# Species-Specific Primers Designed from RAPD Products for *Bithynia funiculata*, the First Intermediate Host of Liver Fluke, *Opisthorchis viverrini*, in North Thailand

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# SPECIES-SPECIFIC PRIMERS DESIGNED FROM RAPD PRODUCTS FOR *BITHYNIA FUNICULATA,* THE FIRST INTERMEDIATE HOST OF LIVER FLUKE, *OPISTHORCHIS VIVERRINI*, IN NORTH THAILAND

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ABSTRACT: *Bithynia funiculata* is the first intermediate host of the human liver fluke *Opisthorchis viverrini* in northern Thailand but its identification through morphological analysis is often problematic due to the shortage of gastropod taxonomists. As a consequence, we focused on the development of species-specific primers for use as an identification tool. Our work involved recovery of a 502-base pair (bp) amplicon of unknown function through species-specific primers whose effectiveness was tested by analyzing specimens of *B. funiculata* from 3 locations in northern Thailand. This primer set did not amplify other species in the Bithyniidae or in other gastropod families. By providing a tool to confirm morphological identifications of *B. funiculata*, and by enabling the identification of juvenile specimens and those with damaged shells, these primers will improve estimates of the prevalence of parasitic infections in this snail.

Opisthorchiasis is a food-borne parasitic infection caused by 3 species (*Opisthorchis viverrini*, *Opisthorchis felineus*, and *Opisthorchis* (*Clonorchis*) *sinensis*) of helminths and which afflicts 10 million people worldwide (WHO, 1995; Schuster, 2010). This disease has a high prevalence in the Greater Mekong region (Cambodia, Lao People's Democratic Republic, Thailand, and the south of Vietnam,), central and eastern Europe (Germany, Poland, Russia, Turkey, and other parts of the former Soviet Union), and in east Asia (China, Korea, Taiwan, and the north of Vietnam) (IARC, 1994, 2011; Keiser and Utzinger, 2005). Approximately 9.4% of the Thai population (5.7 of 61 million) suffers from this infection (Jongsuksuntigul and Imsomboon, 2003), which is a significant risk factor for cholangiocarcinoma as evidenced by both experimental and epidemiological studies (Thamavit et al., 1978; Haswell-Elkins et al., 1992; IARC, 1994; Sithithaworn et al., 1994; Vatanasapt et al., 2000; Watanapa and Watanapa, 2002; Honjo et al., 2005). The infection is also associated with other hepatobiliary diseases including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis, and biliary lithiasis (Harinasuta et al., 1984; Osman et al., 1998; Mairiang and Mairiang, 2003; Sripa et al., 2005, 2007).

Transmission of *O. viverrini* primarily involves *Bithynia funiculata* and cyprinoid fishes as first and second intermediate hosts, respectively (Wykoff et al., 1965). Two of 12 species of bithyniid snails found in Thailand and in other countries in the Greater Mekong region, *B. funiculata* and *Bithynia siamensis* (which includes 2 subspecies, *B. siamensis siamensis* and *Bithynia siamensis goniomphalos*), serve as first intermediate hosts (Brandt, 1974). These snails inhabit wetlands throughout the northeastern, central, and northern parts of Thailand (Wykoff et al., 1965; Brandt, 1974). The *Bithynia* snail habitats in Thailand are typically mixed sand–mud substrata (Chitramvong, 1992). The snail is generally found in shallow, temporary ponds, marshes, and rice fields (Ngern-Klun et al., 2006). The highest *B. s. goniomphalos* population densities are observed in water bodies within a salinity range of 2.5–5.0 ppt (Suwannatrai et al., 2011).

Species of *Bithynia* are very similar morphologically, particularly in their early stages, resulting in frequent misidentification. Definitive recognition requires careful inspection of the size, shape, and color of the shell as well as patterns of sculpture on the shell surface and operculum plus the shape and arrangement of cusps on the radular teeth (Brandt, 1974; Chitramvong, 1992; Adam et al., 1995). However, these features are often not reliable because variation in acidity and water temperature can erode and damage both shells and opercula (Kaewjam, 1987; Rollinson et al., 1998; Liu et al., 2006; Doyle et al., 2010).

Various molecular techniques have been applied for the identification of snail species. Randomly amplified polymorphic DNA (RAPD) analysis is one approach that has gained broad application because it is a relatively fast, cheap, simple technique which can be performed without prior genetic information regarding the specimen being studied (Wilkerson et al., 1993; Williams et al., 1993; Nuchprayoon et al., 2007). However, RAPD suffers from 1 disadvantage, i.e., RAPD results may not be reproducible if PCR conditions are not sufficiently stringent or if template DNA is of low quality. Despite these possible interpretational complexities, the present study focused on the development of primers for the diagnosis of *B. funiculata*.

We sought to design primers for the identification of *B. funiculata* from the DNA sequence of species-specific band(s) isolated through RAPD-PCR. In addition, the sensitivity and specificity of the primers were tested with *B. funiculata* and other snail species, including bithyniids, common in Thailand and the surrounding regions.

## MATERIALS AND METHODS

### Snail collection and screening

In total, 345 specimens of *B. funiculata* adults were collected from 4 localities in Chiang Mai Province, i.e., Mae Taeng, Muang Chiang Mai, Hang Dong, and Sankamphaeng Districts. The average shell size of the collected snails was 10.7 ± 0.1 (from the outer margin of the aperture to the apex of the shell). Snails from Mae Taeng were used for designing species-specific primers whereas those from the other localities were used

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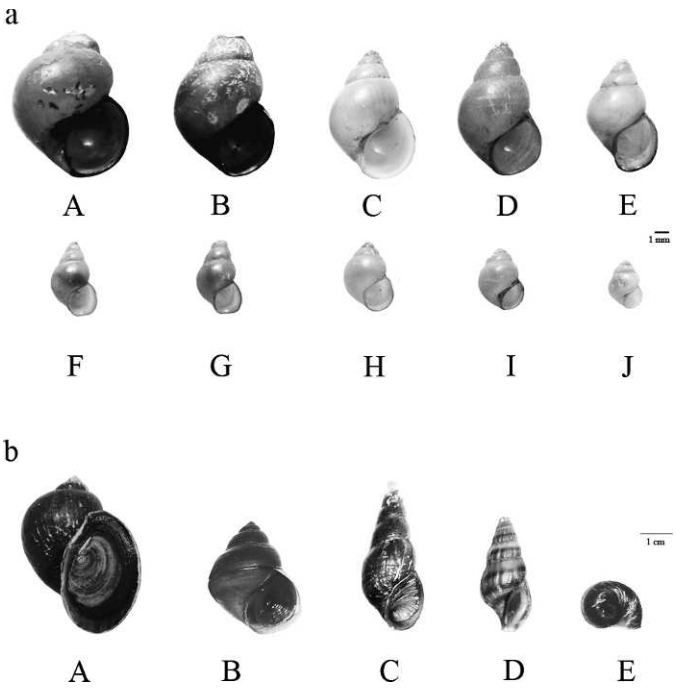


FIGURE 1. (**a**) The shell morphology of bithyniid snails (A) *Bithynia funiculata*; (B) *Bithynia siamensis goniomphalos*; (C) *Hydrobioides nassa*, *Hydrobioides nassa*; (D) *Bithynia siamensis siamensis*; (E) *Wattebledia crosseana*; (F) *Wattebledia siamensis*; (G) *Wattebledia baschi*; (H) *Gabbia wykoffi*; (I) *Gabbia pygmaea*; (J) *Gabbia erawanensis*. Scale bar: A–J = 1 mm. (**b**) The shell morphology of snails in other families. (A) Thiaridae*: Melanoides tuberculata*; (B) Buccinidae: *Clea* (*Anentome*) *helena*; (C) Viviparidae: *Filopaludina martensi martensi*; (D) Ampullaridae: *Pomacea canaliculata*; (E) Bulinidae: *Indoplanorbis exustus*. Scale bar: A–E = 1 cm.

to test the general applicability of the primer set. The primers were also tested to exclude the possibility of cross-reaction with other bithyniids including *Gabbia pygmaea* and *Gabbia wykoffi* from the north, *Gabbia erawanensis*, *Hydrobioides nassa*, *Wattebledia siamensis*, and *B. s. siamensis* from the central region, *B. s. goniomphalos* and *Wattebledia crosseana* from the northeast, and *Wattebledia baschi* from the south (Fig. 1a). Five gastropod species in other families (Ampullaridae: *Pomacea canaliculata*, Buccinidae: *Clea* (*Anentome*) *helena*, Bulinidae: *Indoplanorbis exustus*, Thiaridae: *Melanoides tuberculata*, Viviparidae: *Filopaludina martensi martensi*) were collected in northeast Thailand to further validate the species-specificity of the primer set (Fig. 1b). All snails were identified using standard morphological keys (Brandt, 1974; Chitramvong and Upatham, 1989; Chitramvong, 1992). Representatives of each species were maintained in aquaria containing a layer of soil sediment and dechlorinated tap water. Cercariae shedding (3 times within a month) and the crushing method were performed to exclude the trematode infections from the snails used for this study. To reduce possible bacterial contamination prior to DNA extraction, 10 snails of each species that had released no cercariae were soaked in phosphate-buffered saline (PBS) containing antibiotics (200 unit/ml of penicillin, 100 µg/ml of streptomycin) for 3–4 hr. PBS had no effect on the snails, as they still exhibited active movement.

### DNA extraction

The soft tissue of each snail was then removed from its shell and placed into PBS in a 1.5-ml microtube where it was crushed in CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v β-mercaptoethanol, 20 mM EDTA, 100 mM Tris HCl pH 8, 0.2 mg/ml proteinase K) (Winnepenninckx et al., 1993) and digested at 55 C for 2–3 hr. Protein precipitation was subsequently performed using phenol:chloroform (1:1), followed by phenol:chloroform:isoamyl alcohol (25:24:1) twice, and then centrifuged at 13,306 *g* for 10 min (4 C). The DNA was precipitated in isopropanol (2:3 v/v) for 10–15 min at room temperature (25 C). The DNA pellets were separated by washing twice with 75% ethanol and absolute ethanol and centrifuged at 12,000 *g* for 5 min (4 C). The pellets were dried completely before their use as a DNA template for RAPD-PCR amplification by dissolving each of them in 150 µl Tris-EDTA buffer.

### RAPD-PCR, purification, cloning, and sequencing

The level of genetic diversity in *B. funiculata* from Mae Taeng District was determined using 10 randomly selected specimens in comparison with the pooled DNA from 5 samples collected from the same locality. The pattern of bands was inspected and each band was graded as present (1) or absent (0). Genetic similarities were quantified using the Jaccard's coefficient similarity formula (Jaccard, 1908).

Pooled DNA from 5 specimens of *B. funiculata* from Mae Taeng were amplified by RAPD-PCR using 6 primers to compare with the pooled DNA extract from 5 individuals of each bithyniid species to search for a band unique to *B. funiculata*. Six primers (5′-d[GGTGCGGGAA]-3′, 5′-d[GTTTCGCTCC]-3′, 5′-d[GTAGACCCGT]-3′, 5′-d[AAGAGCCCGT]-3′, 5′-d[AACGCGCAAC]-3′, 5′-d[CCCGTCAGCA]-3′, Amersham Bioscience, London, U.K.) were tested for a unique band. Each primer is a 10-mer of arbitrary sequence that is specifically designed and tested for using in RAPD analysis. Only 1 of the 6 primers, 5′-d[AAG AGC CCG T]-3′, generated a unique band. A tube of RAPD-PCR reaction medium (final volume 25 µl) containing a Ready-To-Go™ bead (GE Healthcare Biosciences, Buckinghamshire, U.K.), 1 µl DNA template (10 ng/µl), 19 µl distilled water, and 5 µl RAPD primer (25 pmol) was prepared. A DNA extract from *Escherichia coli*, BL21 strain (2 µl), was used as a positive control. All PCR reactions were carried out in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, California) using the following cycling regime: initial denaturing at 95 C for 5 min, 45 cycles with denaturing at 95 C for 1 min, annealing at 36 C for 1 min, extension at 72 C for 2 min, and final denaturing at 72 C for 5 min. The unique 872-bp RAPD product from *B. funiculata* was separated by 1.5% TBE agarose gel electrophoresis and the band was excised. The DNA was then purified, cloned, and sequenced in the Biochemistry Department, Faculty of Medicine, Khon Kaen University and at Pacific Science Co. (Bangkok, Thailand).

### Sensitivity and specificity test

The nucleotide sequence of the unique band recovered from *B. funiculata* was used to design a species-specific primer set with Design Primer 3 (Rozen and Skaletsky, 2000) and Oligo analyzer 3. The performance of the species-specific primers was tested with DNA extracts from 15 *B. funiculata* from the 3 localities (5 individuals per locality) in Chiang Mai (Hang Dong, Muang Chiang Mai, and Sankamphaeng Districts). Each PCR was performed in a 25-µl reaction with 1 Ready-To-Go bead, 1 µl DNA template (10 ng/µl), 19 µl distilled water, and 1 µl each of the forward and reverse primers (10 µmol). The PCR Thermo cycle was 5 min at 94 C followed by 45 cycles of 1 min at 94 C, 1 min at 62 C, and 2 min at 72 C, and a final 5 min at 72 C. PCR products were separated by 1.5% TBE agarose gel electrophoresis. Duplicate amplifications were performed to confirm the reliability of band recovery. To prove the amplification products were the same sequence as the amplicon of the origin, the amplified band was cut for DNA sequencing and analysis.

The species specificity of the primers was tested using pooled DNA from 5 individuals for each of 12 species including 9 bithyniid species and 5 from other families: (Thiaridae*: M. tuberculata*, Buccinidae: *C.* (*Anentome*) *helena*, Viviparidae: *F. m. martensi*, Ampullaridae: *P. canaliculata* and Bulinidae: *I. exustus*). DNA of *B. funiculata* was used as a positive control. Each PCR reaction was performed in a 25-µl volume using conditions as described for *B. funiculata*. Duplicate amplifications were performed to confirm the reliability of band recovery.

## RESULTS

### RAPD-PCR

The level of intra-population diversity was assessed in *B. funiculata* from the Mae Taeng District. Comparisons of the unique and shared amplification products between individuals and pooled DNA of *B. funiculata* were performed based on the Jaccard's similarity coefficients (87.5–88.9%) of greater than

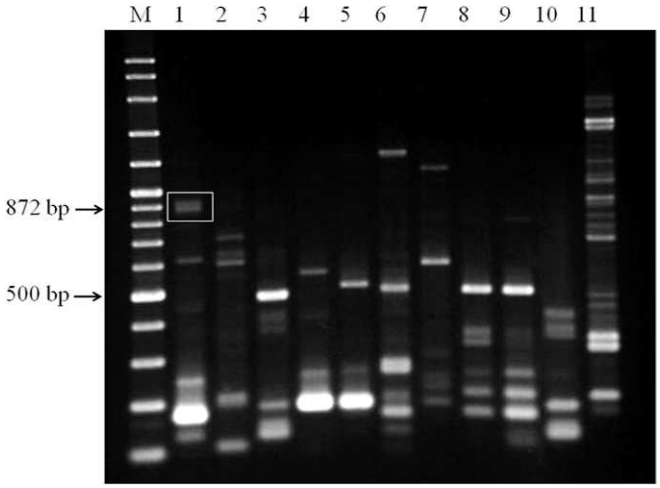


FIGURE 2. RAPD-PCR patterns of bithyniid snails showing the specific band for *Bithynia funiculata* at 872 bp that was excised for DNA sequencing. Lane M: 100 bp DNA ladder, lanes 1–11: PCR products for *B. funiculata*, *Bithynia siamensis goniomphalos*, *Bithynia siamensis siamensis*, *Wattebledia crosseana*, *Wattebledia siamensis*, *Wattebledia baschi*, *Gabbia wykoffi*, *Gabbia pygmaea*, *Gabbia erawanensis*, *Hydrobioides nassa*, and *Escherichia coli*, respectively.

80%, which means they were genetically homogeneous. A unique band in *B. funiculata* with a length of approximately 872 bp was cut from the gel with the amplified RAPD-PCR products (Fig. 2). The DNA sequence of this band of unknown function was deposited in GenBank (JF784168) and used to design a species-specific primer set. The forward primer (BF2F) 5′-GGG ATG CTC GAT TGA AAG TG-3′ and the reverse primer (BF2R) 5′-GAC CTT CCG TGA AAG TCC TG-3′ generated a 502-bp amplicon. DNA sequence comparison (NCBI BLAST) confirmed that the 502-bp amplicon was a segment of the original 872-bp RAPD band.

### Sensitivity assessment

The BF2F and BF2R primer set was found to amplify genomic DNA from *B. funiculata* collected at all 3 of the other localities in Chiang Mai Province (Muang, Hang Dong, and Sankamphaeng Districts) (Fig. 3). Of all the 20 samples tested, 19 were positive, resulting in 95% success.

### Specificity assessment

In contrast, genomic DNA from the 9 bithyniid species (*B. s. siamensis*, *B. s. goniomphalos*, *G. pygmaea*, *G. wykoffi*, *G. erawanensis*, *W. siamensis*, *W. crosseana*, *W. baschi*, and *Hydrobioides nassa*) (Fig. 4a) and 5 species from other families were not amplified (Fig. 4b). The specificity of species-specific primers was found to be 100% without cross-amplification for snails from other families or other bithyniids.

## DISCUSSION

The discrimination of *Bithynia* species has been problematic, especially for researchers with little taxonomic experience, due to their similarity in shell morphology (Rollinson et al., 1998). Moreover, morphological identification is usually only possible for mature snails because juveniles of different species are very

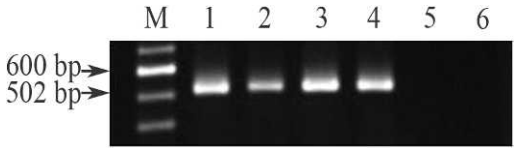


FIGURE 3. Agarose gel with representative PCR products obtained from the genomic DNA of *Bithynia funiculata* from various localities using species-specific primers. The arrow indicates the 502-bp amplicon from *B. funiculata*. Lane M: 100 bp DNA ladder, lanes 1–4 PCR of samples from four localities in Chiang Mai province: Mae Tang, Muang Chiang Mai, Hang Dong, and Sankamphaeng Districts, respectively, and lanes 5–6: distilled water and *Escherichia coli*.

similar. Recently, molecular techniques have been used to aid species identification and disease diagnosis of many organisms (Ortega-Rivas et al., 2003, 2005). RAPD-PCR is a technique that has been applied to assess genetic polymorphisms in many organisms. Moreover, the usefulness of this approach for the differentiation of species through the design of species-specific primers has been well established (Langand et al., 1993; Nagano et al., 1996; Knight et al., 1998; Jones et al., 2001; Wongsawad and Wongsawad, 2009).

Nagano et al. (1996) designed specific primers for the identification of *Dirofilaria immitis* based on unique bands isolated from RAPD-PCR profiles. Ortega-Rivas et al. (2003) analyzed *Acanthamoeba divionensis*, based on the characteristic bands from RAPD-PCR profiles, to design specific primers. RAPD-PCR has also been used to study population diversity or variation between groups. For example, Knight et al. (1998) studied *Biomphalaria glabrata* snails that were resistant to *Schistosoma mansoni* infection by RAPD-PCR and found 2 markers that segregated with resistance in the F1 and F2 progeny. Moreover, RAPD bands may indicate a high degree of polymorphism when screening multiple primers against taxa of interest. This has been noted to be a compromise of quickly identifying species-specific markers (Williams et al., 1990; Arnold et al., 1991). Wongsawad and Wongsawad (2009) designed

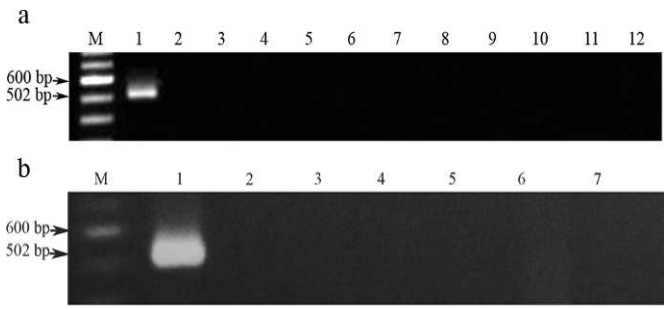


FIGURE 4. (**a**) Agarose gel showing the specificity of species-specific primers when tested against different species of bithyniid snails. The indicated 502-bp amplicon from *Bithynia funiculata* Lane M: 100 bp DNA ladder, lane 1: *B. funiculata*, lanes 2–12: *Bithynia siamensis siamensis*, *Bithynia siamensis goniomphalos*, *Wattebledia siamensis*, *Wattebledia crosseana*, *Wattebledia baschi*, *Gabbia pygmaea*, *Gabbia wykoffi*, *Gabbia erawanensis*, *Hydrobioides nassa*, distilled water, and *Escherichia coli*, respectively. (**b**) Agarose gel showing the specificity of species-specific primers with other species. The indicated 502-bp amplicon from *B. funiculata*. Lane M: 100 bp DNA ladder, lanes 1–7: *B. funiculata*, *Melanoides tuberculata*, *Clea helena*, *Filopaludina martensi martensi*, *Pomacea canaliculata*, *Indoplanorbis exustus*, and distilled water, respectively.

specific primers for *Haplorchis taichui* using a high annealing temperature random amplified polymorphic DNA (HAT-RAPD) method and 18 primers to generate DNA profiles for 13 different parasites. A 256-bp marker generated from 1 primer specific for *H. taichui* was cloned and sequenced. Many reports show that the RAPD-PCR is a good method for accurate identification of the organism. However, in the present study the unique band from RAPD patterns was analyzed and used to design a specific primer pair for identification of *B. funiculata*. The unique band, based on a DNA fragment with high copy number, has an unknown function. For selecting a suitable band product of *B. funiculata* for species-specific primers, the following criteria were considered: (1) the product should be suitable in length (300–1,000 bp) for easy sequence determination; and (2) it should also appear only in *B. funiculata*. At a 65 C annealing temperature, the species-specific primer pairs BF2F and BF2R used in this study could amplify a single product. Our primer set was specific for *B. funiculata* detection and confirmed the band profiles obtained from bithyniid snails as well as from others.

The profile with species-specific bands obtained through PCR was observed in positive *B. funiculata* from diverse localities. Although the RAPD primer allowed specific identification of *B. funiculata*, we decided to develop a species-specific primer set. The use of this primer set was more advantageous than the unique RAPD primer for snail identification because it can decrease low reproducibility (sensitive to changes in the quality of DNA, PCR components, and PCR conditions) and decrease mismatches between the primer and the template through a single band. Nevertheless, a RAPD primer may result in the total absence of a PCR product or in a merely decreased amount of the product. Thus, the RAPD results may be difficult to interpret (Koh et al., 1998; Kumar and Gurusubramanian, 2011).

In fact, the prevalence of *O. viverrini* infection in intermediate host snails is rather low because of misidentification of snail species; thus, parasite-related snails as well as vector control strategies are still ambiguous and neglected. However, this particular finding may be available for identification of intermediate host snails, resulting in under-estimation for prevalence of infected snails. Otherwise, this technique can be used to confirm species of suspected snails due to an eroded shell, or immaturity, or both.

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