species. As a result, they described the presence of an approximately 600 nucleotide 3′ hypervariable coding extension to the *COII* gene in the M genome. Subsequently, Curole (2004) developed two M-specific primers, with opposite polarities, which are complementary to a single location in M *COI* (~200 bp downstream of the 5′ *COI* terminus). Using the UNIOCOII.2 primer with a modified version of the HCO2198 primer, HCO-700dy2 (Fig. 1A, B), Walker *et al.* (2006) were able to amplify the M *COII-COI* junction region from a taxonomically broad array of unionoidean bivalve species. The

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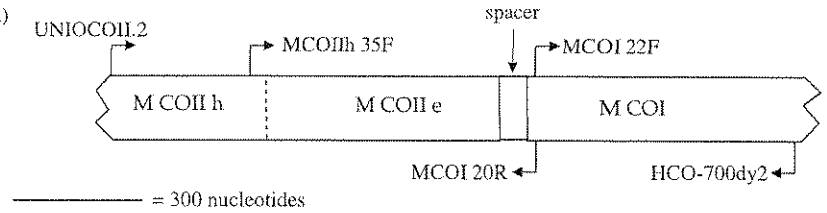
(A)



(B)

	Name	Primer Sequence 5′–3′	Annealing temperature	Extension time	Amplicon size
A.	*MCOIIh 35F *MCOI 20R	TTTATRCCTRTKTGTGTRGARGCTGT GTCCCAATATCYTTATGRTTAGT	55 °C	60 s	~200–800 bp
B.	MCOIIh 35F HCO-700dy2[b]	TCAGGGTGACCAAAAAAYCA	55 °C	120 s	~1.0–1.4 kb
C.	UNIOCOII.2[a] MCOI 20R	CAGTGGTATTGGAGGTATGAGTA	45 °C	60 s	~600 bp–1.0 kb
D.	*MCOI 22F HCO-700dy2[b]	RTGCGTTGRRYDTTTTCBACTA	45 °C	60 s	716 bp

*M genome-specific primer
[a]From Curole and Kocher (2002)
[b]From Walker *et al.* (2006)

Fig. 1 (A) Gene map of the *Ptychobranchus fasciolaris* (GenBank Accession nos EF033301 and EF033321) M *COII-COI* junction region with priming sites indicated and (B) the primer pairings, thermal profiles, and amplicon length ranges of this study.

resulting amplicons had a maximum length of ~1.8 kb (Curole & Kocher 2002; Walker *et al.* 2006). In our hands, the UNIOCOII.2 + HCO-700dy2 primer pairing produced largely M-specific amplicons from amblemine bivalve [Unionoidea: Ambleminae (*sensu* Davis & Fuller 1981)] total DNA templates isolated from testes but amplifications from a number of species yielded either (i) both F and M amplicons (see Curole & Kocher 2002) or (ii) only F amplicons. To facilitate the consistent amplification and sequencing of the M *COII-COI* region, and to increase our ability to recover this region from degraded DNA samples, three new internal, M genome-specific primers were designed. Primer design was facilitated by aligning amblemine F and M *COII-COI* sequences obtained from GenBank with those we generated recently. Alignment of these sequences was done manually and, to target amplification of the M genome, primers were designed in locations where fixed nucleotide differences between amblemine bivalve M and F sequences were noted.

Forward and reverse primers were designed to target amplification of the hypervariable M *COII* extension (M*COII*e)/intergenic spacer region (M*COII*h 35F and M*COI* 20R, Fig. 1). The forward primer, M*COII*h 35F, is located in the 3′ end of the *COII* homologous (M*COII*h) region [i.e. the *COII* region present in both female and male mtDNA genomes (Curole & Kocher 2002)]. The reverse primer, M*COI* 20R, is located in the 5′ end of the *COI* gene slightly downstream of the initiation codon. These extension primers have been successfully used with testes-derived DNA extracts from representatives of 25 amblemine genera (Table 1) and have also produced amblemine M amplicons when used in conjunction with the UNIOCOII.2 and HCO-700dy2 primers (Fig. 1A). A third primer, M*COI* 22F (Fig. 1A, B), has been successfully used, in conjunction with HCO-700dy2, to produce amblemine M *COI* amplicons.

Amplification and sequencing of M amplicons utilized testes-derived total DNA templates (DNeasy Tissue Kit, QIAGEN) obtained from multiple representatives of the species listed in Table 1. PCRs for all primer pairs took place in a 50 μL volume and consisted of 1× QIAGEN PCR buffer, 0.2 mM of each dNTP, 0.5 μM of each primer, 1.0 U of QIAGEN *Taq*, and 2 μL of total DNA extract (~20 ng of DNA). Optimal annealing temperatures for each primer pair were determined using an MJ PTC-200 gradient cycler. Reactions for all primer pairs were cycled using the following basic temperature/time/cycles profile with primer pair-specific changes in annealing temperature and extension times noted in Fig. 1(B): 85 °C for 60 s (94 °C for 60 s, 45 °C for 60 s, and 72 °C for 60 s) × 40 cycles and a final extension time of 10 min. Amplicons were sequenced to confirm both locus and M genome specificities. Template purification and direct sequencing protocols followed Walker *et al.* (2006).

Table 1 Amblemine bivalve species for which M genome amplification and sequence was confirmed using the four primer pairings given in Fig. 1

Actinonaias ligamentina
Amblema plicata
Cyprogenia aberti
Cyrtonaias tampicoensis
Dromus dromas
Ellipsaria lineolata
Epioblasma brevidens
Glebula rotundata
Hamiota subangulata
Lampsilis ovata
Lemiox rimosus
Leptodea fragilis
Ligumia recta
Medionidus conradicus
Obliquaria reflexa
Obovaria olivaria
Plectomerus dombeyanus
Pleurobema sintoxia
Popenaias popeii
Potamilus alatus
Ptychobranchus fasciolaris
Quadrula quadrula
Truncilla truncata
Venustaconcha ellipsiformis
Villosa villosa

All sequences obtained from these amplicons were confirmed as locus-specific M mtDNA sequence via comparisons with GenBank M sequences and in phylogenetic analyses containing both F and M sequences, supporting the hypothesis that these new primers are specific for the amplification of amblemine bivalve M *COII-COI* DNA. We anticipate that these three primer pairings will yield M-specific amplicons from most amblemine bivalve testes-derived total DNA templates and the ability of these new primer pairs to consistently amplify DNA sequences from the independently and more rapidly evolving M genome should greatly facilitate studies of amblemine bivalve phylogeny, phylogeography, and population genetic structure.

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