The identification and characterisation of *Biomphalaria peregrina* (Orbignyi, 1835) from Agua Escondida in northern Patagonia, Argentina

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Abstract

Biomphalaria snails are small, freshwater pulmonates of biomedical importance found throughout South America. Here, we describe the observation and process of identification of a newly-discovered population of *Biomphalaria peregrina* from Agua Escondida, in Mendoza Province, Argentina, by looking at both morphological and molecular characters. A DNA 'barcode' for the population is also presented. *B. peregrina* has been shown to be capable of transmitting *Schistosoma mansoni* under laboratory conditions; considering this potential, increased vigilance for intestinal schistosomiasis is recommended for this site. Furthermore, given the remoteness and aridity of the location, it will help in understanding more about the local ecology and life history of these biomedically important snails.

Key words

Biomphalaria peregrina, Argentina, DNA barcode, COI, 16S, ITS

Biomphalaria snails have long been under scientific scrutiny as the intermediate hosts of *Schistosoma mansoni*, which causes intestinal schistosomiasis in humans. The genus is thought to have evolved in South America, and is currently distributed throughout the continent, Central America, the Caribbean, Africa and the Middle-East (DeJong et al., 2001; Woodruff & Mulvey, 1997). Owing to difficulties in precise identification and discrimination, a suggested 20 species of *Biomphalaria* inhabit the Americas (Malek, 1985; PAHO, 1968), with up to seven being recently observed in Argentina (Paraense, 2004; Rumi et al., 2008). Identification of *Biomphalaria* in the Americas has traditionally focused on internal morphological characters, and specifically features pertaining to the reproductive structures, but more recently, molecular methods have been introduced for identification, and have found to be very successful, particularly in distinguishing between closely related species (Vidigal et al., 2004). The most frequent molecular tool in the literature for American *Biomphalaria* is the use of restriction enzyme digests of the ribosomal Internal Transcribed Spacer (ITS) region of nuclear DNA, whereas for the recently evolved African species, DNA sequence analysis of mitochondrial regions such as 16S (ribosomal RNA) and sub-unit one of the cytochrome oxidase gene (COI) are also commonly used. Sequences of COI in particular are important markers as the region is emerging as that of choice for 'barcoding' higher animals, and reference databases for this marker are growing rapidly. The Consortium for the Barcode of Life (http://www.barcoding.si.edu/), for example, is an organisation dedicated to compiling a dataset of such sequences for the identification of all species.

Much of the interest in identifying *Biomphalaria* stems from the differential ability of different species to act as intermediate hosts for *Schistosoma mansoni*. Of the seven

species of *Biomphalaria* reported from Argentina, *B. tenagophila* and *B. straminea* have been found naturally infected with the parasite, while *B. peregrina*, which has yet to be found naturally infected, has been shown to transmit the parasite in laboratory conditions (Paraense, 2001; Paraense & Correa, 1973). *B. orbignyi*, *B. oligoza*, *B. intermedia* and *B. occidentalis* are the other reported species, which are thought to be refractory to the parasite. Within Argentina itself, most *Biomphalaria* are constrained to the North-east portion of the country, with only *B. peregrina* and *B. orbignyi* found in Mendoza province (Paraense, 2001). Southern Mendoza forms part of the arid pampas, with focalised freshwater bodies often separated by large distances. Biogeographically, the region is included within the Chaqueña Domain, Province of Monte, which is characterized by xerophytic vegetation in a landscape of plains, mountain slopes and plateaus (Cabrera & Willink, 1980). There have been reports of *B. peregrina* as far south as Laguna Llancanelo, but not recently, as far as the authors are aware, in smaller water bodies in the surrounding area.

Here, we describe the use of morphological and molecular techniques, including DNA 'barcoding', to identify a population of *Biomphalaria* collected from a small artificial pond in Agua Escondida in the far south of Mendoza Province, Argentina.

Materials and Methods

The snails were collected from a reservoir pond in the village of Agua Escondida in southern Mendoza Province (GPS coordinates: S 36.156560°, W 68.303670°) in November 2008. The climate in the region is cold and arid, with a mean annual temperature of 12.8°C, ranging between –4.1 and 29.3ºC (daily recorded data from National Meteorological Service of Argentina, 1995-2008). The mean temperature of

winter months is 4.6°C and the mean temperature of summer months is 26.8ºC. The mean annual accumulated precipitation is 255mm. There are scarce water bodies, which are used as water sources by livestock, wild animals and humans (Issia et al., 2008). The local human population is small and houses are scattered over a large area (density less than 1 inhabitant per km^2); goat and sheep rearing is the main economic activity and cattle are raised for domestic use and fascioliasis in livestock can be very common (Issia et al., 2008). Owing to the remoteness of the location, medical facilities are basic and formal screening for intestinal schistosomiasis is not undertaken.

The sampling sites were located along several points of the perimeter of two artificial lagoons connected by a stream, in total covering approximately four hectares. The water was clear and showed a moderate flow; the mean water temperature was 11.5°C (range 2 - 27.2°C) and the mean pH value 8.1, and ranging from 7.6 to 8.3. Snails were collected from the fringes of the pond using metal scoops and a hand-held sieve. For anatomical and molecular analyses, the snails (n = 10) were relaxed overnight in water containing menthol crystals, and then killed in hot (60° C) water. Each snail was removed from its shell with forceps; a piece of the head-foot was cut off and preserved in ethanol, while the rest of the soft tissue was placed in slightly modified Railliet-Henry fluid (Pointier et al., 2005). Shells were dried and labelled accordingly and photographed using an AxioCam attachment to a light microscope (Carl Zeiss Ltd., Welwyn Garden City, UK).

[Table 1]

The soft tissue preserved in Railliet-Henry fluid was dissected to reveal the internal anatomy of each snail, and particularly the reproductive organs. These were sketched using a camera lucida attachment to a dissecting microscope. The pieces of the head-foot were extracted using a standard CTAB extraction with a chloroform-isoamyl alcohol cleaning step. PCR amplifications were made using Promega Go-Taq (Promega UK Ltd, Southampton, UK) for three separate regions of the genome: the cytochrome oxidase sub-unit one (COI) mtDNA gene, the 16S sub-unit of ribosomal DNA, also in the mitochondrion, and whole ribosomal internal transcribed spacer (ITS) region of nuclear DNA. The general PCR cycle used was 2 minutes initial denaturing at 94^{o}C, followed by 35 cycles of 1 minute at 94^{o}C, 45 seconds at the T_M, and 1 minute extension at 72 ^{o}C; primer details and annealing temperatures can be found in Table 1 (Bonnaud et al., 1994; Folmer et al., 1994). Amplifications were performed on a Veriti thermal cycler (Applied Biosystems Inc., Foster City, CA, USA) and visualised on a 2% GelRed^{TM} (Hayward, CA, USA) agarose gel.

PCR products were then purified using PCR Cleanup kit (Millipore, Billerica, MA, USA). For all three DNA markers, purified PCR products were sequenced following Applied Biosystems Big Dye Kit (version 1.1) protocol and run on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Carlsbad, USA). Sequences were visually edited using Sequencher v 4.8 (Gene Codes Corporation, Ann Arbor, Michigan, USA: http://www.genecodes.com) and aligned in MacClade v 4.08 (Sinauer Associates, Sunderland, Massachusetts, USA: http://macclade.org/macclade.html). The computer programme MEGA v 4.0 (Tamura et al., 2007) was used for tree-building and bootstrapping. The ITS amplifications were also used directly for restriction enzyme digests with *HaeIII*, *AluI* and *HpaII*, following published protocol (Spatz et al., 1999). The products were loaded into a

Bio-Experion microgel (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) for quantification of fragment size and visualisation of the banding patterns. Two other snails, a lab-bred *B. glabrata*, and an African *B. choanomphala*, were also included in the ITS amplification and digestion, to compare against the Agua Escondida samples.

Results

[Figure 1]

Analysis of published reports revealed two recent records of *Biomphalaria* species from Mendoza province: Paraense (2001) reported the existence of *B. orbignyi* from a ditch in Mendoza municipality itself, and, approximately 120km to the south, *B. peregrina* from a ditch in San Carlos. Evidence of *B. peregrina* was also observed yet further south, in Laguna Llancanelo (Ciocco et al., 2008). This site is about 100km north of Agua Escondida, where the collections discussed here were made (see Figure 1). These findings fall within the historical latitudinal range of *Biomphalaria*, which has been reported from both sides of the Andes as far south as S 41 ^{o} and S 42^{o} (PAHO, 1968).

[Figure 2]

The conchology and reproductive organ morphology of the snails collected at Agua Escondida (see Figures 2a and 2b) were consistent with published accounts of *B. peregrina* (Paraense, 1966) (Paraense & Deslandes, 1955). Shell heights ranged from 5.33-6.81cm, lengths varied between 6.54 and 8.04cm, and number of whorls was between 4.5 and 5.5.

[Figure 3 and 4]

The 655bp COI and 432 bp 16S mtDNA regions showed little variation within the 10 individuals sequenced, with only one COI and two 16S haplotypes revealed (GenBank acquisition numbers GU168593.1, GU168592.1 and GU168591.1, respectively). A BLAST search of Genbank (www.ncbi.nlm.nih.gov/Genbank) using the COI haplotype resulted in *B. havanensis* as having the highest similarity (100% query coverage, 91% maximum identity), although its COI sequence differed from the Agua Escondida samples at over 50 nucleotide positions across a 655 base pair-long fragment (it is worth noting that the Genbank match was labelled '*B. obstructa'*, which is a synonymous species, and so is referred to here by its correct name of *B. havanensis* (Yong et al., 2001; Yong et al., 1997)). The 16S haplotypes were also subjected to BLAST searches, revealing matches with *B. peregrina* (100% query coverage, 99% maximum identity); these differed from the Agua Escondida samples at 11 nucleotide positions, but grouped together with strong bootstrap support when a neighbour-joining tree was built, also including Genbank sequences from other *Biomphalaria* species (Figures 3 and 4).

[Figure 5]

Sequencing of the ribosomal ITS fragment (approximately 1000bp in length) was problematic in generating a consensus sequence from both the sense and anti-sense strands, but a BLAST search using a 600bp portion of the single strand sense fragment returned *B. peregrina* (100% query coverage, 98% maximum identity). There was a clear distinction between the ITS sequence digests from the South

American and the African species for both *HpaII* and *AluI* (See Figure 5 for *HpaII* results). *HaeIII* did not show clear distinctions between samples and there was some minor intrapopulation polymorphism within the samples from Agua Escondida.

Discussion

The internal morphology of *Biomphalaria peregrina* has been shown to distinguish it even from closely related species, but not from *B. orbignyi* (Spatz et al., 2000); however, *B. orbignyi* has been shown to be refractory to infection by *Schistosoma mansoni*, whereas *B. peregrina* is permissive, which highlights its potential risk of the latter in the transmission of schistosomiasis. In this case, the internal morphology was shown to be typical of that of *B. peregrina*, but this could not rule out *B. orbignyi* as a possible alternative. Moreover, morphological dissection and analysis requires significant expertise, which has led to the rise of molecular tools to assist with identification of field-collected specimens. In the future, it would be useful to provide 'type' representation of *B. orbignyi* DNA barcodes in public-access databases for comparative purposes.

The tree of 16S mtDNA region resulted in strong support for the specimens being *B. peregrina*, and thus supporting the morphological conclusion, as the two Agua Escondida haplotypes clustered closely with *B. peregrina* samples from Genbank, from Brazil and Uruguay, and displayed a deep division even from other South American *Biomphalaria*. The lack of existing COI barcodes for other *Biomphalaria* species hindered the use of this region as an absolute identifier in this case; the BLAST search had returned *B. havanensis* as the closest match (though with only 91% maximum identity), which emphasises the need for as many species as possible

to be represented for this region on open access databases such as Genbank, as it is only with better coverage that the usefulness of the barcoding enterprise will be fully realised. The ITS digest proved somewhat disappointing as a means of specimen identification, particularly as the methodology was not successful for several of the samples. Extra bands on the Bio-Experion image indicate the possibility of intra-individual variation in the ITS sequence, which might also have contributed to the difficulties experienced in sequencing both the sense and anti-sense strands of this fragment. The *HpaII* enzyme digest profiles did not readily distinguish between the Agua Escondida samples and *B. glabrata*. A lack of availability of other South American species of *Biomphalaria* against which to compare directly the Agua Escondida samples prevented further characterisation using these digests, particularly between *B. oligoza* and *B. orbignyi* (Spatz et al., 2000). However, the partial sequencing of the ITS fragment lent strong support to an identification as *B. peregrina*.

The lack of variation within the 10 sampled individuals for the COI region, and the single base transversion between the two 16S haplotypes, points to low genetic diversity within the population of *Biomphalaria* at Agua Escondida. This could suggest a recent founder event, as *Biomphalaria* are hermaphroditic and can quickly colonise a new location from single founding individuals. Further sampling would be required in order to test these hypotheses more thoroughly, but could be useful for understanding the local dynamics of range expansion in this species, especially considering the isolation of this site, and the aridity of the surrounding landscape, neither of which would be considered conducive to the dispersal of freshwater gastropods.

Whilst these snails were not found to be shedding cercariae, the possibility of local transmission should not be ruled out, as *B. peregrina* are known to be capable of transmitting *S. mansoni* in the laboratory (Paraense & Correa, 1973). This information may be important in predicting the potential spread of human schistosomiasis; although the climate in Agua Escondida is now considered unsuitable for the development of the parasite, it may be that conditions will become more favourable in the face of climate change (Mas-Coma et al., 2009), in which case observations of *B. peregrina*, and other permisisve hosts, will become more important. As it stands, the possibility of ephemeral transmission in warmer months in Agua Escondida cannot be discounted, owing to favourable summer temperatures for the development of the parasite. Current work on detection of schistosome DNA from snail tissue (see Abath et al., 2006) is underway (Kane et al., in prep.), which will hope to elucidate further, and more precisely, the transmission status of this locality. From a public health perspective, and especially if schistosome DNA were to be detected in snails, epidemiological screening of the local human populace could be justified, using highly sensitive diagnostics, such as ELISA (Bergquist et al., 2009), as the local next step of this investigation.

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Table 1: Primer names and sequences used for amplification of COI, 16S and ITS regions

Primer name	Region	Sequence (5' - 3')	TM
LCO1490	COI (sense)	GGTCAACAAATCATAAAGATATTGG	45
HCO2198	COI (antisense)	TAAACTTCAGGGTGACCAAAAAATCA	45
16arm	16s (sense)	CTTCTCGACTGTTTATCAAAAACA	50
16brm	16s (antisense)	GCCGGTCTGAACTCAGATCAT	50
ETTS1	ITS (sense)	TGCTTAAGTT AGCGGGT	47
ETTS2	ITS (antisense)	TAACAAGGTTTCCGTAGGTGAA	47

Figure 1: Map of Mendoza province and surrounding area. Agua Escondida, the collection site, is underlined; all other sites where *Biomphalaria* have been observed in the literature are starred: [1]= Paraense (2001); [2]= Ciocco et al. (2008).

Figures 2a and 2b: a) Shell photographs of an Agua Escondida *Biomphalaria*. b) Anatomy of the reproductive system of *Biomphalaria peregrina*, Agua Escondido, Argentina: Ca = carrefour ; ng = nidamental gland ; od = ovispermiduct ; ot = ovotestis ; ov = ovotestis ; po = pouch of the oviduct ; pp = preputium ; pr = prostate ; ps = penis sheath ; sd = sperm duct ; sp = spermatheca ; sv = seminal vesicle ; va = vagina ; vd = vas deferens ; ut = uterus.

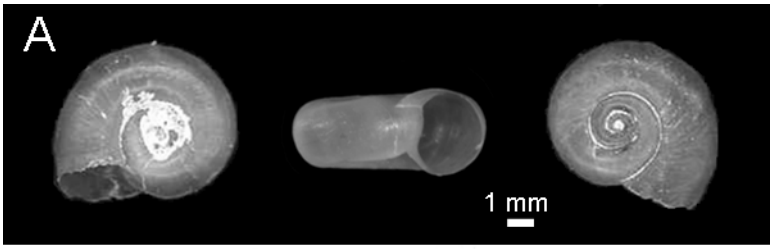
Figure 3: Schematic of the mitochondrial genome, with the relative positions of the COI gene and the 16S region marked against the mitochondrial genome of *B. glabrata* (Genbank accession AY380531.1), along with intraspecific variation found. A = Folmer region; B = 16S 'universal' marker region. * = Genbank acquisition AY030231.1.; ** = Genbank acquisition AY030232.1.

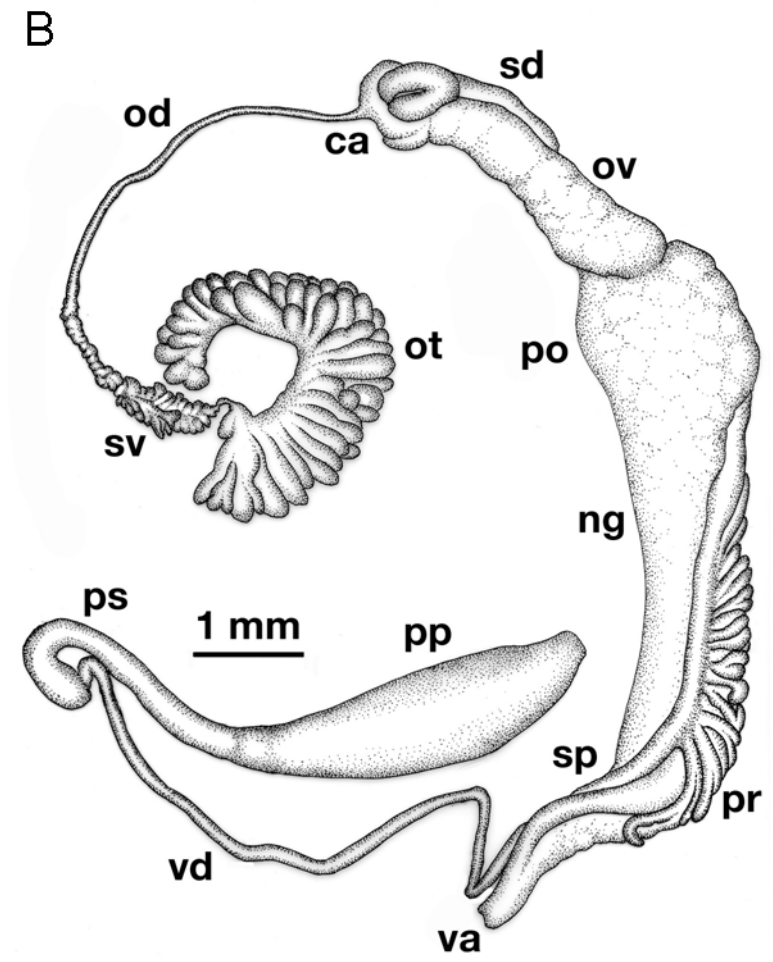
Figure 4: Neighbour-joining tree (with 500 bootstrap replicates) based on the 16S region. Agua Escondida haplotypes (A and B) are called '*B.* cf *peregrina*'.

Figure 5: Gel picture of the *HpaII* enzyme digest of the ribosomal ITS region of nuclear DNA: the Agua Escondida sample is labelled 'Arg-y'; 'B. glab.' stands for *Biomphalaria glabrata* and 'B. cho' stands for *B. choanomphala*.


Pacific Ocean
SAN JUAN
Mendoza[1]
Santiago
SAN LUIS
San Carlos[1]
Andes mountains
CHILE
ARGENTINA
MENDOZA
Laguna Llancanelo[2]
Agua Escondida
LA PAMPA
NEUQUEN
100 km

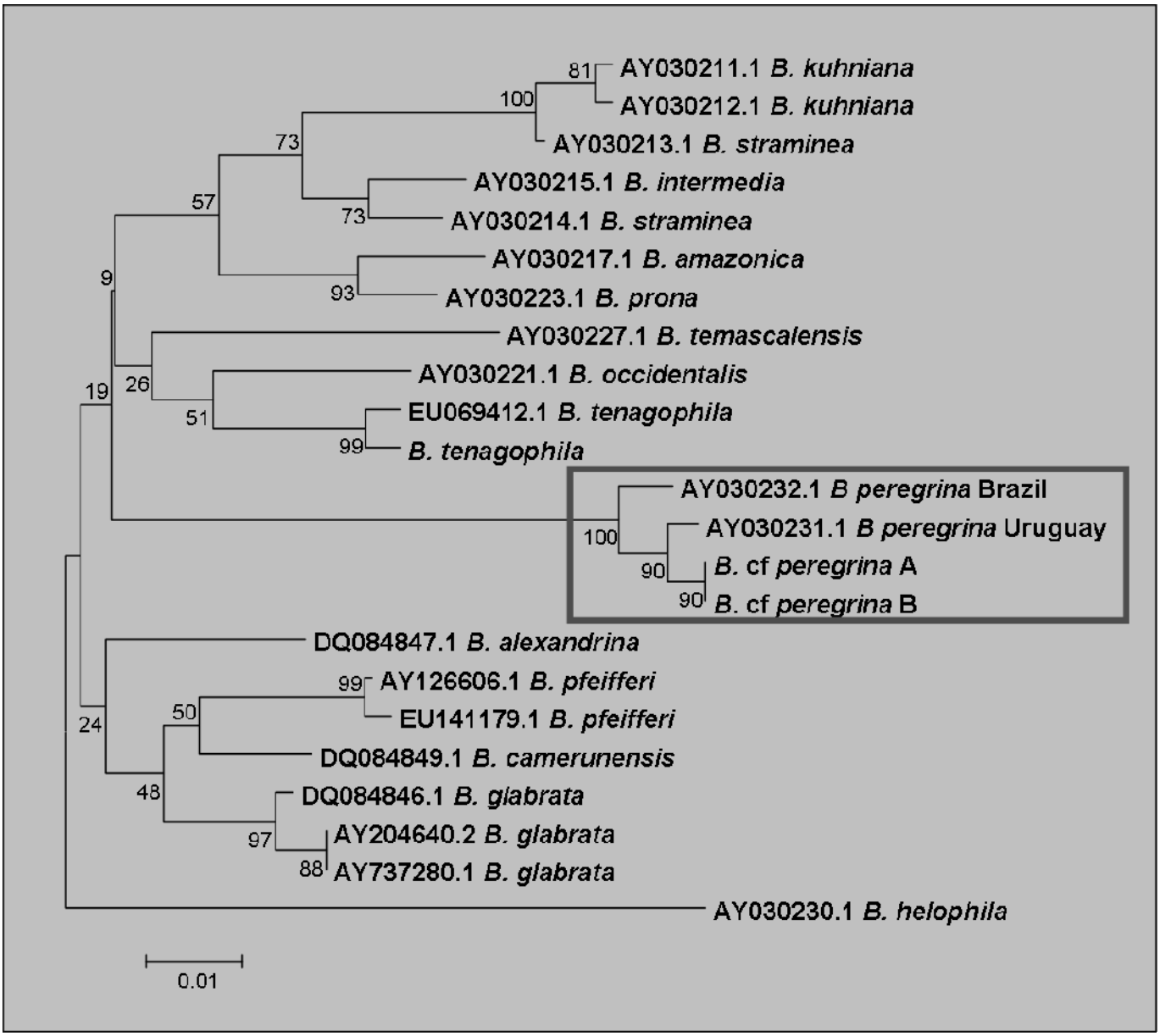
400





No sequence variation within Agua Escondida samples, and no *B. peregrina* haplotypes available on Genbank. Closest species was *B. havanensis*, which had 57 base changes

	12075	12100	12108	12178	12213	12225	12228	12258	12368	12411	12490
B. p. AE A	G	C	T	G	G	G	C	-	-	T	T
B. p. AE B	G	A	T	G	G	G	C	-	-	T	T
B. p Brazil**	A	A	T	A	A	G	T	T	A	A	A
B. p Uruguay*	A	A	-	G	A	A	C	T	-	T	T


AY030211.1 B. kuhniana
AY030212.1 B. kuhniana
AY030213.1 B. straminea
AY030215.1 B. intermedia
AY030214.1 B. straminea
AY030217.1 B. amazonica
AY030223.1 B. prona
AY030227.1 B. temascalensis
AY030221.1 B. occidentalis
EU069412.1 B. tenagophila
B. tenagophila
AY030232.1 B peregrina Brazil
AY030231.1 B peregrina Uruguay
B. cf peregrina A
B. cf peregrina B
DQ084847.1 B. alexandrina
AY126606.1 B. pfeifferi
EU141179.1 B. pfeifferi
DQ084849.1 B. camerunensis
DQ084846.1 B. glabrata
AY204640.2 B. glabrata
AY737280.1 B. glabrata
AY030230.1 B. helophila
81
100
73
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93
19
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99
100
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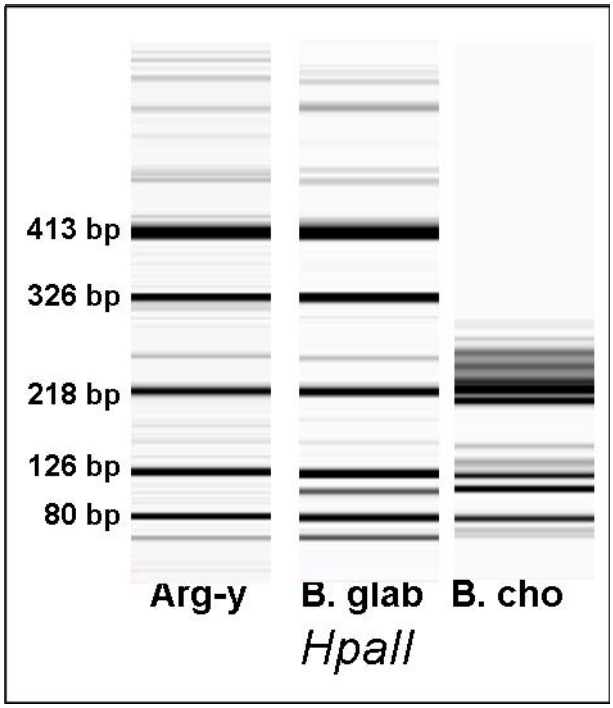
48
97
88
0.01



405