INSIGHTS INTO THE RELATIONSHIPS OF PALEARCTIC AND NEARCTIC LYMNAEIDS (MOLLUSCA: GASTROPODA) BY rDNA ITS-2 SEQUENCING AND PHYLOGENY OF STAGNICOLINE INTERMEDIATE HOST SPECIES OF *FASCIOLA HEPATICA*

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Summary:

Fascioliasis by *Fasciola hepatica* is the vector-borne disease presenting the widest latitudinal, longitudinal and altitudinal distribution known. *F. hepatica* shows a great adaptation power to new environmental conditions which is the consequence of its own capacities together with the adaptation and colonization abilities of its specific vector hosts, freshwater snails of the family Lymnaeidae. Several lymnaeid species only considered as secondary contributors to the liver fluke transmission have, however, played a very important role in the geographic expansion of this disease. Many of them belong to the so-called "stagnicoline" type group. Stagnicolines have, therefore, a very important applied interest in the Holarctic region, to which they are geographically restricted. The present knowledge on the genetics of stagnicolines and on their parasite-host interrelationships is, however, far from being sufficient. The present paper analyses the relationships between Palaearctic and Nearctic stagnicoline species on the base of the new light furnished by the results obtained in nuclear rDNA ITS-2 sequencing and corresponding phylogenetic studies of the lymnaeid taxa *Lymnaea* (*Stagnicola*) *occulta*, L. (S.) *palustris palustris* (topotype specimens) and L. (S.) *p. turricula* from Europe. Natural infections with *F. hepatica* have been reported in all of them. Surprisingly, ITS-2 length and GC content of *L. occulta* were similar and perfectly fitted within the respective ranges known in North American stagnicolines. Nucleotide differences and genetic distances were higher between *L. occulta* and the other European stagnicolines than between *L. occulta* and the North American ones. The ITS-2 sequence of *L. p. turricula* from Poland differed from the other genotypes known from *turricula* in Europe. The phylogenetic trees using the maximum-parsimony, distance and maximum-likelihood methods confirmed (i) the inclusion of *L. occulta* in the branch of North American stagnicolines, (ii) the link between the North American stagnicolines-*L. occulta* group with *Galba truncatula*, and (iii) the location of the *L. p. turricula* genotype from Poland closer to *L. p. palustris* than to other European *L. p. turricula* genotypes. The Palaearctic species *occulta* is included in the genus *Catascopia*, together with the Nearctic species *catascopium*, *emarginata* and *elodes*. The results suggest a potential of transmission capacity for *C. occulta* higher than that of other European stagnicolines or *Omphiscola glabra*. The relatively low genetic distances between *C. occulta* and *G. truncatula* and the clustering of both species in the same clade suggest that *C. occulta* may be potentially considered as the second lymnaeid intermediate host species of *F. hepatica* in importance in eastern and northern Europe, and probably also western and central Asia, after *G. truncatula*. *L. p. turricula* may be considered as a potential secondary vector of *F. hepatica*, at a level similar to that of *L. p. palustris*.

KEY WORDS : Holarctic stagnicolines, *Lymnaea occulta*, *L. palustris turricula*, rDNA ITS-2 sequences, phylogeny, *Fasciola hepatica*.

Résumé : ÉTUDE COMPARATIVE DE LIMNÉES (MOLLUSCA: GASTROPODA) PALÉARCTIQUES ET NÉARCTIQUES PAR SÉQUENSAGE DE L'ITS-2 DE L'ADN RIBOSOMIAL ET PHYLOGÉNIE DES STAGNICOLINES HÔTES INTERMÉDIAIRES DE *FASCIOLA HEPATICA*

La fasciolose à *Fasciola hepatica* est la distomatose présentant la plus large distribution aux plans longitudinal, latitudinal et de l'altitude. *F. hepatica* possède un grand pouvoir d'adaptation à de nouvelles conditions environnementales en raison de ses propres capacités et de celles de ses hôtes intermédiaires, des mollusques d'eau douce de la famille des Lymnaeidae, à coloniser le milieu. Plusieurs espèces de limnées considérées comme ayant un rôle secondaire dans la transmission de la distomatose hépatique ont cependant joué un rôle important dans l'extension géographique de la maladie. Plusieurs appartiennent au groupe des stagnicolines. Celles-ci ont un intérêt pratique important en région holarctique où elles sont cantonnées. Les connaissances actuelles sur la génétique des stagnicolines et sur leurs relations avec le parasite dont elles sont les hôtes sont très limitées. Ce travail étudie les relations entre les espèces paléarctiques et néarctiques de stagnicolines à la lumière des résultats du séquensage de l'ITS-2 de l'ADN ribosomal et de la phylogénie des taxa *Lymnaea* (*Stagnicola*) *occulta*, L. (S.) *palustris palustris* (specimens topotypes) et L. (S.) *p. turricula* d'Europe. Une infestation naturelle par *F. hepatica* a été rapportée chez chacune d'entre elles. Fait surprenant, la longueur de l'ITS-2 et la teneur en GC de *L. occulta* sont les mêmes et parfaitement ajustés avec ceux des stagnicolines nord-américaines. Les différences sont plus importantes entre *L. occulta* et les autres stagnicolines européennes qu'entre *L. occulta* et stagnicolines nord-américaines. Le séquensage de l'ITS-2 de *L. p. turricula* de Pologne diffère des autres génotypes connus de *turricula* d'Europe. Les arbres phylogénétiques obtenus par parcimonie, distances et maximum de vraisemblance confirment : i) l'inclusion de *L. occulta* à la branche des stagnicolines nord-américaines; ii) le lien entre le groupe stagnicolines nord-américaines-*L. occulta* et *Galba truncatula*; et iii) que le génotype de *L. p. turricula* de Pologne est plus proche de celui de *L. p. palustris* que de des autres génotypes de *L. p. turricula* d'Europe. L'espèce paléarctique *occulta* est incluse dans le genre *Catascopia*, avec les espèces néarctiques *catascopium*, *emarginata* et *elodes*. Les résultats suggèrent une plus grande capacité potentielle de transmission de *C. occulta* que de celles des autres stagnicolines européennes et d'*Omphiscola glabra*. La petite différence génétique entre *C. occulta* et *G. truncatula*, ainsi que la proximité des deux espèces dans le même clade suggèrent que *C. occulta* pourrait, en importance, être considéré comme potentiellement la seconde espèce de Lymnaeidae hôte intermédiaire de *F. hepatica*, à un niveau équivalent de celui de *L. p. palustris*.

MOTS CLÉS : stagnicolines holarctiques, *Lymnaea occulta*, *L. palustris turricula*, séquences ITS-2 de l'ADN ribosomique, phylogénie, *Fasciola hepatica*.

INTRODUCTION

Freshwater snails of the family Lymnaeidae are used as intermediate hosts by numerous digenetic trematode species (mainly as first intermediate hosts, but also as second intermediate hosts as for instance in echinostomatids) (Erasmus, 1972; Brown, 1978). Many lymnaeid species are of applied interest because of transmitting several trematode species of large medical and veterinary impact, among which fasciolids (Mas-Coma & Bargues, 1997), schistosomatids of birds and mammals whose cercariae they shed cause dermatitis or swimmer's itch in humans, and echinostomatids (see review in Bargues *et al.*, 2001). Despite the applied interest of lymnaeid snails in epidemiological and control studies concerning the important trematode parasitic diseases they transmit, the present knowledge on the genetics of this gastropod group as well as on their parasite-host interrelationships is far from being sufficient. A good example of this situation is the systematic-taxonomic confusion in which this molluscan family is immersed (see review in Bargues *et al.*, 2001). At lymnaeid species level, the problems are found mainly because of the interspecific morphological and anatomic uniformity numerous species show, usually giving serious difficulties in specimen classification, sometimes even impeding it (e.g. Oviedo *et al.*, 1995). Moreover, intraspecific variation of shell shape is particularly well marked within lymnaeids depending on environmental conditions (Burch, 1968; Burch & Lindsay, 1973), although a genetic component in shell shape has been shown at least in some lymnaeid populations (Samadi *et al.*, 2000). In Europe, there are many specimen classification problems, mainly concerned with species of the "stagnicoline" and "radix" type groups (Glöer & Meier-Brook, 1998). Recent papers suggested that nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) sequences

were able to furnish appropriate markers to clarify the systematics of this snail group, as well as to establish lymnaeid species classification (Bargues & Mas-Coma, 1997; Bargues *et al.*, 1997; Remigio & Blair, 1997a,b). Moreover, a very recent paper has already proved that the sequence of the second internal transcribed spacer (ITS-2) of the rDNA is a useful marker for resolving supraspecific, specific and population relationships in Lymnaeidae (Bargues *et al.*, 2001). The results obtained showed up to which level the lymnaeid systematic confusion can reach. Several populations originally classified as belonging to different species showed identical ITS-2 sequences, and other populations originally classified as pertaining to the same species presented different ITS-2 sequences. Sometimes the sequence differences were very few, suggesting intraspecific variability (different genotypes). But sometimes differences detected among populations classified as pertaining to the same species were numerous, sufficient as to consider different species involved. Finally, the analysis of genetic distances and sequence differences found between the distinct populations and taxa studied allowed us to distinguish the upper limit to be expected within a single species and to how different twin species can be expected to be at rDNA ITS-2 sequence level.

The review of the European lymnaeids by means of ITS-2 sequence comparisons and corresponding maximum-parsimony, distance and maximum-likelihood analyses allowed us to distinguish many genotypes in a total of 11 species (one including two subspecies) distributed in four different genera (one comprising two subgenera) (Bargues *et al.*, 2001): *Lymnaea* (*Lymnaea*) *stagnalis* (Linnaeus, 1758) (including four different genotypes); *L. (Stagnicola) palustris palustris* (Müller, 1774) (one genotype); *L. (S.) palustris turricula* (Held, 1836) (two genotypes); *L. (S.) fuscus* (Pfeiffer, 1821) (= *S. vulnerata* Küster, 1862) (four genotypes); *L. (S.) corvus* (Gmelin, 1791) (one genotype); *Omphicola glabra* (Müller, 1774) (two genotypes); *Radix auricularia* (Linnaeus, 1758) (five genotypes); *R. balthica* (Linnaeus, 1758) [= *R. peregra* (Müller, 1774); = *R. ovata* (Draparnaud, 1805)] (three genotypes); *R. labiata* (Rossmässler, 1835) (= *R. peregra sensu* Ehrmann, 1933; = *R. alpicola* Westerlund, 1875) (two genotypes); *R. lagotis* (Schränk, 1803) (two genotypes); *R. ampla* (Hartmann, 1821) (one genotype); and *Galba truncatula* (Müller, 1774) (two genotypes). In agreement with the recent taxonomic review of Falkner *et al.* (2002), the taxa *R. peregra* and *R. ovata* enter in synonymies. Although used during decades, both taxa are very confusing because of the impossibility to know to which concrete species they actually refer. The resurrection of the taxa *R. balthica*, *R. labiata* and *R. lagotis* becomes thus clarifying.

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In this review paper of Bargues *et al.* (2001), two lymnaeid populations from Turkey were also included, appearing as populations 49 and 57 in Table I (see p. 90). The locality name at population 49 must imply identity of Bozdag and Söke. This is due to a regrettable error by one of the authors (C. Meier-Brook). After publication, the collector of the samples, Dr. Gerhard Falkner, drew his attention upon the fact that localities lie more than 100 km apart from each other. The collector had recognised the species difference as follows: *i*) in the Bozdag Mountains he collected the species then electrophoretically identified by C. Meier-Brook (and also treated in Remigio & Blair, 1997a, unfortunately also there under the erroneous locality name "Söke" instead of Bozdag) as *Radix peregra sensu* Ehrmann (now valid name *R. labiata*, according to Bargues *et al.*, 2001); *ii*) the more southern population of Söke, however, had already been assigned, by Falkner, to a non-European species, which resembles *Radix natalensis* (Krauss), a species otherwise reaching north to Egypt and Israel (the Söke lymnaeid was only classified as *Radix* sp., pending studies of Asian and African lymnaeids, by Bargues *et al.*, 2001). Consequently, to users of the 2001 review paper it may be recommended to cross out, in the line of population 49, the locality names of "Söke, Aydin Sira Daglari" referring to the present paper.

Fascioliasis by *F. hepatica* is the vector-borne disease presenting the widest latitudinal, longitudinal and altitudinal distribution known. *F. hepatica* shows a great adaptation power to new environmental conditions which is the consequence of its own capacities together with the adaptation and colonization abilities of its specific vector hosts, freshwater snails of the family Lymnaeidae (Mas-Coma *et al.*, 2003). Beside the very few lymnaeid species acting as preferential intermediate hosts, several other lymnaeid species only considered as secondary contributors to the liver fluke transmission have, however, played a very important role in the geographic expansion of this disease. Many of them belong to the so-called "stagnicoline" type group (Mas-Coma *et al.*, 2003). Stagnicolines have, therefore, a very important applied interest in the Holarctic region, to which they are geographically restricted. Thus, efforts to clarify this group are welcome, as the general confusion in stagnicolines does not only concern Europe, but also Asia and North America (see Hubendick, 1951).

The present paper has the purpose of analysing the relationships between Palaearctic and Nearctic stagnicoline species on the base of the new light furnished by the results obtained in rDNA sequencing and corresponding phylogenetic studies of the taxa *L. (S.) occulta* (Jackiewicz, 1959), *L. (S.) palustris palustris* and *L. (S.) palustris turricula*. Their parasitological interest lies in the fact that natural infections with the liver fluke

Fasciola hepatica (Trematoda: Fasciolidae) have been reported in populations of all of them. *L. (S.) occulta* is sequenced for the first time; the status of the two *L. (S.) palustris* subspecies could not be definitively established by the previous studies of the respective populations available so far (Bargues *et al.*, 2001).

MATERIALS AND METHODS

LYMNAEID MATERIALS

Specimens of the following lymnaeid populations have been newly sequenced: *Lymnaea (S.) occulta* from Gorzykowo (near the road connecting Wrzesnia and Brzezina), NW Poland; *Lymnaea (S.) palustris palustris* from Fure Sø, Denmark (topotype specimens, laboratory bred, legit G. Falkner); and *Lymnaea (S.) palustris turricula* from Siedzina (near Nysa in Lower Silesia), SW Poland.

The following rDNA ITS-2 sequences present in GenBank-EMBL were used: *Lymnaea (Lymnaea) stagnalis* genotype 1 (GT1) from Germany (Accession No. AJ319614), *L. (L.) stagnalis* GT2 and GT3 from France (AJ319615, AJ319616), *L. (L.) stagnalis* GT4 from Italy, France and Germany (AJ319617); *Lymnaea (Stagnicola) palustris turricula* GT1 and GT2 from Austria (AJ319618, AJ319619); *L. (S.) palustris palustris* from France, Germany and The Netherlands (AJ319620); *L. (S.) fuscus* GT1 from France (AJ319621), *L. (S.) fuscus* GT2 from Germany (AJ319622), *L. (S.) fuscus* GT3 from Austria and France (AJ319623), *L. (S.) fuscus* GT4 from Spain (AJ319624); *L. (S.) corvus* from Austria (AJ319625); *Omphiscola glabra* GT1 from Germany (AJ319626), *O. glabra* GT2 from France (AJ319627); *Radix auricularia* GT1 from Czech Republic, Austria and UK (AJ319628), *R. auricularia* GT2 from Czech Republic (AJ319629), *R. auricularia* GT3, GT4 and GT5 from France (AJ319630, AJ319631, AJ319632); *R. balthica* GT1 from France, The Netherlands, Spain and Iceland (AJ319633), *R. balthica* GT2 from France (AJ319634), *R. balthica* GT3 from France (AJ319635); *R. labiata* GT1 from Czech Republic and Turkey (AJ319636), *R. labiata* GT2 from Germany (AJ319637); *R. lagotis* GT1 from Czech Republic (AJ319638), *R. lagotis* GT2 from Austria (AJ319639); *R. ampla* from Austria (AJ319640); *Radix* sp. from Turkey (AJ319641); *Galba truncatula* GT1 from Spain, Portugal and Switzerland (AJ243017), *G. truncatula* GT2 from Spain, France, Portugal and The Netherlands (AJ296271); *Hinkleyia catascopium* (Say, 1817) from Wisconsin, USA (AJ319642) (Bargues *et al.*, 2001), *H. catascopium* from Au Sable River, Michigan, USA (AF013143), *H. elodes* (Say, 1821) from Zeeb Road, Ann Arbor, Michigan, USA (AF013138), *H. emarginata* (Say, 1821) from Higgins Lake, Michigan, USA (AF013142), and *H. caperata* (Say, 1829) from south-

western Manitoba, Canada (AF013139) (Remigio & Blair 1997b). *H. catascopium*, *H. elodes* and *H. emarginata* were already considered conspecific by Remigio & Blair (1997b) and, in agreement, *H. catascopium* was noted as the valid species, with *H. elodes* and *H. emarginata* as synonyms by Bargues *et al.* (2001); in spite of this, the latter two specific names will be used throughout in this paper to simplify reference to their corresponding ITS-2 sequences.

The rDNA ITS-2 sequences of three species of the family Planorbidae recently available in GenBank-EMBL were also used for outgroup conformation: *Biomphalaria pfeifferi* (Krauss, 1848) (AY030361), *B. amazonica* Paraense, 1966 (AY030385), and *Helisoma trivolvis* (Say, 1817) (AY030403) (DeJong *et al.*, 2001).

MOLECULAR TECHNIQUES

• DNA extraction

Snail feet fixed in 70 % ethanol and maintained at 4°C for several weeks 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 two hours at 55°C with alternate shaking each 15 min. The procedure steps were performed according to methods outlined previously (Bargues & Mas-Coma, 1997; Bargues *et al.*, 2001). Total DNA was isolated according to the phenol-chloroform extraction and ethanol precipitation method (Sambrook *et al.*, 1989). The pellet was dried and resuspended in 30 µl sterile TE buffer (pH 8.0). This suspension was stored at -20°C until use.

• rDNA sequence amplification

The fragment corresponding to the ITS-2 of each lymnaeid was amplified by the Polymerase Chain Reaction (PCR) using 4-6 ml of lymnaeid genomic DNA for each 50 µl PCR reaction, according to methods outlined previously (Almeyda-Artigas *et al.*, 2000). The PCR amplification was performed using primers designed in conserved positions of 5.8S and 28S rRNA genes of several eukaryote Metazoa species. The primers used were as described by Almeyda-Artigas *et al.* (2000). The primer LT1 (forward) 5'-TCGTCTGTGTGAGGGTCG (Bargues *et al.*, 2001) was used for amplification and sequencing purposes. Amplifications were generated in a Peltier thermal cycler (MJ Research, Watertown, MA, USA), by 30 cycles of 30 sec at 94°C, 30 sec at 50°C and one min at 72°C, preceded by 30 sec at 94°C and followed by seven min at 72°C. Ten microliters of the reaction mixture were examined by 1 % agarose gel electrophoresis, followed by ethidium bromide staining.

• Purification and quantification of PCR products

Primers and nucleotides were removed from PCR products by purification on Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's protocol and resuspended in 50 µl of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm.

• DNA sequencing

The sequencing of the ITS-2 of the rDNA was performed on both strands by the dideoxy chain-termination method (Sanger *et al.*, 1977), and was carried out with the Taq dye-terminator chemistry kit for ABI 373A (Perkin Elmer, Foster City, CA, USA), using PCR primers.

SOFTWARE PROGRAMS USED

• For sequence alignment

Sequences were aligned using CLUSTAL-W version 1.8 (Thompson *et al.*, 1994) and introducing sequences in different orders at random to reduce biases (Lake, 1991).

• For phylogenetic analysis

Maximum-parsimony, distance and maximum-likelihood methods were used in phylogeny reconstruction. All these analyses were performed using algorithms provided in PAUP v.4.0b10 (Swofford, 2001).

Maximum parsimony (MP) analysis was performed using the heuristic algorithm. To assess the relative support for internal nodes, a bootstrap resampling approach (with 1,000 replicates) was used. Alignment gaps were treated as missing character states for the analyses. Only minimal length trees were kept. Polytomies were permitted. Accelerated transformation was used for character-state optimization. Starting tree obtained via stepwise addition with addition sequence simple, and branch-swapping algorithm by tree-bisection-reconnection.

For distance analysis, neighbor-joining (NJ) trees (Saitou & Nei, 1987) were generated from Kimura 2-parameter and Kimura 3-parameter (Kimura, 1980) distance matrices. NJ analysis using Kimura distances was performed and statistical support of each NJ tree was assessed with bootstrap-resampling technique (Felsenstein, 1985) over 1,000 replications. Minimum evolution (ME) analyses were also performed using Log-Determinant (Log-Det) for the estimation of distances (Lockhart *et al.*, 1994), and the corresponding ME trees were generated from the Log-Det corrected distances by NJ analysis. A more complex model, TN93, was also applied to verify whether a better fit of the data could be obtained.

Maximum likelihood (ML) trees were also constructed. To generate a ML tree that best fits the ITS-2 data, hie-

rarchical likelihood ratio tests (LRTs) were performed following the procedure outlined in Posada & Crandall (1998). Depending on the model of DNA evolution employed, parameters such as transition/transversion ratio as well as those associated with the assumption of rate heterogeneity were estimated from actual data (Yang, 1996). The maximum-likelihood values for ts/tv, gamma shape parameter α and the proportion of invariable sites were estimated separately for the lymnaeid ITS-2 sequences. Empirical base sequences, where applicable, were used. Analyses were restricted to five different ML nucleotide substitution models, including HKY85 (Hasegawa *et al.*, 1985), HKY85 + Γ (gamma-distributed rates; Yang, 1994b), HKY85 + Γ + I (mixed gamma-distributed rates and invariable sites), GTR (Yang, 1994a), and GTR + Γ + I (mixed gamma-distributed rates and invariable sites; Gu *et al.*, 1995). To provide an assessment of the reliability of the nodes of the trees, a quartet puzzling analysis was employed (with 1,000 puzzling steps); although the quartet puzzling analysis has recently received criticisms (see Ranwez & Gascuel, 2001), it is widely used and appropriate here to allow the comparison with previous results obtained on lymnaeids (Bargues *et al.*, 2001).

RESULTS

SEQUENCE ANALYSES

The rDNA ITS-2 sequences of the two Polish lymnaeids reported in this paper are available in the GenBank/EMBL data bases under the following accession numbers: AJ457042 for *L. occulta*, and AJ457043 for *L. p. turricula*. The lengths and GC contents of these ITS-2 sequences were, respectively, 448 bp and 60,7 % for *L. occulta*, and 473 bp and 58,1 % for *L. p. turricula*. The 473 bp-long ITS-2 sequence of the *L. p. palustris* topotype from Denmark proved to be identical to that known from other populations of the same nominal subspecies from France, Germany and The Netherlands (AJ319620) (Bargues *et al.*, 2001).

When comparing the ITS-2 sequence of *L. occulta* with those known of other European stagnicoline lymnaeid species, as *L. p. palustris* (one genotype), *L. p. turricula* (two genotypes), *L. fuscus* (four genotypes), and *L. corvus* (one genotype), two differences appear: *i*) the length in *L. occulta* (448 bp) is shorter and does not fit into the range shown by European stagnicolines (468-484 bp); *ii*) the GC content in *L. occulta* (60,7 %) is higher and does not enter within the range of the other European stagnicolines (57,9-58,5 %). Surprisingly, both ITS-2 length and GC content of *L. occulta*

are similar and perfectly fit within the respective ranges known in North American stagnicoline lymnaeid species, as *H. caperata*, *H. catascopium*, *H. elodes* and *H. emarginata* (length: 444-449 bp; GC content: 60,3-60,8 %).

Genetic distances in pairwise comparisons according to Kimura's two-parameter model between different populations of European and American stagnicoline lymnaeid species are shown in Table I. Interestingly, both total and mean character differences are higher between *L. occulta* and the European stagnicolines (108-177 and 0.26601-0.28329, respectively) than between *L. occulta* and the North American ones (27-76 and 0.06490-0.18095, respectively).

In the different alignments performed between *L. occulta* and the North American stagnicolines (Table II), ITS-2 sequence differences appear markedly more numerous when compared to *H. caperata* than when compared to the group *H. emarginata*, *H. elodes* and *H. catascopium*. A total of 103 absolute nucleotide differences (22.59 % in the respective two-sequence alignment of 456-bp length), including 43 transitions (9.43 %), 30 transversions (6.58 %) and 30 insertions plus deletions (6.58 %) were detected between the sequences of *L. occulta* and *H. caperata*, whereas those were only 87-90 absolute nucleotide differences (18,28-18.75 % in two-sequence alignments of 476-481 bp lengths), including 11-14 transitions (2,29-2.92 %), 14-17 transversions (2,92-3.57 %) and 58-63 insertions plus deletions (12.18-13.10 %) between *L. occulta* and the group *H. emarginata*, *H. elodes* and *H. catascopium*.

When comparing the ITS-2 sequence of *L. p. turricula* from Poland with those known of other European populations of *L. p. palustris* (one genotype present in Denmark, France, Germany and The Netherlands) and *L. p. turricula* (two genotypes, both from Salzburg, Austria), very few differences were found. In pairwise sequence alignments (473-bp long), the following differences between *L. p. turricula* from Poland and the above-mentioned genotypes were detected: only one transition (0.2 %) versus *L. p. palustris*, two transitions plus one transversion (0.6 %) versus *L. p. turricula* GT1, and two transitions plus two transversions (0.8 %) versus *L. p. turricula* GT2. As the ITS-2 sequence of *L. p. turricula* from Poland is not equal to any of the previous two genotypes known, it is here designed as genotype 3 (GT3).

In the multiple, 473-bp long ITS-2 sequence alignment including all the three genotypes of *L. p. turricula* plus *L. p. palustris*, only five absolute nucleotide differences were detected (1.1 %). When the *L. fuscus* genotypes are included in this alignment, 33 nucleotide differences appear (6.9 % in the 480-bp long alignment), and when *L. corvus* is added to the latter alignment

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 <i>L. fuscus</i> GT1	–	0.00212	0.00857	0.01285	0.03219	0.03433	0.03004	0.02790	0.04925	0.28266	0.28199	0.28504	0.28297	0.27094	0.28101
2 <i>L. fuscus</i> GT2	1	–	0.00857	0.01285	0.03219	0.03433	0.03004	0.02790	0.04925	0.28266	0.28199	0.28504	0.28297	0.27094	0.28101
3 <i>L. fuscus</i> GT3	4	4	–	0.00427	0.02784	0.02998	0.02570	0.02355	0.04915	0.28029	0.27962	0.28504	0.28058	0.26667	0.27848
4 <i>L. fuscus</i> GT4	6	6	2	–	0.02784	0.02998	0.02998	0.02784	0.05342	0.27791	0.27725	0.28266	0.27818	0.26667	0.27848
5 <i>L. p. turricula</i> GT1	15	15	13	13	–	0.00634	0.00634	0.00423	0.05085	0.28404	0.28337	0.28873	0.28436	0.26601	0.27273
6 <i>L. p. turricula</i> GT2	16	16	14	14	3	–	0.00846	0.00634	0.05297	0.28404	0.28337	0.28873	0.28436	0.26847	0.27525
7 <i>L. p. turricula</i> Pol	14	14	12	14	3	4	–	0.00211	0.04873	0.28169	0.28103	0.28638	0.28199	0.26601	0.27020
8 <i>L. p. palustris</i>	13	13	11	13	2	3	1	–	0.04661	0.28404	0.28337	0.28873	0.28436	0.26847	0.27273
9 <i>L. corvus</i>	23	23	23	25	24	25	23	22	–	0.29792	0.29493	0.29561	0.29604	0.28329	0.29777
10 <i>C. emarginata</i> Mi	119	119	118	117	121	121	120	121	129	–	0.00446	0.01116	0.01126	0.06731	0.15633
11 <i>C. catascopium</i> Wis	119	119	118	117	121	121	120	121	128	2	–	0.00670	0.00676	0.06490	0.15633
12 <i>C. elodes</i> Mi	120	120	120	119	123	123	122	123	128	5	3	–	0.00676	0.06971	0.15881
13 <i>C. catascopium</i> Mi	118	118	117	116	120	120	119	120	127		3	3	–	0.06971	0.15881
14 <i>C. occulta</i>	110	110	108	108	108	109	108	109	117	28	27	29	29	–	0.18095
15 <i>H. caperata</i> Man	111	111	110	110	108	109	107	108	120	63	63	64	64	76	–

Below diagonal: total character differences; above diagonal: mean character differences. Pol = Poland; Mi = Michigan, USA; Wis = Wisconsin, USA; Man = Manitoba, Canada.

Table I. – Pairwise comparisons of nucleotide divergences according to Kimura's two-parameter model for the whole set of European and North American stagnicoline lymnaeid ITS-2 sequences.

Compared species	Alignment length	Nucleotide differences		Substitutions				Insertions + deletions		
				Transitions		Transversions				
		Number of bp	No.	%	No.	%	No.	%	No.	%
<i>C. occulta</i> vs <i>C. emarginata</i>	480	89	18.54	12	2.50	15	3.12	62	12.92	
<i>C. occulta</i> vs <i>C. elodes</i>	480	90	18.75	14	2.92	14	2.92	62	12.92	
<i>C. occulta</i> vs <i>C. catascopium</i> Wis	481	90	18.71	11	2.29	16	3.33	63	13.10	
<i>C. occulta</i> vs <i>C. catascopium</i> Mi	476	87	18.28	12	2.52	17	3.57	58	12.18	
<i>C. occulta</i> vs <i>H. caperata</i>	456	103	22.59	43	9.43	30	6.58	30	6.58	

Wis = Wisconsin; Mi = Michigan

Table II. – ITS-2 sequence differences detected in pairwise comparisons between *Catascopia occulta* and other proximal stagnicoline lymnaeid species.

differences increase up to 40 (8.2 % in the 485-bp long alignment). If *L. occulta* is included in the multiple alignment of the sequences of the European stagnosticolines *L. p. palustris*, *L. p. turricola*, *L. fuscus* and *L. corvus*, the number of absolute nucleotide differences increases considerably up to 188 (35.8 %) in a 525-bp long ITS-2 alignment.

PHYLOGENETIC ANALYSES

Alignments of the 37 rDNA ITS-2 sequences representing a total of 68 populations of 14 European and four North American lymnaeid species (and subspecies), including the genus/subgenus taxa *Lymnaea*, *Stagnicola*, *Omphiscola*, *Radix*, *Hinkleyia*, and *Galba*, were used to infer phylogenies.

Because of the inclusion of a species as *L. occulta*, which appears building a bridge between European and North American lymnaeids, the assay of a especially selected external outgroup appears to be convenient to verify the main branching of the phylogenetic trees, even with the risk of somewhat losing resolution in small branch differentiation. Thus, for phylogenetic reconstruction, analyses were carried out using three Planorbidae, *B. pfeifferi* (representing the African *Biomphalaria* clade), *B. amazonica* (representing the Neotropical *Biomphalaria* clade) and *H. trivolvis* (to include another planorbid genus) (DeJong *et al.*, 2001) as outgroups.

When using the three planorbids as outgroup, a 581-position-long alignment was obtained. Of these, 160 sites were constant and 390 were parsimony-informative. All MP, NJ and ML analyses yielded similar trees, where *L. occulta* did not appear in or linked to the European stagnosticoline branch, but clustered together with the North American stagnosticoline group.

MP analysis, using the heuristic option, yielded a single most-parsimonious tree (Fig. 1A). The MP tree obtained was 1,002 steps long. CI and HI were 0.726 and 0.274, respectively. RI, RC and G-fit were 0.918, 0.666 and – 333.850, respectively. In distance analysis, very similar topologies and bootstrap values were obtained in the NJ trees constructed using Kimura 2-parameter (tree not shown) and LogDet distances (score = 2.6073). TN93 provided a topology identical to that obtained with NJ using LogDet distances, showing slightly higher support values for some branch nodes (Fig. 1B).

For the ITS-2 sequences, the following values were estimated: ts/tv of 1.09 for all lymnaeids and 1.4 for only stagnosticolines, α of 0.8, and the proportion of invariable sites I of 0.1275. A ts/tv ratio of 2 was also applied because this ratio had previously shown the best fit for lymnaeid ITS-2 (BARGUES *et al.*, 2001). The ML model best fitting the ITS-2 data of lymnaeids was HKY85 using the ts/tv ratio of 2, in which the log likelihood was 4865.40131, puzzle values obtained were

very high (of 100 in all main branches except one with 96) and the number of quartets examined was 91390 using least-squares method with ML distances (Fig. 1C). When Γ and I were introduced to HKY85, slightly better – lnL were obtained, but puzzle values always appeared lower and topologies usually showed incongruent branching results (trees not shown). Using settings corresponding to the GTR model, the likelihood was worse (– lnL = 4893.1753) and the puzzle values were lower in the branches of *Radix*, American stagnosticolines and outgroup planorbids. In GTR + Γ + I, the likelihood was better (– lnL = 4785.7400) but puzzle values were lower than in HKY85 and given branches did not fit appropriately (trees not shown).

All MP, NJ and ML trees showed main branches supported by very high bootstrap and puzzle values (all higher than 90, most of them of 100). The ingroup taxa, representing all lymnaeids studied, are divided into two big clades, one for *Radix* species and another for all other genera. In this second clade, two branches are well defined: one branch for the European lymnaeids including *Lymnaea* (*Lymnaea*), *Lymnaea* (*Stagnicola*) and *Omphiscola*, and another branch comprising the European *Galba* and *L. occulta* together with the North American stagnosticolines. In the HKY85 tree obtained with ts/tv of 1.09 and without gamma correction and in that obtained with the GTR model, the *Omphiscola* branch becomes basal to the European stagnosticolines, as in the MP and NJ trees, whereas *Omphiscola* appears only basal to *Lymnaea* (*Lymnaea*) in all other trees obtained under the HKY85 model (Fig. 1C).

Summing up, the phylogenetic trees obtained showed similar trees and confirmed i) the inclusion of *L. occulta* in the branch of *H. emarginata*, *H. elodes* and *H. cata-scopium*, ii) the link between the North American stagnosticolines-*L. occulta* group with *G. truncatula*, and iii) the location of *L. p. turricula* GT3 from Poland closer to *L. p. palustris* than to *L. p. turricula* GT1 and GT2.

DISCUSSION

LYMNAEA OCCULTA AND HOLARCTIC STAGNICOLINES

The lengths of the lymnaeid ITS-2 vary within a very large range. Three different lymnaeid species groupings according to their ITS-2 length could be distinguished (Bargues *et al.*, 2001): *Radix* and *Galba* may be considered the oldest taxa (370–406 bp lengths), and *Lymnaea* s. str., European *Stagnicola* and *Omphiscola* (468–491 bp lengths) the most recent, North American stagnosticolines being intermediate (434–450 bp lengths). This hypothesis agrees with the phylogeny of lymnaeids based on palaeontological data, chromosome numbers and radular dentition (Inaba, 1969). That is why the sequence results obtained on *L. occulta*

Fig. 1. – Phylogenetic trees of the lymnaeid species studied, obtained using the three planorbids *B. pfeifferi*, *B. amazonica* and *H. trivolvis* as outgroup: A) based on maximum parsimony (MP) analysis using the heuristic option; numbers above the line indicate branch lengths (steps); numbers below the line represent the percentage of 1,000 bootstrap replicates; B) minimum evolution (ME) tree based on Log-Det corrected distances using the neighbor-joining (NJ) method; scale bar indicates the number of substitutions per sequence position; numbers indicate the frequency of a particular branch cluster in 1,000 bootstrap replicates; bootstrap values obtained with the TN93 model noted in parentheses only when higher; C) derived from the maximum likelihood (ML HKY85) model; scale bar indicates the number of substitutions per sequence position; numbers represent the percentage of 1,000 puzzling replicates. All lymnaeid species included in the trees are Palearctic excepting *Catascopia catascopium*, *C. elodes*, *C. emarginata* and *Hinkleyia caperata* which are Nearctic.

are so interesting: both ITS-2 length and its GC content fit in the ranges of the American stagnicolines. Moreover, MP, NJ and ML analyses are all furnishing phylogenetic trees in which *L. occulta* is always clustering with the American stagnicolines.

In all phylogenetic analyses performed previously, European stagnicolines always appeared in a clade different from that of North American ones. These phylogenetic results, together with the great nucleotide differences and genetic distances, suggested that North American and European stagnicolines do not belong to the same supraspecific taxon, despite shell morphology and visceral anatomy similarities which may be homoplastic (Bargues *et al.*, 2001). Similar conclusions were reached in trees inferred from 16S mitochondrial rDNA sequences (Remigio & Blair, 1997a; Remigio, 2002).

The results obtained in the present work suggest that *L. occulta* may be considered an evolutionary bridge between Palaearctic and Nearctic stagnicolines. In this sense, it is worth mentioning that *L. occulta* shows a geographical distribution in Europe restricted to eastern and northern regions, as eastern Germany (Glöer & Meier-Brook, 1998), Poland, former Czechoslovakia, former Yugoslavia, Ukraine and Sweden (Jackiewicz, 1998a). Moreover, it has been found in Jenisejsk and the Selenga river delta at the Baikal Lake, Siberia (Jackiewicz, 1992, 1998b), and its high resistance to dryness and water freezing (Jackiewicz, 1998a) suggest that it may be largely expanded up to the Asian Far East where it may have been confused with other stagnicoline species because of its shell morphology. Stagnicoline materials, originally ascribed to different species and later put together within the taxon *L. palustris sensu lato* by Hubendick (1951), have been reported up to the Peninsula of Kamchatka, in Alaska, Canada, USA and central Mexico, and even introduced into Panama (see review by Hubendick, 1951). The future sequencing of these American stagnicolines may largely clarify our present knowledge on this interesting Holarctic lymnaeid group.

The species *L. occulta* presents a well known morpho-anatomy which includes a singular combination of features of the reproductive organs (see review in Jackiewicz, 1998a). Although the results obtained with the random amplified polymorphic DNA (RAPD) technique should be taken into account with a great caution in taxonomical studies (Backeljau *et al.*, 1995), RAPD studies were already suggesting the separate taxonomic status of *L. occulta*, electrophoretic band analyses being unable to clarify its taxonomic position in *Stagnicola* or *Lymnaea s. str.* (Rybska *et al.*, 2000).

Absolute differences in the rDNA ITS-2 sequences and the genetic distances between *L. occulta* and the other stagnicoline species do not only prove the validity of this species, but also show that it cannot be included in

the European subgenera *Lymnaea* (*Lymnaea*) Lamarck, 1799 (type species: *stagnalis*) and *Lymnaea* (*Stagnicola*) (Jeffreys, 1830) (= *Stagnicola* Leach, 1830; = *Limnophysa* Fitzinger, 1833) (type species: *palustris*), nor in the closest North American genus *Hinkleyia* Baker, 1928 (type species: *caperata*), nor in the neighbouring European genus *Galba* Schrank, 1803 (type species: *truncatula*). The genetic distances detected between *L. occulta* and *H. caperata* (0.18095) or between *L. occulta* and *G. truncatula* (0.15426-0.15691) are higher-similar than those detected between the genera *Omphiscola* and *Lymnaea* (*Lymnaea*) or *Lymnaea* (*Stagnicola*) (0.15217-0.17577) (see Bargues *et al.*, 2001).

The inclusion of the *L. occulta* evolutionary bridge in the phylogenetic trees has largely contributed to clarify the status of the different Holarctic stagnicolines. Ribosomal DNA ITS-2 sequence analyses and phylogenetic results now agree with the conclusion reached by Remigio & Blair (1997b) after the study of the ITS-1, 5.8S and ITS-2 nuclear rDNA sequences about the convenience of ascribing *catascopium-elodes-emarginata* and *caperata* to two different genera. Moreover, present results suggest that *L. occulta* and the American *catascopium*, *emarginata* and *elodes* belong to the same supraspecific taxon, genetic distances between them being very low (0.06490-0.06731).

Consequently, the nomination of a new genus *Catascopia* with *catascopium* as type species and also including the species *emarginata*, *elodes* and *occulta*, has been proposed (Meier-Brook & Bargues, 2002).

LYMNAEA PALUSTRIS SUBSPECIES

The results in the rDNA ITS-2 sequence analyses and respective phylogenetic trees confirm the previous results on *L. palustris* and *L. turricula* obtained by Bargues *et al.* (2001). All ITS-2 sequences from lymnaeids, classified as belonging to these two species, studied up to the present, correspond to the same species and, consequently, molecular results suggest that *L. turricula* must be considered conspecific with *L. palustris*. RAPD distances and electrophoretic bands were already suggesting that both were very close (Rybska *et al.*, 2000).

If the morpho-anatomic differences in the reproductive organs, as the ratio of the praeputium to the penis sheath length and prostate shapes, especially in the length ratio of its proximal part to the distal one (see Jackiewicz, 1998a), would show a relationship to populations showing different geographical/ecological distributions, their distinction at subspecies level, *L. p. palustris* and *L. p. turricula*, would be possible.

The present knowledge gives *L. turricula* a distribution comprising the Danube valley in Germany, southern Poland, Czech Republic, Austria, Hungary, and Bulgaria (Falkner, 1984, 1985; Jackiewicz, 1989, 1996, 1998a;

Kilias, 1992; Glöer & Meier-Brook, 1998; Rybska *et al.*, 2000). This distribution includes overlapping areas, which does not necessarily mean coexistence of both *palustris* and *turricula* in the same localities.

Worth mentioning is that all *L. p. palustris* populations studied up to the present, even from localities very far away ones from the others (Denmark, France, Germany, The Netherlands), showed identical ITS-2 sequence. Such a monomorphic phenomenon is unique among European lymnaeids, as in all other species from which more than one population could be studied, an intraspecific variability in the ITS-2 sequence was found (Bargues *et al.*, 2001). Thus, in this case, one wonders whether the presence of only a very few mutations, as those detected in the different *L. p. turricula* populations studied, would be sufficient to correlate with the phenotypic differences at the base of their species/subspecies distinction. The sequencing of additional nuclear ribosomal and mitochondrial DNA markers evolving faster than the ITS-2 is here needed.

STAGNICOLINE VECTORS OF THE LIVER FLUKE

Fascioliasis is an important disease in humans and livestock caused by two digenetic trematode species of the genus *Fasciola*: *F. hepatica* and *F. gigantica*. Whereas in Europe, the Americas and Oceania only *F. hepatica* is concerned, the distributions of both species overlap in many areas of Africa and Asia (Mas-Coma & Bargues, 1997; Mas-Coma *et al.*, 1999). *Fasciola hepatica* is believed to be of European origin, with *Galba truncatula* as the original intermediate host species (Mas-Coma *et al.*, 2001). *F. gigantica* appears to be restricted to areas of Africa and Asia where *Radix* species are present, mainly forms of *R. auricularia* in Asia and of *R. natalensis* in Africa. *Radix* is not present in the New World, the only species of this genus known in North America, *R. auricularia* and *R. peregra*, having been introduced from Eurasia (Hubendick, 1951; Clarke, 1981). Although parasitological data support an origin of *Fasciola* in the Old World, probably Palaearctic, hypotheses suggest an evolutionary origin for lymnaeids in the Nearctic region, where this group of freshwater gastropods shows its greatest number of species (Hubendick, 1951).

The marked *Fasciola*/lymnaeid species specificity suggests an old parasite/host relationship of *F. hepatica* with *Galba* and of *F. gigantica* with *Radix*. The monophyletisms shown by both *Galba* and *Radix* in the phylogenetic trees of the European lymnaeids are in agreement with known *Fasciola*/lymnaeid species relationships and suggest a parallel evolution of each liver fluke species with both different lymnaeid branches towards an increasing specificity (Bargues *et al.*, 2001). The phylogenetic trees obtained from the ITS-2 sequences are consequently able to differentiate between

lymnaeids transmitting and those non-transmitting fasciolids, as well as between those transmitting *F. hepatica* and those transmitting *F. gigantica*, similarly as trees inferred from 18S rDNA sequences did (Bargues & Mas-Coma, 1997; Bargues *et al.*, 1997).

There are lymnaeid species belonging to taxa other than *Galba* able to play the role of intermediate host of *F. hepatica* in given circumstances. In Europe, *F. hepatica* has the lymnaeid *G. truncatula* as the preferred transmitting snail species. However, *O. glabra*, *L. (S.) palustris* and recently *R. ovata* have also been found transmitting it under natural conditions (Kendall, 1970; Bouix-Busson & Rondelaud, 1985, 1986; Dreyfuss *et al.*, 1994, 2002). Moreover, experimental studies have shown that *O. glabra*, *L. (S.) palustris* and *L. (S.) fuscus*, and even *L. (L.) stagnalis*, *R. peregra* and *Myxas glutinosa* (Müller, 1774) can be extremely infected if miracidium infection takes place during the first few days of the snail's life, although a high mortality level is the consequence (Kendall, 1970; Boray, 1978; Brown, 1978; Vareille-Morel *et al.*, 1994; Dreyfuss *et al.*, 2000, 2002).

In North America, among the numerous lymnaeids incriminated in the transmission of *F. hepatica*, several belong to the stagnicoline group too: *L. palustris* from Southwestern USA could be experimentally infected (Wilson & Samson, 1971), although the same species could not be infected in Wisconsin (Foreyt, 1978); *L. palustris nutalliana* Lea, 1841 was found to be the most important vector, both naturally and experimentally, and *L. proxima proxima* Lea, 1856 to be an additional natural and experimental intermediate host, both in Washington (Lang, 1977); *L. traskii* Tryon, 1863 and *L. montanensis* Baker, 1913 could both only be experimentally infected when very young (Krull, 1934; Rowan *et al.*, 1966). These lymnaeids were all synonymised with *L. palustris* by Hubendick (1951) and *L. montanensis* appears to be very close to *Hinkleyia caperata*, a species which could not be experimentally infected in Wisconsin (Foreyt, 1978).

According to its close relationship to *L. (S.) p. palustris* and position in the phylogenetic trees obtained, the stagnicoline *L. (S.) p. turricula* may be considered as a potential secondary intermediate host of *F. hepatica*. As in the case of *L. (S.) p. palustris*, a natural infection with *F. hepatica* has already been reported in a population of this lymnaeid in Poland (Czapski, 1977), where the main vector species *G. truncatula* is also present (Jackiewicz, 1998a).

Results of DNA sequences and phylogenetic trees suggest a potential of transmission capacity for *C. occulta* higher than that of European stagnicolines or *O. glabra*. The relatively low genetic distances between *C. occulta* and *G. truncatula* and the clustering of both species in the same clade suggest that *C. occulta* may be poten-

tially considered as the second lymnaeid intermediate host species of *F. hepatica* in importance in eastern and northern Europe after *G. truncatula*. Unfortunately, no conclusion can be drawn from the only known report of a natural infection of *C. occulta* with the liver fluke in Poland (Czapski, 1962). Both experimental transmission studies in the laboratory and epidemiological research in fascioliasis endemic areas, where *C. occulta* is present, are needed to know whether this lymnaeid species only plays the role of intermediate host sporadically or may be considered a lymnaeid developing an important role in the transmission. This question will attain high interest, owing to the large distribution of this species in eastern Europe and Asia.

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