Molecular Ecology (2001) **10**, 1333–1340

Lack of molluscan host diversity and the transmission of an emerging parasitic disease in Bolivia

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Abstract

Fasciolosis is a re-emerging parasitic disease that affects an increasing number of people in developing countries. The most severe endemic affects the Bolivian Altiplano, where the liver fluke (*Fasciola hepatica*) and its hermaphroditic snail host, *Lymnaea truncatula*, have been introduced from Europe. To achieve a better understanding of the epidemiological situation and the consequences of the colonization event of this invasive species, genetic analysis of Bolivian snail populations was needed. Here we compare the genetic diversity and population structure of snail samples from the Bolivian Altiplano with samples from the Old World at six polymorphic microsatellite loci. Whereas some variability exists in the snail populations from the Old World, we observe only a single genotype of *L. truncatula* in the Bolivian Altiplano. We discuss the possible explanations for such a reduction in genetic variability, and, given the high natural parasitism pressures exerted on the snail populations, we discuss the relevance of this result for host–parasite interactions.

Keywords: *Fasciola hepatica*, host–parasite interaction, human disease, invasive species, *Lymnaea truncatula*, microsatellites

Received 2 October 2000; revision received 4 January 2001; accepted 4 January 2001

Introduction

Fasciolosis is a re-emerging, highly pathogenic human infection, today considered an important parasitic disease, whose epidemiological picture has changed in recent years (Chen & Mott 1990; World Health Organization 1995; Mas-Coma *et al*. 1999). As many as 17 million people, mostly children, are thought to be infected by the liver fluke *Fasciola hepatica*, including human endemics in Europe, Asia, Africa and America (Hopkins 1992; Esteban *et al*. 1998). The most striking example of a human fasciolosis endemic is in the Northern Bolivian Altiplano, where the highest prevalence and intensity reports to date have been found (mean prevalence of 40%, but up to 100% in some localities; Hillyer *et al*. 1992; Mas-Coma 1998; Esteban *et al*. 1999).

The intermediate host of the liver fluke is a hermaphroditic mollusc of the genus *Lymnaea*, which lives in freshwater ponds or ditches. Recent morphological, isoenzymatic and molecular studies have demonstrated that the snail present in Bolivia belongs to the European species *Lymnaea truncatula* (Bargues *et al*. 1997; Jabbour-Zahab *et al*. 1997; Samadi *et al*. 2000). Moreover, morphological studies have shown that the liver fluke found in Bolivia also seems to be of European origin (Valero *et al*. 1999). Thus, we find a rarely observed joint invasion of both parasite and vector, responsible for the introduction of a human disease from Europe. Interestingly, the invaded habitat presents new environmental conditions, as there are substantial variations in daily and night-time air and water temperature (3–28 °C) and high evapotranspiration, due to the extremely high altitude of the Bolivian Altiplano (4000 m) (Fuentes *et al*. 1999). Moreover, *L. truncatula* is under strong selection pressures, because the liver fluke, like many trematodes, castrates its host.

This situation raises important evolutionary issues: the colonization event, or other bottleneck events due to the peculiar population dynamics of freshwater snails (see Städler & Jarne 1997), are likely to reduce the genetic

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variability in the snail populations (for a review see Frankham 1997). The time since the end of the bottleneck will determine the amount of genetic diversity retained. Genetic variability and population structure of lymnaeid snails may then give insights about the number of colonization events and the time since the last bottleneck event. The first genetic study was conducted using isoenzymes (Jabbour-Zahab *et al*. 1997) and showed an absence of within population polymorphism. Consequently, we used more variable molecular markers for this study, which describes the population genetic structure of *L. truncatula* at six polymorphic microsatellite loci (see Trouvé *et al*. 2000), both in Bolivia and in the Old World.

The results are discussed with relevance to host–parasite interactions, given the high parasitic pressures undergone by the snail host in Bolivia.

Materials and methods

Sampling

Snails were hand-collected and stored in 80% ethanol.

The Bolivian samples cover the endemic disease area in the northern Bolivian Altiplano, between the city of La Paz and Lake Titicaca (Fig. 1), and were chosen to allow detailed analysis of population structure at a small geographical scale. A total of 245 individuals were sampled from 13 sites in the Bolivian Altiplano, with eight sites sampled twice (in 1995 and 1996). Samples from the Old World (a total of 122 individuals) come from France (three sites), Morocco (three sites), Spain (four sites) and Portugal (two sites) (see sample sizes on Table 1).

Parasite prevalence

All snails collected in Bolivia (a total of 1197) were tested for the presence of trematode larvae (*Fasciola hepatica* or other genus) through dissection of their gastrogenital tracts.

Genetic analysis

DNA extraction was performed using the phenol–chloroform method, modified for molluscs (Jarne *et al*. 1992). Polymerase chain reactions (PCR) were performed following Trouvé *et al*. (2000) at six polymorphic and impure microsatellite loci (GenBank Accession nos AF226976, AF226977, AF226978, AF226980, AF226985 and AF226986). PCR products were denatured and separated on 5% polyacrylamide and 7 M

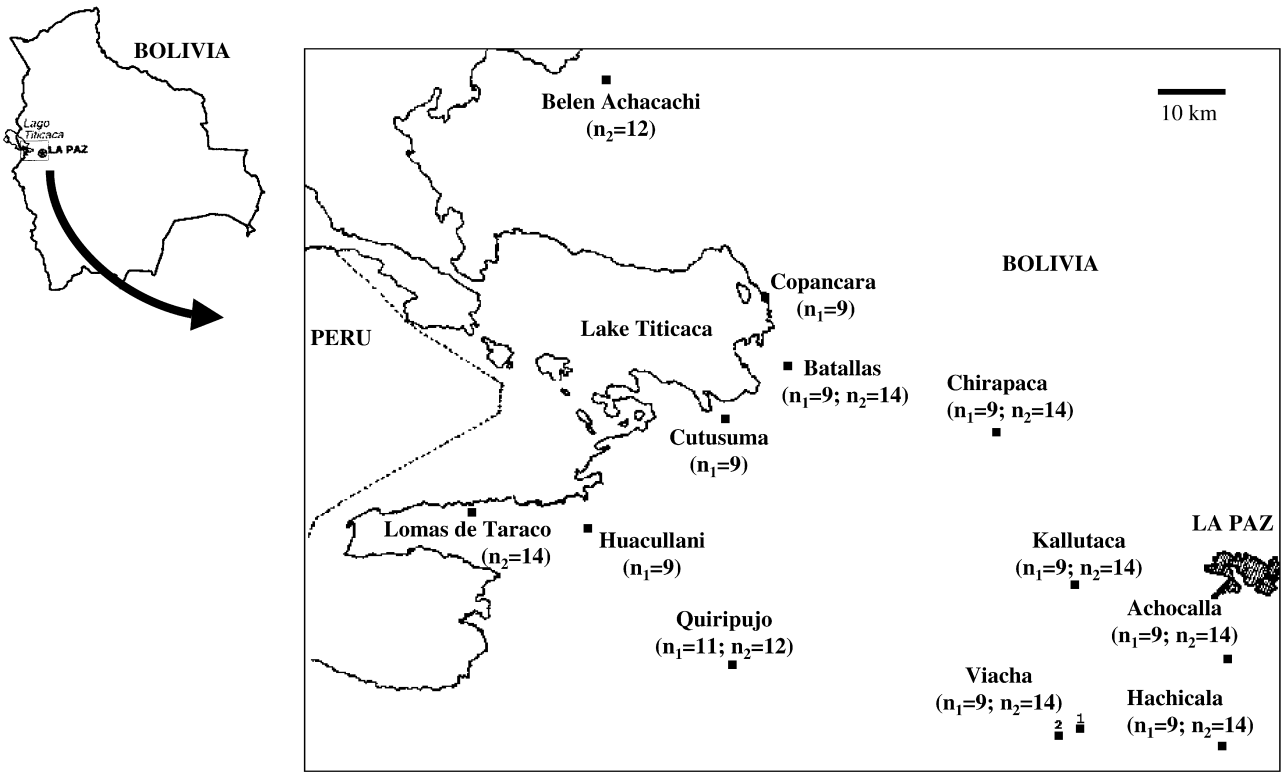

Fig. 1 Sampling map of the northern Bolivian Altiplano, showing the 13 sites sampled. Sites Viacha 1 and 2, Kallutaca, Hachicala, Batallas, Chirapaca, Quiripujo and Achocalla were sampled twice, in 1995 and 1996. Sites Huacullani, Cutusuma and Copancara were only sampled in 1995. Belen and Lomas de Taraco were sampled only in 1996. A total of 1197 snails was collected and screened for trematode infection. A total of 245 snails was genotyped. n_1, n_2: number of snails sampled in each site for microsatellite genotyping in 1995 and 1996, respectively.

Table 1 Sample size in the Old World. Mean observed heterozygosity (H_O) and mean expected heterozygosity (H_E): H_O, H_E averaged over all loci for each population

Country	Morocco			France			Spain				Portugal		Bolivia
Site	Oudaya	Sidi Allal Tazi	Zahir Gharb	Adriers	Berneuil	Opoul	Mampodre	Devesa	Cabornera	Villaverde	Beira	Minho	All populations
Sample size	18	5	5	17	17	10	5	5	10	10	10	10	245
Mean H_O	0	0	0	0.02	0	0	0	0	0.07	0.12	0	0	0
Mean H_E	0.21	0	0	0.21	0.07	0	0.23	0.40	0.22	0.37	0.28	0	0
Mean no. of alleles ± SD	1.7 ± 0.5	1	1	1.7 ± 0.5	1.2 ± 0.4	1	1.5 ± 0.5	1.7 ± 0.5	2 ± 0.9	2.2 ± 0.8	1.8 ± 1	1	1

Mean nos. of alleles: mean number of alleles per population. SD, standard deviation.

urea gels. Bolivian and Old World same-size alleles were sequenced (Genome Express, Grenoble, France) in order to check for size homoplasy. However, for technical reasons, sequences could be compared at three loci only (21, 37, 43). For each locus two sequences from Bolivia were compared with 3–5 sequences taken from the Old World populations.

Within population polymorphism and linkage disequilibrium

Within population polymorphism was studied using FSTAT 2.9 (Goudet 1995, 1999 available from http://www.unil.ch/izea/softwares/fstat.html). The observed (H_O) and expected heterozygosities (H_E) (Nei 1987) were calculated for each sample and averaged over all loci. Deviations from Hardy–Weinberg equilibrium (HWE) were tested for each sample as follows: a statistic, f (Weir & Cockerham 1984), is calculated on randomised data sets and its value compared with the statistic obtained from the observed data set. The randomised data sets are obtained through permutations of alleles among individuals, within sites. The procedure provides an unbiased estimate of the probability to obtain a statistic as large or larger than the observed one. The P-values obtained were corrected for multiple tests using a sequential Bonferroni procedure (Holm 1979; Rice 1989).

Estimators of F_{IS} values, f (Weir & Cockerham 1984), within samples were then computed. Selfing rates (s) were estimated from the classical formula (Crow & Kimura 1970):

$$F_{IS} = \frac{s}{2-s}$$

A linkage disequilibrium analysis was conducted using GENEPOP 3.1d. (Raymond & Rousset 1999). This software computes an exact test on genotypic contingency tables created for each pair of loci. An unbiased estimation of the exact P-value is obtained using a Markov chain algorithm as described in GENEPOP. Sequential Bonferroni corrections were applied for the multiple tests conducted in the same sample.

Population differentiation

Pairwise population differentiation overall loci was tested using FSTAT (Goudet 1995, 1999). Hardy–Weinberg equilibrium within samples was not assumed for the computation of the test (i.e. contingency tables of alleles by samples were created through permutations of genotypes among samples). An analogue of an exact test was computed as above, unless the statistic used is the G-log-likelihood ratio (Goudet *et al*. 1996). A sequential Bonferroni correction was computed on the P-values obtained for the multiple paired tests.

With FSTAT, three of 66 tests were not significant, while the θ-values between the three population pairs were high. These tests involved very small samples; the permutation

procedure used by FSTAT may not be valid in such cases. We re-tested the global genotypic differentiation of these three population pairs with GENEPOP, which uses Markov chains instead of permutations to compute the *P*-values.

Estimators of F_{ST}, θ (Weir & Cockerham 1984), between pairs of samples were also computed.

Bottleneck events in Bolivia

Coalescent processes are useful for estimating the time (T) since the last bottleneck event for a given population (Donnelly 1999). A simplifying assumption is that $2N \gg T$, where N is the effective size of the population. It means that the growth of the population after the bottleneck event was exponential: the coalescent is then a star-like tree. Thus Tt, the total time of coalescence (sum of the branches of the tree) is directly related to T:

$$T = \frac{Tt}{n},$$

where n is the number of genes sampled (twice the number of individuals sampled). The mutation rate, μ, is considered identical across all l loci. The number of mutations observed, X, follows a Poisson distribution. We applied this method in the case of Bolivia, where no mutation was detected (see below). The probability of no mutation occurring is then taken from the zero term of a Poisson distribution. Thus $P(X = 0) = \exp(-\mu nT)$. To find an upper boundary on T we solved for $P \leq 0.05$, such that:

$$T \leq \frac{-\ln 0.05}{\mu nl}$$

(see also Provan *et al*. 1999).

Results

Genetic variability

We found no genetic variability in Bolivia. In other words, of the 245 individuals screened at six loci, only one snail multilocus genotype was observed (Table 1). For three loci we checked that the unique Bolivian microsatellite allele has the same sequence in several snails (data available upon request). Therefore, the total absence of variability that we observe does not seem to be due to size-homoplasy between alleles, albeit that homoplasy may be undetectable even at the sequence level.

A similar procedure showed that the shared alleles have the same sequence in Bolivia and the Old World. Microsatellite allele sharing between Bolivian and Old World snail samples is consistent with the introduction of *Lymnaea truncatula* from Europe, as already shown using the isoenzyme method (Jabbour-Zahab *et al*. 1997).

In contrast, genetic variability exists in Old World snail populations. The total number of alleles detected per microsatellite locus in all *L. truncatula* samples ranges from two (locus 11) to nine (locus 16). Observed and expected heterozygosities and mean number of alleles per population are given for each population in Table 1. The sample populations from the Old World show a rather low within-population polymorphism: four populations are fixed for one allele at all loci (two in Morocco, one in France and one in Portugal), and the other samples show a low average observed allelic diversity (mean number of alleles per sample: 1.2 ± 0.4 SD to 2.2 ± 0.8 SD).

Thus, population genetics parameters are computable only in Old World samples.

F_{IS}, *selfing rates and linkage disequilibrium in the Old World*

Consistent with the very low observed heterozygosities, multilocus heterozygote deficiencies are high and significant for each sample (Table 2).

Linkage disequilibrium is significant in one population between all possible pairs of non fixed locus (Morocco, Oudaya).

Thus a predominant selfing mode of reproduction seems to be the rule in the hermaphroditic snail *L. truncatula*. Estimated selfing rates are presented in Table 2.

Among-population structure in the Old World

We observe a generally highly significant population structure between pairs of populations. All the pairwise tests are significant, when using FSTAT together with GENEPOP (see

Table 2 F_{IS} per population averaged over all loci and selfing rates computed from F_{IS}

Country	Morocco	France		Spain				Portugal
Site	Oudaya	Adriers	Berneuil	Mampodre	Devesa	Cabornera	Villaverde	Beira
Sample size	18	17	17	5	5	10	10	10
All loci: F_{IS}	1**	0.906**	1**	1*	1*	0.69**	0.691**	1**
Selfing rates	1	0.95	1	1	1	0.82	0.82	1

Levels of significance for F_{IS} after sequential Bonferroni correction: $^{**}P < 1\%$; $^{*}P < 5\%$. Only polymorphic populations are shown.

Table 3 F_{ST} between pairs of populations in the Old World

	Morocco			France			Spain				Portugal	
	Oudaya (1)	Sidi Allal Tazi (2)	Zahir Gharb (3)	Adriers (4)	Berneuil (5)	Opoul (6)	Mampodre (7)	Devesa (8)	Cabornera (9)	Villaverde (10)	Beira (11)	Minho (12)
1	0	0.81***	0.77***	0.68***	0.78***	0.75***	0.62**	0.66***	0.70***	0.62***	0.55***	0.81***
2		0	1+++	0.81***	0.94***	1*	0.85*	0.80*	0.82***	0.70*	0.78***	1*
3			0	0.72**	0.90*	1*	0.83***	0.81***	0.81***	0.69***	0.73*	1***
4				0	0.64**	0.77***	0.63***	0.59*	0.58***	0.60***	0.62***	0.81***
5					0	0.89***	0.80***	0.80***	0.73***	0.65***	0.74***	0.92***
6						0	0.89***	0.89***	0.82***	0.74***	0.80***	1***
7							0	0.34+	0.62*	0.47*	0.33*	0.89***
8								0	0.58***	0.23++	0.44*	0.89***
9									0	0.54***	0.63***	0.82***
10										0	0.53***	0.72***
11											0	0.73***
12												0

Levels of significance computed with FSTAT after a sequential Bonferroni correction: ***$P < 0.1\%$; **$P < 1\%$; *$P < 5\%$; +++, ++, +;
Levels of significance computed with GENEPOP. M, Morocco; F, France; S, Spain; P, Portugal.

Table 4 Infestation rates of Bolivian *Lymnaea truncatula* by trematodes

Bolivian Altiplano sample site	Date of collection	No. of snails dissected	% of snails infected with trematodes
Viacha 1	2/95	114	3.5
Viacha 1	2/96	44	88.3
Viacha 2-River	2/95	123	27.6
Viacha 2-River	2/96	20	15.0
Kallutaca	2/95	95	0.0
Kallutaca	2/96	46	2.1
Hachicala	2/95	30	6.7
Hachicala	2/96	67	0.0
Batallas	1/95	58	1.7
Batallas	2/96	87	1.2
Chirapaca	2/95	33	0.0
Chirapaca	2/96	53	9.4
Lomas de Taraco	2/96	37	10.8
Huacullani	2/95	16	6.3
Cutusuma	1/95	29	3.5
Quiripujo	2/95	77	70.1
Quiripujo	2/96	58	5.2
Copancara	2/95	21	0.0
Achocalla	2/95	82	1.2
Achocalla	2/96	53	1.9
Belen Achacachi	2/96	54	14.8
All samples	—	1197	13.6 ± 23.3 SD

All the snails collected in the Altiplano were dissected. SD: Standard deviation.

Materials and Methods). Extremely high F_{ST} values are obtained (from 0.23 to 1) (Table 3).

The Bolivian case

Parasite prevalence. We dissected 1197 snails from different areas in the Altiplano in order to estimate trematode prevalence. Parasite prevalence displayed high mean and variance [13.6% (± 23.3 SD); Table 4]. These results suggest a highly variable selection pressure exerted by parasites on the different snail populations.

Date of the last bottleneck event in Bolivia. We estimated T, the time since the snail population underwent its last

bottleneck event (see Materials and Methods). The number of snail generations since the last bottleneck depends on the mutation rate of the six microsatellite loci. As this mutation rate is unknown, we obtained a range for T according to the usually accepted range of mutation rates for microsatellite loci (between 10^{-3} and 10^{-5} mutations per individual and generation; Hancock 1999). For this range of mutation rate, the upper bound of T lies between 1 and 102 generations.

Discussion

Biology of European Lymnaea truncatula

Our study of the genetic variability of 12 samples of *Lymnaea truncatula* from Europe and North Africa revealed a low level of polymorphism. For comparison, Viard *et al*. (1997) found a higher gene diversity (H_E) in another freshwater snail (*Bulinus truncatus*) (generally > 0.5). However, this result gives important insights into the biology of *L. truncatula*, because such a reduction in genetic polymorphism could be explained in two ways: (i) mating system; and (ii) population dynamics.

Mating system. Our results show high deviations from HWE within populations, towards heterozygote deficiencies. Because *L. truncatula* is an hermaphrodite, these results may indicate very high selfing rates in this species. The significant linkage disequilibrium found in the population of Oudaya (Morocco) might reflect genotypic structures that are maintained by selfing. The strong differentiation observed between populations may reflect the large distances between the samples (30–10 000 km), preventing gene flow. Because it accelerates genetic drift, selfing may also increase differentiation between the populations (Viard et al. 1996). We conclude from all these observations that *L. truncatula* is an almost exclusive selfer. Such high selfing values (0.82–1) are encountered in other hermaphroditic mollusc species (see Viard *et al*. 1996, 1997), but are uncommon in other species of the genus *Lymnaea*, including one of the closest species to *L. truncatula*, *L. peregra* (Jarne 1995; Bargues & Mas-Coma 1997).

Predominant selfing may also explain the low amount of polymorphism observed in our snail populations; selfing actually reduces the effective size of the population, leading to loss of variability (Nei 1987; Slatkin 1995b; Viard *et al*. 1997). These theoretical expectations are consistent with our results and with other findings in plants, in which the correlation between mating system and genetic diversity has been documented more. Extensive reviews of plant isozyme data (Hamrick & Godt 1989, 1996) indeed show a lesser amount of genetic diversity (H_E) at the population and species level in selfing plants. Furthermore, a much larger proportion of genetic variation in selfing species is found among, rather than within, populations.

Population dynamics. Lymnaea truncatula lives in ponds or ditches connected with meadows (Rondelaud 1993). These habitats are subject to temporary flooding or droughts. Such bottlenecks are particularly frequent in populations of freshwater snails that usually live in temporary habitats (Städler & Jarne 1997). Bottlenecks decrease the amount of genetic variability and are thus the second main explanation for our observation of low genetic polymorphism (Chakraborty & Nei 1976; Slatkin 1995a).

The Bolivian issue

No genetic variability was found in Bolivian snail populations. This result is remarkable with relevance to theoretical expectations of host–parasite interactions. First of all, through dissection of the gastrogenital tract of all the snails collected, we found a high mean and variance of prevalence of infection (mean ± SD of 13.6 ± 23.3%) (see Jokela & Lively 1995). Second, preliminary results (S. Hurtrez-Boussès, unpublished data) indicate the existence of microsatellite polymorphism in Bolivian liver flukes. Theoretically, both genetic variability in the parasite and strong selection pressures exerted on its host are prerequisites for the expectations of the 'Red Queen hypothesis'. (i) The coselection between host and parasite should lead to a maintenance of sex on both sides (Lively 1987) which should contribute indirectly to maintenance of polymorphism on host–parasite interaction loci. However, there is no expectation for maintenance of polymorphism on neutral loci such as microsatellites. (ii) Alternatively, in a selfing host population, linkage disequilibrium between selected and neutral loci could lead to the maintenance of overall host genetic diversity (Howard & Lively 1994; Dybdahl & Lively 1998).

We do not have clues about the mating system of *L. truncatula* in Bolivia, because of the lack of genetic variability. However, in the Old World, this snail is an almost exclusive selfer. If we assume that Bolivian snails also are selfers, some 'clonal' diversity should have been maintained in Bolivia.

A stronger effect seems to have impaired the expectations of the Red Queen hypothesis. The most probable explanation is that a bottleneck event removed the host genetic variability and has prevented coevolution between host and parasite since then. This bottleneck event must have been recent enough to hinder restoration of the snail host genetic variability. The computation of the time since this event gives an upper bound of 100 generations, for a relatively low microsatellite mutation rate (1×10^{-5}) (Dallas 1992; Weber & Wong 1993; Schug *et al*. 1997). As the available observations report two generations of snails per year in France (Morel-Vareille 1973), our result thus suggests a very recent bottleneck effect in the Altiplano, as expected.

This bottleneck effect may reflect the introduction from Europe. Malacological surveys give arguments for this hypothesis. The presence of lymnaeid snails on the Bolivian Altiplano was first reported in by Ueno *et al*. (1975). Before that time several malacological surveys had been carried out in the Lake Titicaca area, the last being the Percy Sladen Trust Expedition of 1937 (Haas 1955). Curiously lymnaeids are always absent from the species lists established during these surveys suggesting that the introduction of these snails into the Altiplanic area is perhaps relatively recent.

However, phylogenetic results seem not to be in accordance with this hypothesis. We sequenced the internal transcribed spacers (ITS1, ITS2; GenBank Accession nos: AJ272052, AJ272051, AJ243017, AJ243018) of Bolivian Altiplano and European lymnaeids. ITS are for use in species-level or even deeper phylogenetic studies (Hershkovitz & Lewis 1996) having a lower mutation rate than microsatellites. The sequence results showed two parsimony-informative mutations in the 504 bp ITS1 (0.39% divergence), and one in the 433 bp ITS2 (0.23%), between Bolivian and European lymnaeids (two snails from the Altiplano and eight from populations from Portugal, Spain, France and Switzerland). This ITS divergence is compared with the existence of a private allele in Bolivia at the microsatellite locus 11. Despite the low ITS sampling in Europe, these results might suggest that the introduction of *L. truncatula* from Europe is older than the last bottleneck effect detected in the Altiplano with microsatellites loci. This apparent discrepancy between the different observations could be the result of the historical colonization events of *L. truncatula* from Europe. Indeed, this species might have been first introduced in different areas of South America, and could have recently invaded the Bolivian Altiplano. Further phylogenetical surveys of lymnaeid snails in Bolivia and neighbouring countries might give further arguments for this last hypothesis.

Epidemiological consequences

Finally, we would like to stress the importance of our findings from an epidemiological point of view. Monomorphic lymnaeid populations susceptible to *Fasciola hepatica* may explain such high transmission rates. Moreover, our results could greatly facilitate the control of the liver fluke in the Bolivian Altiplano, through an easier elimination of the transmitting snail, ensured by its present lack of variability.

Acknowledgements

J. G. Esteban, R. Angles, D. Rondelaud, G. Dreyfuss, G. Chlyeh, K. Khallaayoune and M. A. Conceição collaborated in snail collections. We thank T. Bataillon, N. Galtier, T. de Meeûs, Y. Michalakis and two anonymous referees for helpful discussions or comments on the manuscript. This work was partially supported by funding from the STD program of the Commission of the European Community (DG XII: Science, Research and Development) (Contract no. TS3-CT94-0294). Brussels, EC.

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