

NOTE / NOTE

Comparison between shell morphology and genetic diversity in two sympatric lymnaeid snails, vectors of fasciolosis

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Abstract: Species determination of freshwater snails is particularly important in the case of disease vectors. In central France, for example, it has been shown that *Galba truncatula* (Müller, 1774) and *Omphiscola glabra* (Müller, 1774), which can be found in sympatry, are intermediate hosts for fasciolosis. Although these two species are distinguishable based on their shell morphology, some snails present in sympatric zones possess shell characteristics that are common to both species, suggesting possible gene flow. To test this hypothesis, we carried out multilocus enzyme electrophoreses on individuals sampled in one zone of sympatry. Our results clearly show that all snails with shell characteristics common to both species are *O. glabra*. We also note an absence of hybridization between *G. truncatula* and *O. glabra* in the study area. Remarkably, we observe a total monomorphism at the six studied loci among individuals of *G. truncatula*. Similarly, we find a total lack of heterozygotes in the *O. glabra* sample (with classic or intermediate shell characteristics). These results suggest a predominance of selfing in the reproductive mode of both species. Our results suggest that conchological characteristics can provide information about the taxonomic position of lymnaeids. However, they are insufficient to confidently differentiate species.

Résumé : Le problème de la systématique des mollusques dulçaquicoles se pose de façon d'autant plus cruciale que de nombreuses espèces sont vectrices de parasites largement répandus. En particulier, il a été montré dans le Centre de la France que *Galba truncatula* (Müller, 1774) et *Omphiscola glabra* (Müller, 1774), tous deux hôtes intermédiaires de la grande douve du foie, pouvaient se trouver en sympatrie. Les deux espèces peuvent être distinguées par la morphologie de leur coquille mais il existe, dans les zones de sympatrie, des individus possédant des caractéristiques communes aux deux espèces suggérant ainsi la possibilité d'hybridations. Pour tester cette hypothèse, nous avons effectué des électrophorèses enzymatiques multi-locus sur des individus échantillonnés dans une zone de sympatrie. Tous les individus présentant un morphotype intermédiaire ont un génotype comparable aux individus *O. glabra*. Les résultats montrent clairement qu'il n'existe aucun phénomène d'hybridation entre *G. truncatula* et *O. glabra* dans la zone d'étude. De façon remarquable, tous les individus *G. truncatula* présentent une absence totale de polymorphisme aux six loci étudiés. Parmi les échantillons de l'espèce *O. glabra* (en incluant les individus de morphotype classique et de morphotype intermédiaire), aucun hétérozygote n'a été détecté. Ces résultats suggèrent que les deux espèces seraient majoritairement autotélocantes. Les critères morphologiques des coquilles peuvent donc aider à déterminer la position systématique des individus, mais ne sont toutefois pas suffisants pour trancher entre deux espèces.

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Introduction

The growing interest in biodiversity and its evaluation has highlighted the importance of species identification (Ronquist and Gärdenfors 2003; see review in De Meeus et al. 2003). The determination of taxa is particularly important in the case of organisms that are involved in the spread of diseases. Indeed, prophylactic strategies require a thorough knowledge of the biology and ecology of parasites and their vectors (Hurtrez-Boussès et al. 2001).

One such example is that of fasciolosis, a cosmopolitan disease caused by liver flukes (*Fasciola hepatica* L., 1758 and, to a lesser extent, *Fasciola gigantica* Cobbold, 1885) that is responsible for severe human health and veterinary problems (see reviews in Dalton 1999 and Hurtrez-Boussès et al. 2001). The life cycle of liver flukes involves a freshwater snail intermediate host, in which an important asexual multiplication of the parasite occurs (for details on the life cycle see Andrews 1999). *Galba truncatula* (Müller, 1774) (= *Lymnaea truncatula*) is recognized as the predominant intermediate host in Europe (Boray 1966; Graczyk and Fried 1999) and in some parts of South America where European strains have most probably been introduced (Bolivian Aliplano: Jabbour-Zahab et al. 1997; Meunier et al. 2001). In other parts of the world, *F. hepatica* has adapted to local intermediate hosts, including *Austropeplea tomentosa* (Pfeiffer, 1855) in New Zealand and Australia (Boray 1966), and *Lymnaea cubensis* (Pfeiffer, 1839) and *Lymnaea viatrix* (Orbigny, 1835) in South America and Central America (Bargues et al. 1997; Samadi et al. 2000). Moreover, in Europe, several molluscan species have been infected by *F. hepatica*: *Anisus leucostoma* Millet, 1813 (= *Planorbis leucostoma*) (Abrous et al. 2000); *Radix ovata* Draparnaud, 1805 (Dreyfuss et al. 2002); and *Omphiscola glabra* Müller, 1774 (= *Lymnaea glabra* = *Stagnicola glaber*) (Rondelaud et al. 2001; Dreyfuss et al. 2003). The situation is particularly interesting in central France, where fasciolosis is common in cattle (Mage et al. 2002). Indeed, in this region, the prevalences of *F. hepatica* in *O. glabra* have seriously increased during the last decade (Dreyfuss et al. 2003).

In a retrospective survey of human fasciolosis in central France, Rondelaud et al. (2000) showed that *G. truncatula* and *O. glabra* can be found in sympatry (9.2% of the sites in which *F. hepatica* is present; $N = 65$). These two species are distinguishable based on shell morphology (Fig. 1). The shell of *G. truncatula* (Fig. 1A) is recognizable by its pyramidal form (on average, 12 mm high), a spire whose whorls are set out like stairs (in French populations) and whose aperture is approximately one-half the length of the shell. In contrast, *O. glabra* (Fig. 1B) has a narrow, elongated shell (on average, 20 mm high) with a smaller aperture that is approximately one-third the length of the shell (Hubendick 1951). However, it has been shown that lymnaeid snails can present considerable variability in conchological characters (Oviedo et al. 1995). Moreover, Remigio and Blair (1997) have suggested that morphological homoplasy is frequent among lymnaeids. Indeed, in zones of sympatry between *G. truncatula* and *O. glabra*, some snails have shell characteristics that are common to both species (Fig. 1C), suggesting possible gene flow and confusing species identification. Such snails harbouring intermediate morphology

have been observed neither in zones with only *G. truncatula* nor in zones with only *O. glabra* (D. Rondelaud, personal observation).

The aim of this study is to compare snail identification based on conchological parameters to that deduced from genetic variability. For this purpose, we carried out multilocus enzyme electrophoreses on individuals sampled in a zone of sympatry between *G. truncatula* and *O. glabra*, using loci that proved to be discriminatory between taxa of lymnaeid snails (Jabbour-Zahab et al. 1997; Durand et al. 2002).

Materials and methods

Sampling

The study site is located in central France, 108 km north of Limoges (Chézeau Chrétien, Migné, department of Indre: $x = 524.7$ km, $y = 2186.2$ km, Lambert conical projection, zone III). A total of 93 lymnaeid snails were collected on April 2002, within a 5 m long segment of the same ditch. Since isoenzymatic markers were previously isolated in *G. truncatula* (Jabbour-Zahab et al. 1997; Durand et al. 2002), we used samples of *G. truncatula* as reference specimens for this study. For this purpose, 15 *G. truncatula* were also collected from three other sites where *O. glabra* was absent (Berneuil, department of Haute Vienne: $x = 504.6$ km, $y = 2119.6$ km, $N = 6$; Rignac-Belcastel, department of Aveyron: $x = 600.2$ km, $y = 1931.5$, $N = 6$; Montarnaud, department of Hérault: $x = 710.9$ km, $y = 1850.3$ km, $N = 3$). All the specimens were, after morphological identification (see below), stored at -80 °C until protein extraction.

Morphological identification

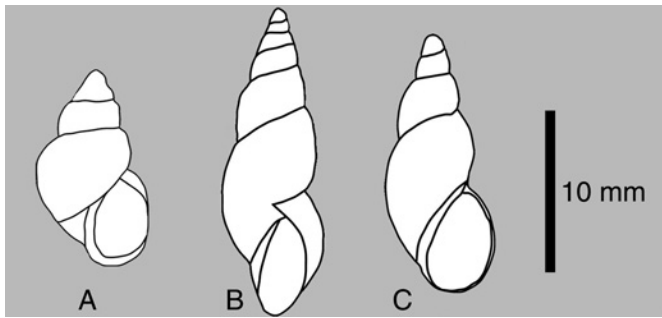
Firstly, each snail was identified based on conchological characteristics according to Hubendick (1951). Identification was made "by eye" (Fig. 1) by a researcher (D. Rondelaud). Three groups were recognized as follows: (1) individuals with morphological characteristics of *G. truncatula* (hereinafter called G.t. group), (2) individuals with morphological characteristics of *O. glabra* (hereinafter called O.g. group), and (3) individuals sharing morphological characteristics of both species (hereinafter called I (intermediate) group).

Secondly, each snail was identified based on the conchological characteristics of height of the mouth and breadth of the last whorl according to Kerney and Cameron (1979). Since these parameters increase with the size of the animal, they were divided by the total height of the shell. Measurements were taken with the aid of a stereomicroscope that was attached to an image processor running the Esilab software (Aries, Prolabo, France).

Multilocus enzyme electrophoresis

Enzyme extraction and electrophoresis were performed according to protocols described in Jabbour-Zahab et al. (1997), Pasteur et al. (1987), and Ben Abderrazak et al. (1993). Six enzyme systems were used: aspartate amino transferase (AAT, E.C. 2.6.1.1), glucose phosphate isomerase (GPI, E.C. 5.3.1.9), leucine aminopeptidase (LAP, E.C. 3.4.11.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), malic enzyme (ME, E.C. 1.1.1.40), and mannose phosphate isomerase (MPI, E.C. 5.3.1.8).

Fig. 1. Three morphological shell categories. Shells with characteristics of (A) *Galba truncatula* (G.t. group), of (B) *Omphiscola glabra* (O.g. group), and characteristics common to (C) *G. truncatula* and *O. glabra* (I group).



Statistical and population genetics analyses

Statistical analyses were made using SYSTAT® version 8.0 (SPSS Inc. 1998). Differences in conchological parameters among the three groups were tested with a nonparametric Kruskal–Wallis test.

Genetic analyses were conducted using FSTAT version 2.9.3.2 (Goudet 1995; 2002). Genetic diversity was assessed by computing observed (H_o) and expected (H_e) heterozygosities (Nei 1987). Heterozygote deficiencies (F_{IS}) were assessed (Weir and Cockerham 1984) and were used to estimate selfing rate (s) according to Crow and Kimura's (1970) equation $F_{IS} = s/(2 - s)$. Linkage disequilibrium between pairs of loci was also computed. Differentiation between pairs of morphological groups was assessed by estimating F_{ST} values.

Finally, a sequential Bonferroni correction was applied to the p values in the case of multiple tests (Rice 1989).

Results

Morphological identification

Based on conchological parameters (for details see Fig. 1), 31 individuals were identified “by eye” as *O. glabra* (O.g. group), 18 as *G. truncatula* (G.t. group), and 44 as an intermediate morph (I group). Height of the mouth and breadth of the last whorl are plotted in Fig. 2. Both parameters significantly differed between the three groups — height of the mouth/height of the shell: 0.372 ± 0.028 (mean \pm SD) for O.g. group, 0.513 ± 0.021 for the G.t. group, 0.429 ± 0.022 for the I group (Kruskal–Wallis test, $H = 70.168$, $df = 2$, $p < 0.002$ after a Bonferroni correction); mean breadth of last whorl/height of the shell: 0.337 ± 0.017 for O.g. group, 0.518 ± 0.039 for G.t. group, 0.426 ± 0.025 for I group (Kruskal–Wallis test, $H = 76.857$, $df = 2$, $p < 0.002$ after a Bonferroni correction).

Enzyme electrophoreses

All 15 reference specimens of *G. truncatula* harboured only a single allele at each of the six loci. The same multilocus genotype was found for 17 of the 18 individuals of the G.t. group (Table 1). The 18th individual harboured genotypes similar to those found in the other two groups, i.e., O.g. and I groups. The O.g. and I groups shared the same alleles at the six studied loci (Table 1). In these two

groups, the locus *LAP* was monomorphic, whereas two alleles were found at each of the other loci. For four of the five polymorphic loci, we did not find any heterozygote, whereas for the locus *GPI* the proportion of heterozygotes reached 37% in the O.g. group ($N = 31$) and 48% in the I group ($N = 44$). The most probable explanation for such high heterozygosity levels at only one locus despite a total lack of heterozygotes at all other loci is the existence of two different loci for the *GPI* system, hereinafter called *GPI-1* and *GPI-2* (Pasteur et al. 1987). In such a case, it is impossible to determine individual genotypes at loci *GPI-1* and *GPI-2*. For this reason, population genetic analyses were only performed with the other four polymorphic loci (*AAT*, *ME*, *MDH*, *MPI*).

Population genetics

Each of the four polymorphic loci presents two alleles in the O.g. group, as well as in the I group. In both of these groups, there is a total lack of heterozygotes ($H_o = 0$, $F_{IS} = 1$, $p < 0.01$ in both groups). The mean expected heterozygosity (H_e) is also low (0.294 ± 0.195 for O.g. group, 0.313 ± 0.188 for I group). Among all the possible pairs of loci, one presents a significant linkage disequilibrium (*MDH* \times *ME*; $p < 0.05$ after a Bonferroni correction). There is no significant genetic differentiation between the O.g. group and the I group ($F_{ST} = 0.033$, nonsignificant).

Discussion

Taxonomic status

All six studied systems were diagnostic, since they distinguished two different genetic entities (Table 1): (1) *G. truncatula* (all individuals collected in Berneuil, Rignac-Belcastel, and Montarnaud and 17 individuals of the G.t. group) and (2) *O. glabra* (all the individuals of the I and O.g. groups and 1 individual of the G.t. group). These two entities do not share any allele and are therefore clearly separated, showing the absence of hybridization between *G. truncatula* and *O. glabra* within these samples.

All the individuals harbouring conchological characters common to both species (I group) proved to be *O. glabra*. As previously shown by Samadi et al. (2000) and Durand et al. (2002), our results suggest that conchological characteristics can provide information on the taxonomic position of lymnaeids, but are insufficient to confidently differentiate species. Contrary to these findings, Ward et al. (1997) have shown that shell morphology, as well as electrophoretic analyses, consistently discriminates between natural populations of two closely related lymnaeid species: *Radix peregra* (Müller, 1774) (= *Lymnaea peregra*) and *R. ovata*. However, Wullschleger and Jokela (2002) have shown that, under laboratory conditions, these two species can converge to a similar shell morphology.

Population genetics

The total lack of polymorphism in *G. truncatula* and the very low polymorphism found in *O. glabra* are consistent with previous studies on *G. truncatula* (Jabbour-Zahab et al. (1997) with enzymatic systems and Meunier et al. (2001) with microsatellite markers) and other lymnaeid species (Durand et al. 2002): *L. cubensis*, *L. viatrix*, and *Pseudo-*

Fig. 2. Breadth of the last whorl as a function of height of the mouth of 93 snails from the study site and of 15 *G. truncatula*, which served as the reference (+) taxon. To control for the size of the snail, both parameters were divided by the total height of the shell. The three morphological categories were determined “by eye” (see text and Fig. 1).

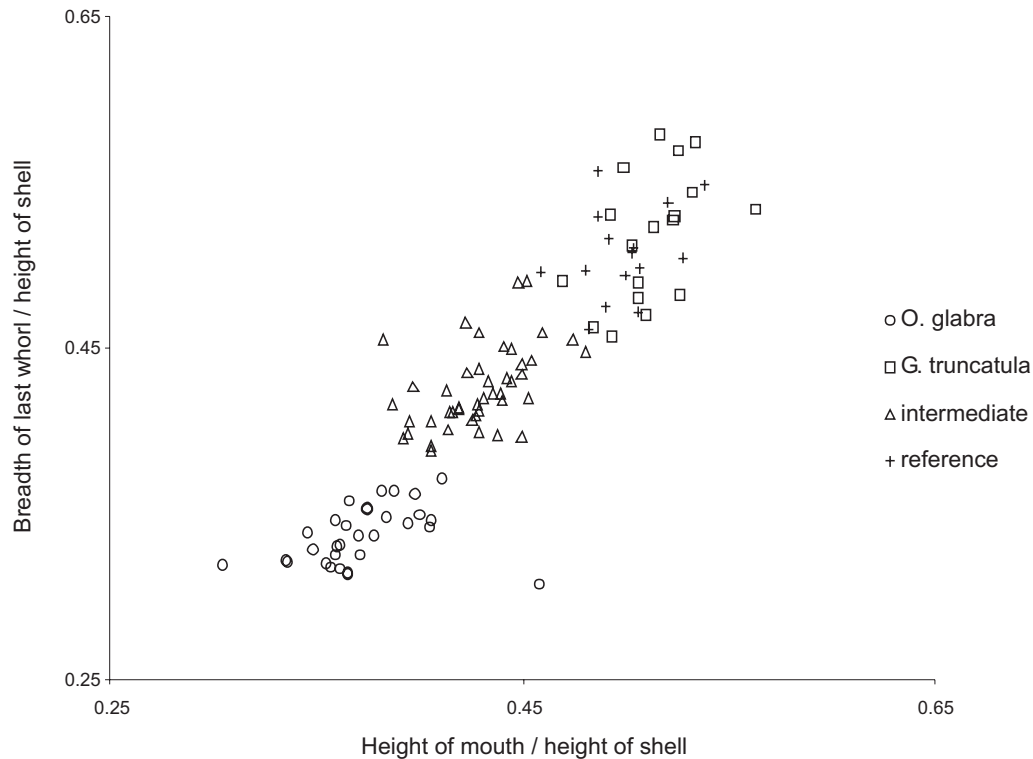


Table 1. Allelic frequencies of the five electrophoretic loci from the three groups of samples obtained from Chézeau Chrétien and other sites.

Locus	Allele	G.t. group*		O.g. group [†]	I group [‡]
		Chézeau Chrétien (n = 17) and other sites (n = 15)	Chézeau Chrétien (n = 1)	Chézeau Chrétien (n = 31)	Chézeau Chrétien (n = 44)
AAT	a	1	0	0	0
	b	0	1	0.889	0.667
	c	0	0	0.111	0.333
LAP	a	1	0	0	0
	b	0	1	1	1
MDH	a	1	0	0	0
	b	0	1	0.759	0.829
	c	0	0	0.241	0.171
ME	a	1	0	0	0
	b	0	1	0.519	0.676
	c	0	0	0.481	0.324
MPI	a	1	0	0	0
	b	0	1	0.963	0.972
	c	0	0	0.037	0.028

Note: Groups are determined on the basis of conchological characteristics (for more details see text and Fig. 1). The locus *GPI* is excluded from this table, since the exact determination of genotypes was not possible (see text for details).

*G.t. group represents individuals harbouring morphological characteristics of *Galba truncatula*.

[†]O.g. group represents individuals harbouring morphological characteristics of *Omphiscola glabra*.

[‡]I group represents individuals sharing morphological characteristics of *G. truncatula* and *O. glabra*.

succinea columella Say, 1817 (= *Lymnaea columella*). Consistent with these results, we also found a low level of polymorphism in *O. glabra* (1.86 ± 0.38 alleles per locus).

Two main hypotheses can explain such low genetic variability: (1) predominance of selfing as a reproductive mode and (2) population dynamics inducing genetic drift.

Mating system

By combining empirical and experimental approaches, Meunier et al. (2004a) have shown that *G. truncatula* is a preferential selfer. Since selfing reduces the effective population size, it induces a loss of genetic variability (Nei 1987; Viard et al. 1997). Concerning *O. glabra*, our results revealed (assuming the existence of two loci for the *GPI* system) a total lack of heterozygotes in the study sample ($N = 75$). Such high deviations from Hardy–Weinberg equilibrium can be explained by the importance of selfing in this hermaphrodite species and (or) by the Wahlund effects, i.e., the existence in the total sample of subpopulations that are genetically differentiated. Since sampling was made at a very small scale (5 m along the ditch), such a substructure seems unlikely in our study. Therefore, *O. glabra* is probably also a predominant selfer.

Population dynamics

Both studied species live in small drainage ditches. Such habitats experience temporal variations in water level that are probably responsible for frequent extinction and recolonization events in snail populations (Goumghar et al. 2001). Meunier et al. (2004b) have shown that a combination of high selfing rates and important bottleneck effects induces drastic reductions of effective population size in *G. truncatula*, leading to a loss of genetic diversity (see Chakraborty and Nei 1976). Our results suggest a similar situation for *O. glabra*.

Shell variation

We did not find a significant genetic differentiation between two morphological entities of the same species (O.g. group and I group), suggesting that the observed differences in shell characteristics do not have a genetic basis. It has been suggested that selection may favour high levels of phenotypic plasticity in freshwater snails living in temporary habitats (for a discussion see Ward et al. 1997). This hypothesis is supported by a study conducted by Wullschleger and Jokela (2002). Indeed, these authors have shown that *R. peregra* and *R. ovata* displayed considerable morphological plasticity, since they obtained a convergence in shell morphology for these two species after having reared two generations under similar laboratory conditions. Moreover, since our study populations live in temporary habitats and frequently experience bottleneck effects (see above), one possible source of morphological variation is the existence of different founder effects. In that case, shell characteristics of colonizers, which are most probably selfers, might be transmitted to lineage of descendants.

Finally, our study highlights the problem of species identification, which was part of a recent debate on the concepts of species (e.g., Hey 2001; Schilthuisen 2001; De Meeus et al. 2003). Indeed, in the case of organisms that reproduce mainly by selfing, as our lymnaeid snails, or in the case of parthenogenetic organisms (Schilthuisen 2001), lineages of individuals can represent morphological groups which can be classified as different species. Moreover, the biological concept of species (i.e., group of individuals that can interbreed to produce viable and fertile descendants and that are reproductively isolated from other groups; Mayr 1942) is not

applicable in the case of organisms that do not outbreed (for a discussion on this issue see De Meeus et al. 2003).

Our study highlights the importance of studies on genetic variability to complete morphological identifications of the liver fluke vectors and to address their biological and ecological characteristics.

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