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TECHNICAL NOTE

A rapid molecular method to detect the invasive golden apple snail *Pomacea canaliculata* (Lamarck, 1822)

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Abstract The golden apple snail, *Pomacea canaliculata* has become a major agricultural and environmental pest across Asia. Here, using the mitochondrial cytochrome oxidase I as a diagnostic, we develop a multiplex PCR, that discriminates *P. canaliculata* from the most common non-invasive *Pomacea* species, thereby providing a fast and reliable diagnostic tool.

Keywords Invasive species · Multiplex PCR · Ampullariidae · COI

Technical note

One of the major threats to global biodiversity is invasive exotic species. The golden apple snail, *Pomacea canaliculata* (Lamarck, Gastropoda, Ampullariidae) is native to South America but was introduced to East and South-East Asia during the 1980s for commercial production and as a dietary protein supplement (Anderson 1993; Matienzo 1984; Mochida 1991). *P. canaliculata* feed on aquatic flora and inhabit slow-moving rivers, dams and irrigation channels. They are tolerant to variable temperatures, salinities and humidity making them eminently suited for successful dispersal (Cowie 2002). Since their introduction to East and South-East Asia, *P. canaliculata* have escaped, becoming a major pest to rice crops and other freshwater environments across Asia reaching Japan, the Philippines, New Guinea and Malaysia (Pain 1946; Carlsson et al. 2004; Hirai 1988; Rejesus et al. 1990; Halwart 1994). There are serious concerns that *P. canaliculata* will also establish and spread throughout much of northeast Australia, Bangladesh and India (Department of Agriculture, Forestry and Fisheries, Australia).

One of the major challenges in an effective management strategy across Australasia has been the development of a definitive diagnosis of exotic ampullarids. Indeed, there are no clear taxonomic criteria for distinguishing among species since the external differences are subtle; the within-species variation is high (e.g. Estebenet and Martin 2003), and separation of the highly invasive *P. canaliculata* from other ampullarid snails can only be accomplished by a trained malacologist. Yet, for urgent diagnoses, such as quarantine operations, timely and correct identifications are critical.

Molecular diagnostic techniques such as DNA 'barcoding' can circumvent problems associated with morphological identification (e.g. Herbert et al. 2003). For example, Cytochrome Oxidase Subunit I (COI) is easily amplified with universal primer sets across different animal species and has high phylogenetic resolution between species compared to other mitochondrial genes (Folmer et al. 1994). Here, using COI as the marker, we develop a rapid DNA-based diagnostic protocol that identifies *P. canaliculata*, from the non-invasive but phenotypically similar species *P. insularum* and *P. bridgesii* without the use of DNA sequencing.

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Table 1 Primers used in multiplex PCR to amplify the mtDNA COI gene in *Pomacea* sp.

Name	Annealing temperature C°	Sequence 5′–3′	References
LCO1490[a]	52	GGT CAA CAA ATC ATA AAG ATA TTG	Folmer et al. (1994)
HCO2198[a]	52	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer et al. (1994)
PcanCOI	55	TGG GGT ATG ATC AGG CC	Matsukura et al. (2008)
PinsCOI	55	ATC TGC TGC TGT TGA AAG	Matsukura et al. (2008)
PbridCOI	55	CTC TAT CCT AGG TGC GGT AAA TT	Designed by the authors

[a] Universal COI primers

DNA was extracted from 16 *Pomacea* samples from 10 to 50 mg of frozen tissue or eggs using the DNeasy® Blood and Tissue kit (Qiagen). COI was amplified by PCR in *P. canaliculata*, *P. insularum* and *P. bridgesi*, in reactions containing 1× CL buffer (Qiagen), 0.5× *Q* solution (Qiagen), 3.5 mM $MgCl_2$, 0.05 mM of each dNTP, 10 µm of each primer LCO1490 and HCO2198 (Table 1), 2× BSA, and 5U of *Taq* polymerase (Qiagen). PCR cycling conditions were 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and a final extension of 72°C for 5 min. PCR product was checked using 1% TAE agarose gel electrophoresis and purified using ExoSap-IT® (USB Corporation, USA). Purified PCR product was sequenced by the Australian Genome Research Facility (AGRF) using an AB-3730xl (Applied Biosystems, USA).

Sequences were aligned using SEQUENCHER (Gene Codes Corporation, USA) and checked for the absence of stop codons. The universal COI primers amplified ~658 base pairs (bp) fragments for all tested snails with suitable DNA quality. It should be noted that DNA extracted from degraded tissue samples did not amplify or produced low quality sequence data that was unsuitable for further analyses. *Pomacea* species identities were established or confirmed using GenBank® BLAST and the Barcode of Life Data System (BOLD). Samples that were identified with 99–100% confidence were used for Neighbour-joining phylogentic analysis using PAUP v.4.0 to assess the resolution of COI to distinguish between *Pomacea* species of interest.

Generally, nucleotide divergence was low within-species (0–5%) and moderate between species ranging from 10% between *P. insularum* and *P. canaliculata* to 17% between *P. insularum* and *P. bridgesii*. Neighbor-joining phylogenetic analysis resulted in three well supported monophyletic clades representing each species. Species

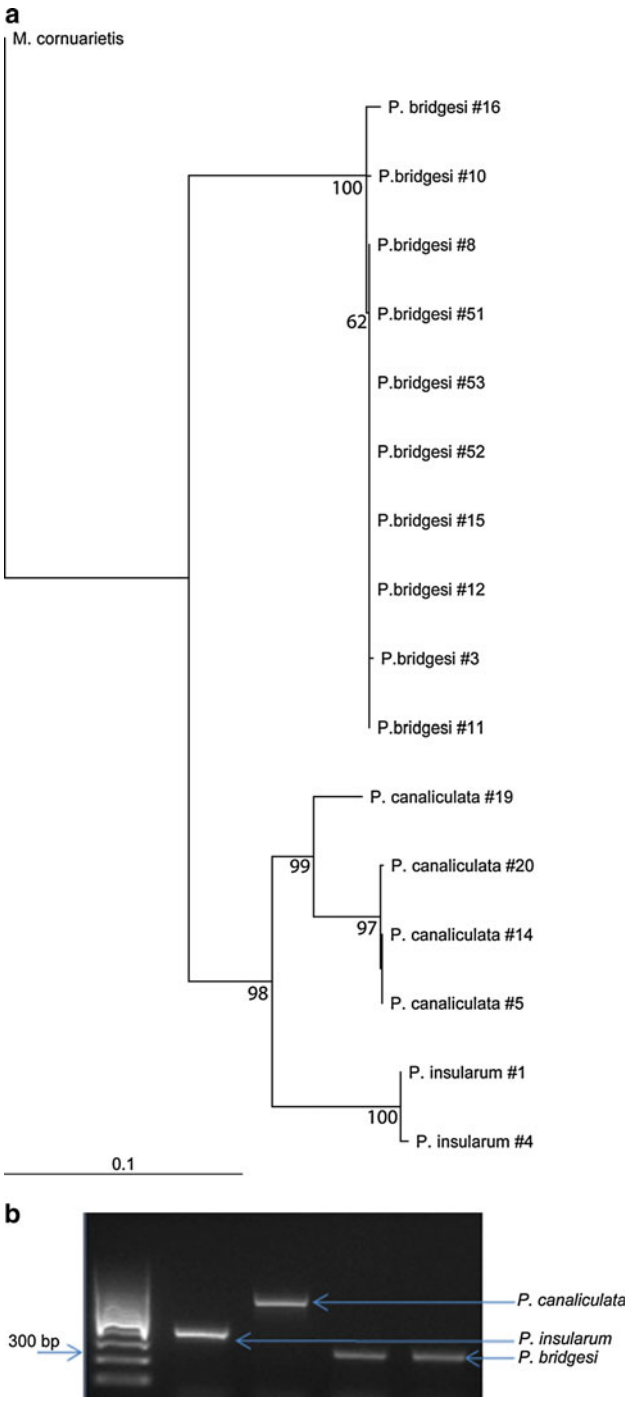

Fig. 1 **a** Neighbour-joining phylgenetic tree showing relationships between *P. canaliculata*, *P. insularum*, and *P. bridgesii* inferred from 658 bp of COI mtDNA gene. Numbers below branches are bootstrap support values based on 1,000 bootstrap replicates, **b** Image of agarose gel electrophoresis showing length differences of the amplified fragments between *P. canaliculata*, *P. insularum*, and *P. bridgesii*. Fragments amplified were 666, 390 and 250 bp, respectively

identities within the phylogenetic tree were consistent with GenBank and BOLD database results.

Visual comparison of the nucleotide sequences between each species, in combination with genetic distance and phylogenetic analysis suggested that nucleotide differences in COI were valid differentiators between species (Fig. 1). To create a single rapid molecular method to distinguish *P. canaliculata*, *P. insularum* and *P. bridgesi*, we developed a multiplex PCR protocol employing species specific forward primers in combination with the universal reverse primer. Here, forward primers from Matsukura et al. (2008) for *P. canaliculata* and *P. insularum* that amplify a 666 bp and a 390 bp fragment of the COI, respectively were used (Table 1). For *P. bridgesi*, a forward primer was designed in OLIGO® v.7 (MBI, USA) for a 250 bp fragment of COI. The universal reverse primer HCO2198 was used in the multiplex PCR. Amplification of COI fragments in the multiplex PCR used the same reaction conditions as above with 10 μm of each primer HCO2198, PcanCOI, PinsCOI and PbridCOI (Table 1). Multiplex PCR products were then assessed using 1% TAE agarose gel electrophoresis. In all amplifications, multiple fragments were not detected simultaneously in any snail. It must be noted however, if another ampullariid species is sampled, this multiplex PCR will produce uninformative results. Here, the new sample should be identified via PCR and DNA sequencing using primers LCO1490 and HCO2198 and sequence results can be used to design a new primer that is compatible with the multiplex PCR described here.

We have developed a fast and reliable molecular method that easily discriminates *P. canaliculata* from its most common congeners without requiring DNA sequencing. This approach is superior to traditional barcoding which also requires DNA purification, COI sequencing and sequence analysis. Thus, for rapid diagnostic purposes, the use of species-specific amplified fragments of COI in a multiplex PCR provides suitable resolution between target species without the need for DNA sequencing.

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