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Characterisation of fascioliasis lymnaeid intermediate hosts from Chile by DNA sequencing, with emphasis on *Lymnaea viator* and *Galba truncatula*

Patricio Artigas [a], M. Dolores Bargues [a], Roberto L. Mera y Sierra [b], Veronica H. Agramunt [a], Santiago Mas-Coma [a,*]

[a] Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain
[b] Cátedra de Parasitología y Enfermedades Parasitarias, Facultad de Ciencias Veterinarias y Ambientales, Universidad J.A. Maza, Av. de Acceso Este - Lateral Sur 2245, S. José, Guaymallén, Mendoza, Argentina

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ABSTRACT

In South America, *Fasciola hepatica* infection poses serious health problems in both humans and livestock. In Chile, the medical impact appears yearly stable and mainly concentrated in central regions, where the veterinary problem is highlighted by higher animal prevalences. Studies were undertaken by rDNA ITS-2 and ITS-1 and mtDNA *cox*1 sequencing to clarify the specific status of the lymnaeids, their geographical distribution and fascioliasis transmission capacity in Chile, by comparison with other American countries and continents. Results change the lymnaeid scenario known so far. The lymnaeid fauna of mainland Chile shows to be poor, including only two authochthonous species, *Lymnaea viator* and *Pectinidens diaphana*, and a third introduced species of Palaearctic origin *Galba truncatula*. Both *Lymnaea lebruni* and *Lymnaea patagonica* proved to be synonyms of *P. diaphana*. *G. truncatula* appears to have always been confused with *L. viator* and seems distributed from Región VI to Región IX, overlapping with human endemic areas. DNA sequencing results suggest that the absence of correlation between remote sensing data and disease prevalences could be due to transmission capacity differences between *L. viator* and *G. truncatula*. Results furnish a new baseline on which to undertake future appropriate studies on transmission, epidemiology and control.

1. Introduction

Freshwater lymnaeid snails transmit fascioliasis, a highly pathogenic liver parasitosis caused by trematode species of the genus *Fasciola* which affects humans and livestock species almost everywhere (Mas-Coma et al., 2009a). Distribution, both in space (latitudinal, longitudinal and altitudinal) and time (seasonal, yearly), of fascioliasis depends on the presence and population dynamics of the specific intermediate host species in its turn linked to the presence of the appropriate water bodies and on adequate climate characteristics enabling fluke development. In the last two decades, this disease is emerging in many countries of Latin America, Europe, Africa and Asia. This emergence phenomenon has partly been related to climate change, given the high dependence of both fasciolid larval stages and their freshwater lymnaeid snail hosts on climatic and environmental characteristics (Mas-Coma et al., 2008, 2009b). The increasing importance of human fascioliasis also relies on recent results showing a great morbidity impact on children in long-term infection (Valero et al., 2003, 2006, 2008).

Within the several human fascioliasis hotspot regions known, South America stands out due to the human hyperendemic areas caused by *Fasciola hepatica* in many Andean countries, such as Bolivia (Esteban et al., 1997, 1999) and Peru (Esteban et al., 2002; Gonzalez et al., 2011). In Argentina the human fascioliasis situation seems to be underestimated (Mera y Sierra et al., 2011), in Colombia appropriate studies are still pending (Bargues et al., 2011a), and in Venezuela a potential underestimation of the situation has recently been highlighted (Bargues et al., in press-a).

In Chile, human fascioliasis reports have been numerous, including individual case descriptions (e.g., Venturelli et al., 2003; Lopez et al., 2004; Rosas et al., 2008; Morales et al., 2009), epidemic situations and familiar outbreaks (Subercaseaux et al., 1985; Borie et al., 1990), and even studies on human fascioliasis endemic areas (Apt et al., 1992, 1993). A large random survey in the provinces of Curico, Talca and Linares showed prevalences of 0.6%, 0.75% and 0.71%, respectively, with an estimation of 2000 people infected in the area analysed (Apt et al., 1993). Only in the capital Santiago,

Abbreviations: rDNA, nuclear ribosomal DNA; mtDNA, mitochondrial DNA; ITS-1, first internal transcribed spacer; ITS-2, second internal transcribed spacer; COX1, cytochrome c oxidase subunit I nucleotide sequence (*cox*1) and amino acid sequence.

* Corresponding author. Tel.: +34 96 3544905; fax: +34 96 3544769.
E-mail address: S.Mas.Coma@uv.es (S. Mas-Coma).

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between 20 and 30 liver fluke infected patients were diagnosed yearly and this only concerned symptomatic subjects (Apt, 1987; Alcaino and Apt, 1989).

Fascioliasis is also a great veterinary problem in Chile (Alcaino and Apt, 1989; Morales and Luengo, 1995; Alcaino and Gorman, 1999). Official data from slaughterhouses indicate that the disease affects livestock in all regions, with countrywide high yearly prevalences in cattle (28.5–31.8%, mean 30.1%), sheep (1.8–2.75, mean 1.9%), goats (11.2–18.3%, mean 14.0%), pigs (1.0–2.1%, mean 1.4%), equines (9.1–14.1%, mean) and camelids (0.03–8.37%, mean 0.99%) according to data from the 1989 to 1995 period (Morales et al., 2000).

Despite the importance of fascioliasis in Chile, lymnaeid snails have been only the focus of a very few studies in that country. A total of seven lymnaeid species have been described or noted to be present in Chile (Hubendick, 1951; Paraense, 1982, 1984; Sielfeld, 2001; Valdovinos, 2006):

- *Lymnaea diaphana* King and Broderip, 1832: originally described from Cape Gregory, Strait of Magallanes (King and Broderip, 1832) and later also cited from Argentina and Peru (Bargues et al., in press-b); the genus *Pectinidens* proposed by Pilsbry (1911) for *diaphana* as type species has recently been molecularly demonstrated to be valid (Bargues et al., in press-b);
- *L. viator* D'Orbigny, 1835 (=*L. viatrix sensu* Paraense, 1976): originally described under var. A ventricosa from Rio Negro in Argentina as well as surrounding Santiago and Casablanca in Chile, and under var. B elongata from Rimac river canals around Callao and Lima in Peru (D'Orbigny, 1835, 1837; Paraense, 1976; Bargues et al., 2007a);
- *Lymnaea chilensis* Beck, 1838: originally noted from Chile without further geographical detail (Beck, 1838);
- *Lymnaea lebruni* Mabille, 1884: originally described from Punta Arenas, Patagonia (Región XII) (Mabille, 1884);
- *Lymnaea pictonica* Rochebrune and Mabille, 1885: originally described from Isla Picton (Rochebrune and Mabille, 1885);
- *Lymnaea cousini* Jousseaume, 1887: cited once in Valdivia (Hubendick, 1951), but originally described from Ecuador and later also from Colombia (Bargues et al., 2011a);
- *L. patagonica* Strebel, 1907: originally described from Agua Fresca, Strait of Magallanes (Strebel, 1907).

However, *L. chilensis* may be deleted from that list because neither a description nor a locality were provided by Beck (1838: species name noted as "*L. Chilensis* B. – Chili" in page 112 but without description in the Appendix where the descriptions of all the new species were included), so that it is taxonomically considered a *nomen nudum*, as already stated by Hubendick (1951). Moreover, different synonymies have been proposed between these species, authors sometimes not in agreement one another. For instance, *L. lebruni* is considered a synonym of *L. diaphana* by Pilsbry (1911) because Mabille (1884) overlooked the article of King and Broderip (1832). *Lymnaea patagonica* is considered a synonym of *L. pictonica* by Hubendick (1951), whereas *L. patagonica* is a valid species according to Malek (1985).

The species noted to be involved in fascioliasis transmission in Chile is *L. viator*, specimens of which collected from Macul and Bucalemu proved to be susceptible to experimental infection already long ago (Tagle, 1944).

The classification of individual lymnaeids poses serious difficulties when only applying malacological methods, due to anatomic similarities and large intraspecific variation of shell shape and size (Bargues et al., 2001; Bargues and Mas-Coma, 2005). Although shell shape may help in particular species and populations (Samadi et al., 2000), there are groups, as the "fossarine" or *Galba*/*Fossaria* group, in which specimen classification may be very difficult when based only on phenotype, as is the case of the aforementioned *L. viator* and *L. cousini* (Bargues et al., 2007a, 2011a,b, in press-a, in press-b). The implications of lymnaeids for fascioliasis transmission, epidemiology and control urged to develop new tools to facilitate specimen classification, genetic characterisation of natural populations and laboratory strains, and to elucidate the systematics and taxonomy of the Lymnaeidae. This is the purpose of the worldwide lymnaeid molecular characterisation initiative (Mas-Coma et al., 2009a). Nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) markers proved to be useful for this endeavour in invertebrates in general, although disadvantages and limitations depending on each marker should be taken into account (Mas-Coma and Bargues, 2009). Their application also showed their usefulness in lymnaeid snails for specific, generic and suprageneric taxon levels (Bargues and Mas-Coma, 2005).

The internal transcribed spacers of the rDNA, mainly ITS-2 and secondarily ITS-1, are the most useful sequences for lymnaeid species classification (Bargues and Mas-Coma, 2005). A fragment of the cytochrome c oxidase subunit I gene (*cox*1) of mtDNA also proved to be useful in lymnaeids (Bargues et al., 2007a), although mtDNA markers should be used with great caution in lymnaeids (Bargues et al., 2011b), due to (i) problems of nucleotide saturation making it unappropriate for comparison of genera and even well separated species within the same genus and (ii) biased information furnished by only a gene fragment (Mas-Coma and Bargues, 2009).

The aim of the present article is to expose the results of ITS-2, ITS-1 and *cox*1 sequencing of lymnaeid species present in Chile and to ascertain their systematic status. The final analysis has the purpose to offer a new baseline on which to design and launch further lymnaeid studies and appropriate assessments on human and animal fascioliasis in Chile from now on. The implications of the new intermediate host scenario on the disease in that country are finally discussed.

2. Materials and methods

2.1. Lymnaeid snail materials

The snail specimens studied were collected in the field, from lymnaeid populations present in geographical areas with human infection and/or animal fascioliasis endemicity. In order to be systematically conclusive, sequenced specimens of the species *L. diaphana*, *L. viator*, *L. lebruni*, and *L. patagonica* were from their respective type localities. Unfortunately, the present impossibility to visit Picton island due to the distribution of anti-personnel mines throughout the island did not allow for the collection of *L. pictonica*. An effort to find *L. cousini* was made in Valdivia and its surroundings. Localities and their coordinates and altitudes furnishing the lymnaeid specimens sequenced are noted in Table 1 and Fig. 1. The number of specimens analysed for each species is noted in Table 1.

2.2. Molecular techniques

2.2.1. DNA extraction

DNA was extracted from more than one specimen of a given population when this was deemed necessary for sequence verification. DNA was only isolated from the foot of each snail (Bargues et al., 1997, 2007a). Snail feet fixed in 70% ethanol were used for DNA extraction procedures. After dissection under a microscope, half of the foot was suspended in 400 μl of lysis buffer (10 mM Tris–HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate SDS) containing 500 μg/ml Proteinase K (Promega, Madison, WI, USA) and digested for 2 h at 55 °C with alternate shaking each 15 min. Total DNA was isolated according to the