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RAPD MARKERS AS A TOOL FOR ANALYSIS OF RELATIONSHIPS AMONG SELECTED SPECIES OF LYMNAEIDAE (GASTROPODA: PULMONATA)‡

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ABSTRACT: Total DNA samples, isolated from several dozen individuals identified on the basis of morphological and anatomical features as *Lymnaea stagnalis* (L.), *Stagnicola corvus* (Gmelin), *S. palustris* (O. F. Müller), *S. turricula* (Held), and *S. occulta* (Jackiewicz) were used for amplification of DNA fragments by RAPD. In this way, molecular characters of each individual were identified. Pairwise distances were the smallest (0.0008–0.0188) among conspecific individuals from the same population. Conspecifics from different populations were generally less similar (pairwise distances 0.0177–0.0589). Greater differences were observed among individuals of different species (distances 0.1257–0.6505, i.e. at least 10-fold larger than distances within and between populations). RAPD markers are useful for analyses of taxonomic relationships within the Lymnaeidae. The results confirm the specific status of *L. stagnalis*, *S. corvus* and *S. occulta*. A separate group is formed by *S. palustris/turricula*, although these two taxa are very similar, and RAPD analysis does not explain if they are distinct species or subspecies.

KEY WORDS: Lymnaeidae, *Stagnicola*, *Lymnaea*, taxonomy, RAPD

INTRODUCTION

One of the methods based on polymerase chain reaction (PCR) is a rapid DNA amplification technique, called RAPD (Random Amplified Polymorphic DNA) (WILLIAMS et al. 1990). Short primers (single or in pairs), each with an arbitrary nucleotide sequence, enable amplification of some DNA fragments, which after electrophoretic separation can be interpreted as sets of molecular characters. The RAPD technique enables detection of DNA polymorphism even if there is no information on nucleotide sequences of the analysed DNA and the sites on the strand where primers are annealed (CAETANO-ANOLLES 1994, PRIMROSE 1999). The polymorphism of PCR products results from changes in the nucleotide sequence at the sites of annealing of the primer to the DNA template, which results in elimination or formation of a new site of annealing of the primer, and thus to the absence or presence of a product of the reaction. In such a situation the set of RAPD markers can be used for taxonomic analysis (WILLIAMS et al. 1990, KLEIN-LANKHORST et al. 1991, ARMBRUSTER 1997, PRIMROSE 1999). This method was successfully used to determine taxonomic relations among molluscs, such as snails of the genera *Littorina* (Littorinidae) (CROSSLAND et al. 1993), *Bulinus* (Planorbidae) (LANGAND et al. 1993, STOTHARD & ROLLINSON 1996, 1997, STOTHARD et al. 1997), or *Cochlicopa* (Cochlicopidae) (ARMBRUSTER 1997). The RAPD method served also as a tool for detecting coevolution between blood flukes (*Schistosoma* spp.) and their hosts, i.e. snails of the genus

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Biomphalaria from Zimbabwe (WEBSTER et al. 2001), or for verification of the hypothesis that the colonisation success of *Potamopyrgus antipodarum* (Gray), which reproduces mainly parthenogenetically, is in Europe a result of hybridization between several clones (JACOBSEN et al. 1996).

During the last 20 years, the classification of European snails of the genera *Lymnaea* Lamarck, 1799, and *Stagnicola* Jeffreys, 1830, was under debate. JACKIEWICZ (1959, 1993, 1998, 2000) distinguished five species, and assigned them to the following subgenera: *Lymnaea (Lymnaea) stagnalis, L. (L.) corvus, L. (Stagnicola) palustris, L. (S.) turricula*, and *L. (S.) occulta*. This classification was generally accepted by FALKNER (1995), although he suggested that the subgenera distinguished by JACKIEWICZ should be raised to the generic status and *L. corvus* should be assigned to the genus *Stagnicola* (FALKNER 1995, FALKNER et al. 2001). A completely different view was presented by KILIAS (1992), who regarded *S. corvus, S. palustris, S. turricula* and *S. occulta* as ecomorphs of *L. palustris*. In turn, BARGUES et al. (2001, 2003) suggested, based on an analysis of nuclear ribosomal DNA ITS-2 sequences, that *S. palustris* and *S. turricula* should be treated as subspecies within *S. palustris*. Moreover, MEIER-BROOK & BARGUES (2002) included *S. occulta* in the genus *Catascopia*, newly established by them. A new light on the classification of the species listed above can be thrown by an analysis of other molecular characters, including RAPD markers.

Numerical analysis of RAPD results is associated with some limitations of taxonomic and phylogenetic conclusions (BACKELJAU et al. 1993, 1995). Genetic distances (or similarities) calculated on the basis of RAPD markers do not enable unequivocal determination whether the observed differences are at the level of species, subspecies, or even lower (interpopulation variation). Thus our preliminary RAPD analysis of European lymnaeid snails of the genus *Stagnicola* Jeffreys, 1830 (RYBSKA et al. 2000) needed to be supplemented by an analysis of intrapopulation and interpopulation variation in RAPD results.

MATERIALS AND METHODS

SNAIL MATERIAL

Snails of five lymnaeid species recorded in Poland were studied. The collected individuals were identified on the basis of morphological and anatomical characters according to JACKIEWICZ's (1998, 2000) keys. The snails originated from the following localities:

Lymnaea stagnalis (Linnaeus, 1758) – Lake Budzyńskie near Mosina, W. Poland (population 1.1); and a pond near Klecie, SE. Poland (population 1.2);

Stagnicola corvus (Gmelin, 1791) – a small water body near Ostrów Wielkopolski, NW. Poland (population 2.1); a pond near Luboń, close to Poznań, W. Poland (population 2.2); and a drainage ditch near Nysa, SW. Poland (population 2.3);

Stagnicola palustris (O. F. Müller, 1774) – ponds near Morasko, close to Poznań, W. Poland (population 3.1); flood plains of the Nida River, Chroberz near Kielce, S. Poland (population 3.2); and Lake Wilczyńskie near Konin, central Poland (population 3.3);

Stagnicola turricula (Held, 1836) – water body near the Krościenko-Ustrzyki Dolne road, Bieszczady Mts., SE. Poland (population 4.1); and a drainage ditch near Nysa, SW. Poland (population 4.2);

Stagnicola occulta (JACKIEWICZ, 1959) – drainage ditch at Gorzykowo, near the Września–Gniezno road, W. Poland (population 5.1).

The snails caught in the wild were next kept in glass containers until DNA isolation from their tissues. Room temperature was maintained in the containers. The snails were sporadically fed with lettuce leaves.

MOLECULAR TECHNIQUES

The experiment involved at least 15 individuals from each of the studied populations. Total DNA was isolated individually from the foot muscle of each snail, with the use of DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions.

The isolated DNA was subjected to amplification in a PTC-200 thermocycler (MJResearch), according to the RAPD method. In the reactions, we used primers with an arbitrary sequence of 10 nucleotides each (sources of the sequences are given in brackets):

P2, 5'-TGCACACTGA-3' (KLEIN-LANKHORST et al. 1991);

P3, 5'-TGGTGACTGA-3' (KLEIN-LANKHORST et al. 1991);

P4, 5'-TGGTCACTGT-3' (KLEIN-LANKHORST et al. 1991);

P5, 5'-GTCCCGACGA-3' (HOSAKA & HANNEMAN 1994);

P6, 5'-CTCGTTTGGG-3' (JAKUBOWICZ, unpublished).

The primers were obtained from TibMol-Biol (Germany). They were applied in the following combinations: P2+P2, P2+P5, P5+P5, P2+P6, P3+P6, P6+P6, P4+P6, P3+P5, and P2+P3.

DNA amplification by PCR was carried out according to the manufacturer's instructions (Qiagen), in a final reaction volume of 10 µl, containing 7.5 ng total DNA, the pair of primers (0.5 µM each), all dNTPs (200 µM each), 1 mM spermidine (FIEDOROW & SZWEYKOWSKA-KULIŃSKA 1997), PCR buffer (Qiagen, 1.5 mM $MgCl_2$, Tris/HCl, KCl, $(NH_4)_2SO_4$) and 0.25 U

Taq DNA polymerase. The PCR reactions were carried out according to the following procedure (KLEIN-LANKHORST et al. 1991): initial denaturation (95°C, 5 min), 50 cycles (each composed of denaturation at 92°C, 1 min; annealing at 35°C, 2 min; and extension at 72°C, 2 min), and final extension (75°C, 5 min).

Amplification products were next subjected to electrophoresis in 10% polyacrylamide gel in 1% TBE buffer (90 mM Tris, 2 mM EDTA, borate, pH 8. 3) at 30 mA and 150 V. In the gel, DNA was stained with ethidium bromide (0.5 µg/ml). Next, the gels were photographed in UV light on Polaroid film.

DATA ANALYSIS

The RAPD products separated on the gel, for the five lymnaeid species, were coded in the binary system, where 1 denoted presence of a band on the gel, and 0 denoted its lack. The data matrix served as the basis to calculate the distance matrix with the programme RESTDIST within the Phylogeny Inference Package PHYLIP, version 3. 6, using the method of NEI & LI (1979), modified by FELSENSTEIN (1995).

RESULTS

VARIATION WITHIN AND BETWEEN POPULATIONS

To assess and analyse differences within and between populations of each species, the material used for DNA amplification was selected so that the electrophoretic separation enabled comparison of images for at least 10 individuals from each population, and an outgroup composed of 2–3 individuals from another, conspecific population. Examples of results of these experiments are illustrated in Fig. 1 for *L. stagnalis* (populations 1.1 and 1.2), Fig. 2 for *S. corvus* (populations 2.1 and 2.2), Fig. 3 for *S. palustris* (populations 3.1 and 3.2), Fig. 4 for *S. turricula* (populations 4.1 and 4.2), and Fig. 5 for *S. occulta* (population 5.1). Such comparisons were made for at least two populations of each species, except *S. occulta*, which was found in only one locality. The experiments were repeated several times in various combinations of populations of the studied species.

The amplified DNA fragments visualised on the gel were encoded in the binary system (0–1). In this way, 561 molecular characters were obtained, which made it possible to calculate pairwise distances between individuals representing the studied populations of those species, with the use of the RESTDIST programme (Tables 1–5).

In the case of *L. stagnalis* (Table 1), the smallest pairwise distance between individuals from the same population (1.1) was 0.0056, and the largest was

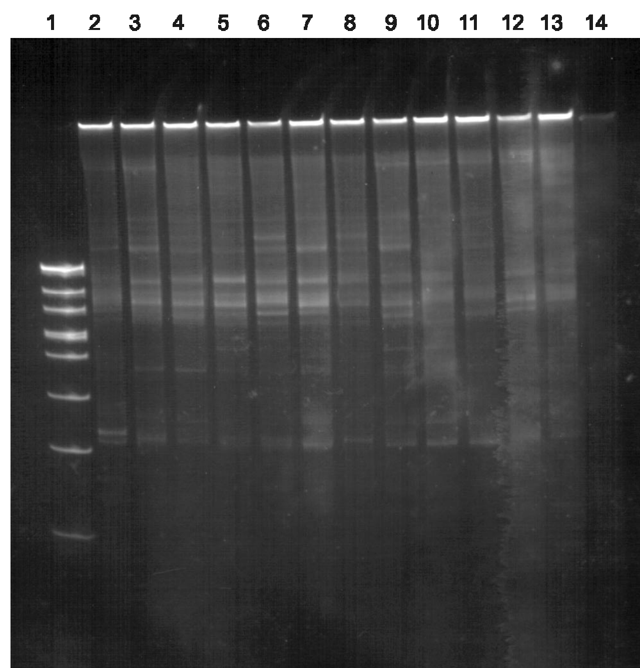

Fig. 1. Variation within and among populations of *L. stagnalis*, on the basis of RAPD analysis with primers P3+P6: 1 – GeneRuler 100-bp DNA Ladder (Fermentas); 2–11 – individuals from population 1.1; 12–13 – individuals from population 1.2; 14 – H_2O

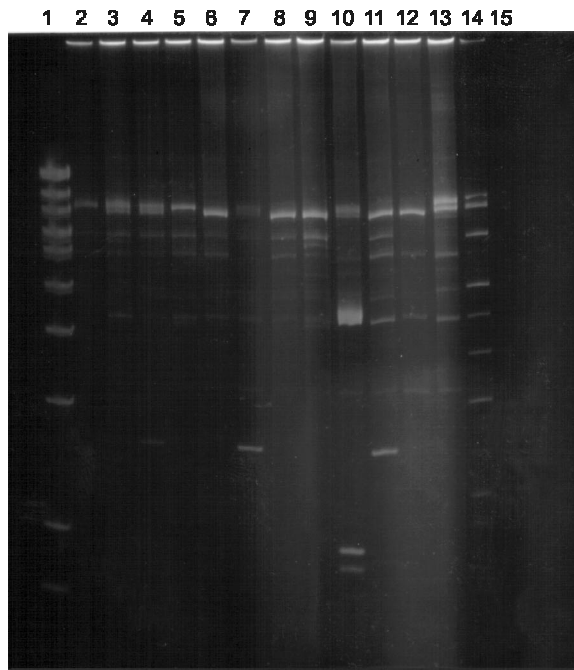

Fig. 2. Variation within and among populations of *S. corvus*, on the basis of RAPD analysis with primers P3+P5: 1 – GeneRuler 100-bp DNA Ladder (Fermentas); 22–11 – individuals from population 2.1; 12–14 – individuals from population 2.2; 15 – H_2O

0.0150. The comparison of these snails with individuals from another population (1.2) revealed a greater variation, which was reflected in slightly larger distances, ranging from 0.0253 to 0.0357 (the distance between individuals within population 1.2 was 0.0073).

In the case of *S. corvus* (Table 2), the smallest distance between individuals from the same population (2.1) was 0.0049, while the largest within that population was 0.0165. Within the other analysed population (2.2), the pairwise distance ranged from 0.0065 to 0.0127. Distances between individuals from those two

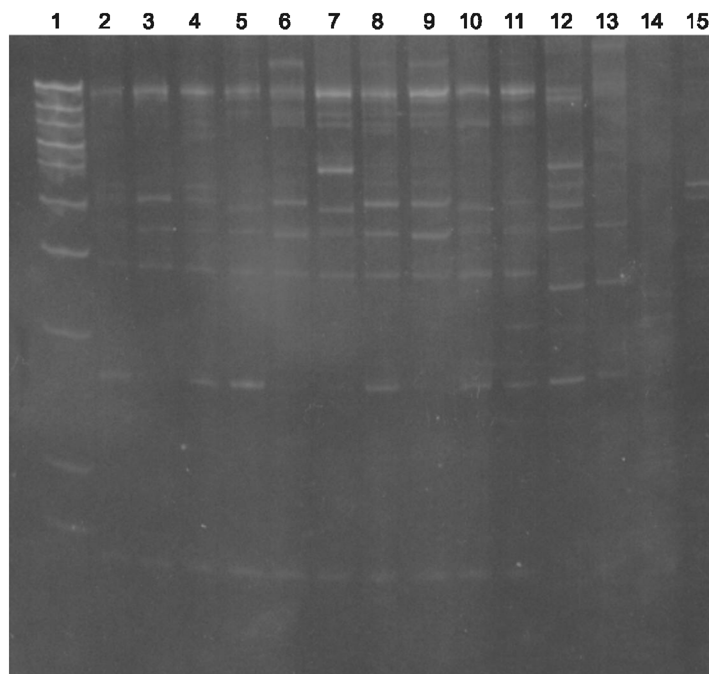

Fig. 3. Variation within and among populations of *S. palustris*, on the basis of RAPD analysis with primers P3+P5: 1 – GeneRuler 100-bp DNA Ladder (Fermentas); 2–11 – individuals from population 3.1; 12–14 – individuals from population 3.2; 15 – H_2O

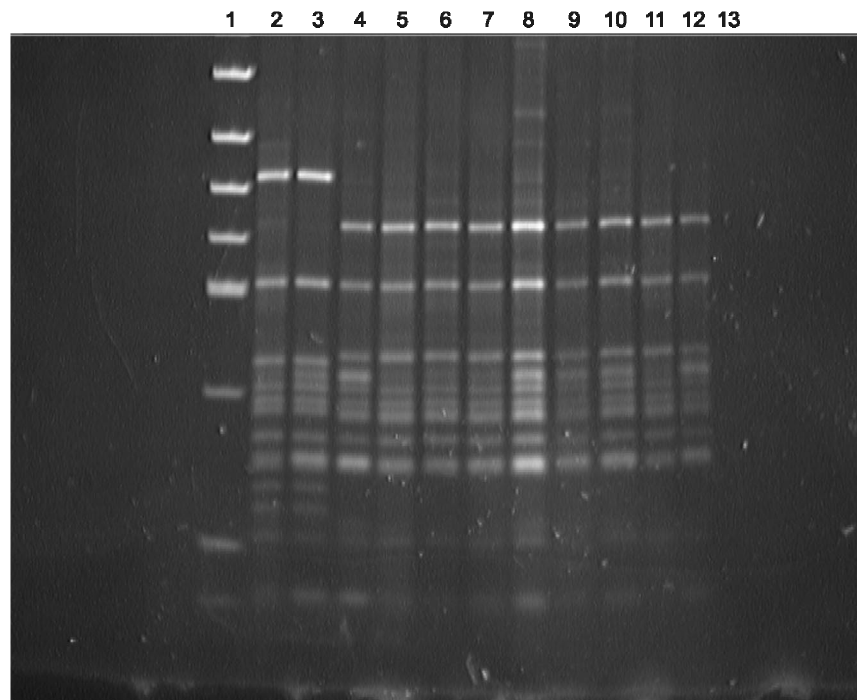

Fig. 4. Variation within and among populations of *S. turricula*, on the basis of RAPD analysis with primers P2+P5: 1 – GeneRuler 100-bp DNA Ladder (Fermentas); 2–10 – individuals from population 4.2; 11–12 – individuals from population 4.1; 13 – H_2O

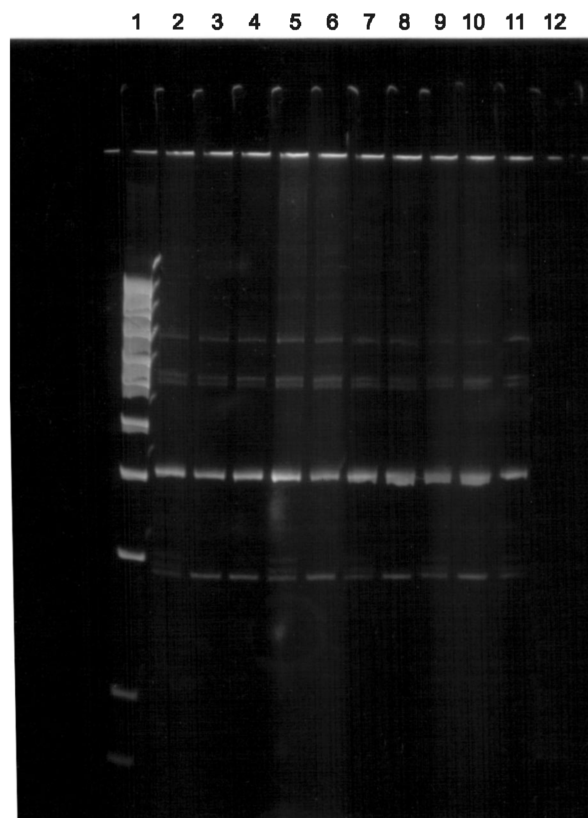

Fig. 5. Variation within and among populations of *S. occulta*, on the basis of RAPD analysis with primers P3+P6: 1 – GeneRuler 100-bp DNA Ladder (Fermentas); 2–11 – individuals from population 5.1; 12 – H_2O

Table 1. Distances between individuals from two populations of *Lymnaea stagnalis*

	A	B	C	D	E	F	G	H	I	J	K	L
A	x	0.0075	0.0110	0.0130	0.0137	0.0115	0.0099	0.0101	0.0094	0.0092	0.0313	0.0300
B		x	0.0080	0.0111	0.0120	0.0084	0.0105	0.0082	0.0075	0.0085	0.0297	0.0266
C			x	0.0074	0.0095	0.0073	0.0092	0.0094	0.0076	0.0086	**0.0357**	0.0287
D				x	0.0089	0.0101	0.0122	0.0125	0.0130	0.0104	0.0303	**0.0253**
E					x	0.0087	0.0095	0.0132	**0.0150**	0.0125	0.0354	0.0269
F						x	0.0073	0.0086	0.0091	0.0065	0.0315	0.0265
G							x	0.0106	0.0123	0.0122	0.0337	0.0287
H								x	**0.0056**	0.0075	0.0307	0.0313
I									x	0.0058	0.0313	0.0281
J										x	0.0266	0.0253
K											x	0.0073
L												x

A–J – 10 individuals from population 1.1; K-L – 2 individuals from population 1.2. Boldface marks the lowest (0.0056) and the highest (0.0150) values of pairwise distance between individuals from the same population, as well as the lowest (0.0253) and the highest (0.0357) between individuals from different populations

Table 2. Distances between individuals from two populations of *Stagnicola corvus*

	A	B	C	D	E	F	G	H	I	J	K	L	M
A	x	0.0085	0.0104	0.0110	0.0080	0.0098	0.0134	0.0152	0.0159	0.0148	0.0243	0.0268	0.0236
B		x	0.0072	0.0094	0.0113	0.0098	0.0101	0.0152	0.0124	0.0131	0.0223	0.0249	0.0216
C			x	**0.0049**	0.0099	0.0085	0.0138	0.0120	0.0094	0.0101	0.0209	0.0236	0.0203
D				x	0.0073	0.0141	0.0145	0.0110	00101	0.0108	**0.0177**	0.0262	0.0191
E					x	0.0110	0.0131	0.0113	0.0138	0.0110	0.0202	0.0270	0.0196
F						x	0.0114	0.0184	0.0137	0.0144	0.0197	0.0224	0.0211
G							x	0.0101	0.0124	**0.0165**	0.0223	0.0268	0.0257
H								x	0.0108	0.0114	0.0265	**0.0289**	0.0279
I									x	0.0074	0.0191	0.0236	0.0243
J										x	0.0179	0.0224	0.0211
K											x	0.0127	0.0065
L												x	0.0084
M													x

A–J – 10 individuals from population 2.1; K-M – 3 individuals from population 2.2. Boldface marks the lowest (0.0049) and the highest (0.0165) values of pairwise distance between individuals from the same population, as well as the lowest (0.0177) and the highest (0.0289) between individuals from different populations

populations were slightly larger, as they ranged from 0.0177 to 0.0289.

For the studied individuals of *S. palustris* (Table 3), the smallest pairwise distance between individuals from one population (3.1) was 0.0060, and the largest was 0.0169. Within the other population (3.4), the distance between individuals was 0.0062. Distances between individuals from those two populations varied from 0.0310 to 0.0441.

For the analysed individuals identified as *S. turricula* (Table 4), differences within the first population (4.1) were illustrated by distances of 0.0051 to 0.0188. Within the other population (4.2), differences between individuals reached a distance of 0.0074. Variation between individuals from different populations of this species was reflected in distances of 0.0392 to 0.0590.

In the studied population of *S. occulta*, the smallest pairwise distance (Table 5) between individuals of this species was 0.0056, and the highest was 0.0186.

VARIATION BETWEEN SPECIES

To assess the variation between species, PCR reactions (RAPD) and electrophoretic separation were carried out, selecting for analysis two individuals of each species, from two different populations: *L. stagnalis* (populations 1.1 and 1.2), *S. corvus* (populations 2.1 and 2.3), *S. palustris* (3.1 and 3.2 or 3.3). In the case of *S. turricula* and *S. occulta*, two individuals

Table 3. Distances between individuals from two populations of *Stagnicola palustris*

	A	B	C	D	E	F	G	H	I	J	K	L
A	x	0.0073	0.0079	0.0098	0.0130	0.0142	0.0137	0.0120	0.0105	0.0130	0.0346	0.0365
B		x	0.0073	0.0103	0.0111	0.0123	0.0142	0.0162	0.0097	0.0135	0.0417	0.0417
C			x	0.0098	0.0106	**0.0169**	0.0137	0.0157	0.0117	0.0142	0.0346	0.0365
D				x	0.0092	0.0125	0.0157	0.0151	0.0125	0.0137	0.0348	0.0367
E					x	0.0123	**0.0169**	0.0150	0.0086	0.0099	0.0373	0.0329
F						x	0.0130	0.0151	0.0084	0.0097	0.0411	**0.0441**
G							x	0.0108	0.0144	0.0171	0.0359	0.0404
H								x	0.0138	0.0164	0.0383	0.0405
I									x	**0.0060**	0.0357	**0.0310**
J										x	0.0344	0.0299
K											x	0.0062
L												x

A–J – 10 individuals from population 3.1; K-L – 2 individuals of from population 3.2. Boldface marks the lowest (0.0060) and the highest (0.0169) values of pairwise distance between individuals from the same population, as well as the lowest (0.0310) and the highest (0.0441)

Table 4. Distances between individuals from two populations of *Stagnicola turricula*

	A	B	C	D	E	F	G	H	I	J	K	L
A	x	0.0083	0.0110	0.0158	0.0130	0.0172	0.0130	0.0158	0.0124	0.0143	0.0528	0.0503
B		x	**0.0051**	0.0159	0.0147	0.0124	0.0085	0.0097	0.0172	0.0161	**0.0590**	0.0564
C			x	0.0140	0.0143	0.0137	0.0097	0.0110	0.0184	0.0174	0.0570	0.0544
D				x	0.0130	**0.0188**	0.0130	0.0127	0.0097	0.0112	0.0500	0.0475
E					x	0.0112	0.0149	0.0146	0.0113	0.0115	0.0488	0.0490
F						x	0.0143	0.0140	0.0152	0.0157	0.0539	0.0544
G							x	0.0061	0.0099	0.0161	0.0545	0.0520
H								x	0.0084	0.0112	0.0500	0.0503
I									x	0.0081	0.0459	0.0461
J										x	0.0393	**0.0392**
K											x	0.0074
L												x

A–J – 10 individuals from population 4.1; K-L –2 individuals from population 4.2. Boldface marks the lowest (0.0051) and the highest (0.0188) values of pairwise distance between individuals from the same population, as well as the lowest (0.0392) and the highest (0.0590)

Table 5. Distances between individuals from a population of *Stagnicola occulta*

	A	B	C	D	E	F	G	H	I	J
A	x	0.0067	0.0071	0.0113	0.0127	0.0149	0.0173	0.0130	0.0171	0.0150
B		x	0.0079	0.0112	0.0138	0.0161	0.0162	0.0152	0.0173	0.0162
C			x	0.0094	0.0130	**0.0186**	0.0176	0.0143	0.0163	0.0176
D				x	0.0101	0.0101	0.0154	0.0134	0.0164	0.0132
E					x	0.0105	0.0136	0.0149	0.0115	0.01360
F						x	0.0084	0.0097	0.0115	0.0075
G							x	0.0076	0.0075	0.0063
H								x	0.0087	0.0066
I									x	**0.0056**
J										x

A–J – 10 individuals from population 5.1. Boldface marks the lowest (0.0056) and the highest (0.0186) values of pairwise distance between individuals from the same population

from one population each were compared (4.1 and 5.1, respectively). Examples of electrophoretic patterns for such a system of compared individuals are shown in Fig. 6.

Due to the application of various primer pairs, 253 PCR products were obtained, distinguished as DNA bands on the polyacrylamide gel. In further calculations they were treated as molecular characters, reflecting interspecific differences. The analysis of those characters (program RESTDIST) resulted in a distance matrix for all individuals of the pairs representing individual taxa (Table 6). Pairwise distances between two individuals of *S. turricula* and *S. occulta*, deriving from the same populations, reached 0.00079 and 0.02171, respectively. For the other species, represented by individuals from different populations, the distances reached 0.00446 for *L. stagnalis*, 0.02509 for *S. corvus*, and 0.02941 for *S. palustris*. All these values were within the ranges of intrapopulation and interpopulation differences for those species, presented above (Tables 1–5).

The distances indicate distinct differences between species. They were the smallest (0.1257–0.1845) between *S. palustris* and *S. turricula*, and the largest (0.6451–0.6505) between some individuals of *L. stagnalis* and *S. palustris* as well as between *S. occulta* and *S. palustris*. For all other pairs of species, the distances ranged from about 0.42 to 0.57.

The distance matrix for RAPD characters in the interspecific analysis (Table 6) made it possible to construct dendrograms reflecting the similarity between the studied lymnaeids. Among the obtained results, the most interesting were the dendrograms constructed with three methods: Dollo (Fig. 7), neighbour-joining (Fig. 8), and UPGMA (Fig. 9). All the dendrograms based on RAPD analysis suggest a taxonomic distinctness of the studied snail species. The results indicate that *S. palustris* and *S. turricula* are the most closely related. Also *S. occulta* is located in branches closer to *S. palustris* and *S. turricula*. In contrast, *S. corvus*, depending on the applied method of dendrogram construction, appears closer to *Lymnaea stagnalis* (Figs. 7 and 8), or to other *Stagnicola* species (Fig. 9).

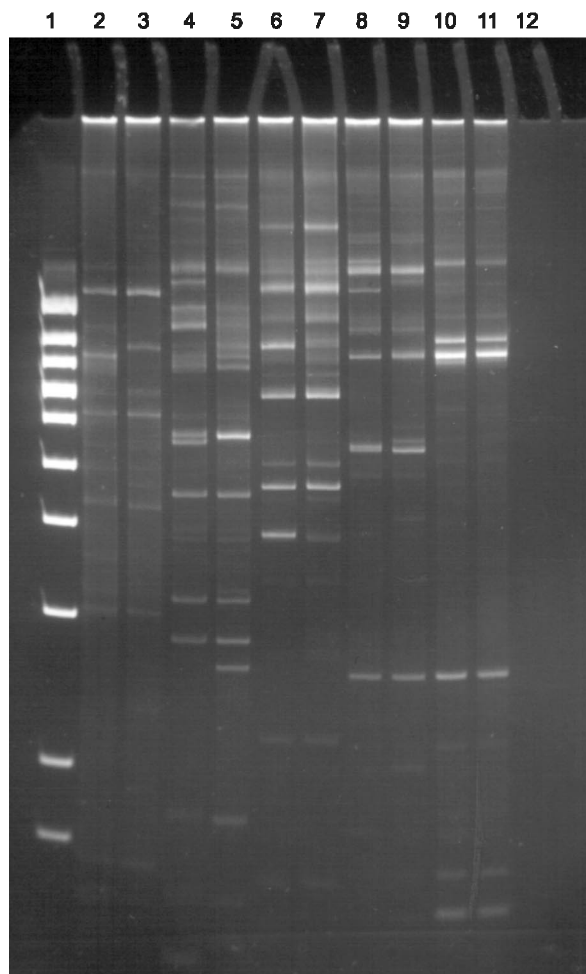

Fig. 6. Analysis of variation among species on the basis of RAPD analysis with primers P3+P5: 1 – GeneRuler 100-bp DNA Ladder (Fermentas); 2 and 3 – *S. corvus* (individuals from populations 2.1 and 2.3); 4 and 5 – *L. stagnalis* (individuals from populations 1.1 and 1.2); 6 and 7 – *S. occulta* (individuals from population 5.1); 8 and 9 – *S. palustris* (individuals from populations 3.1 and 3.3); 10 and 11 – *S. turricula* (individuals from population 4.1); 12 – H_2O

Table 6. Distances reflecting the magnitude of differences between the five studied snail species of the genera *Lymnaea* and *Stagnicola*, calculated by the program RESTDIST

	A	B	C	D	E	F	G	H	I	J
A	x	0.004460	0.421203	0.434926	0.639706	0.624758	0.592884	0.553944	0.573477	0.544867
B		x	0.463083	0.446618	0.592194	0.645161	0.549605	0.511548	0.558503	0.528919
C			x	0.025090	0.623762	0.558140	0.603093	0.570534	0.508207	0.523364
D				x	0.571865	0.590164	0.535714	0.514131	0.393258	0.471204
E					x	0.029412	0.125701	0.149277	0.594494	0.430065
F						x	0.156904	0.184502	0.650549	0.451634
G							x	0.000790	0.529520	0.518497
H								x	0.540580	0.533499
I									x	0.021709
J										x

A – *L. stagnalis,* first individual, population 1.1; B – *L. stagnalis,* second individual, population 1.2; C – *S. corvus,* first individual, population 2.1; D – *S. corvus*, second individual, population 2.3; E – *S. palustris*, first individual, population 3.1; F – *S. palustris*, second individual, population 3.2; G – *S. turricula,* first individual, population 4.1; H – *S. turricula,* second individual, population 4.1; I – *S. occulta,* first individual, population 5.1; J – *S. occulta*, second individual, population 5.1

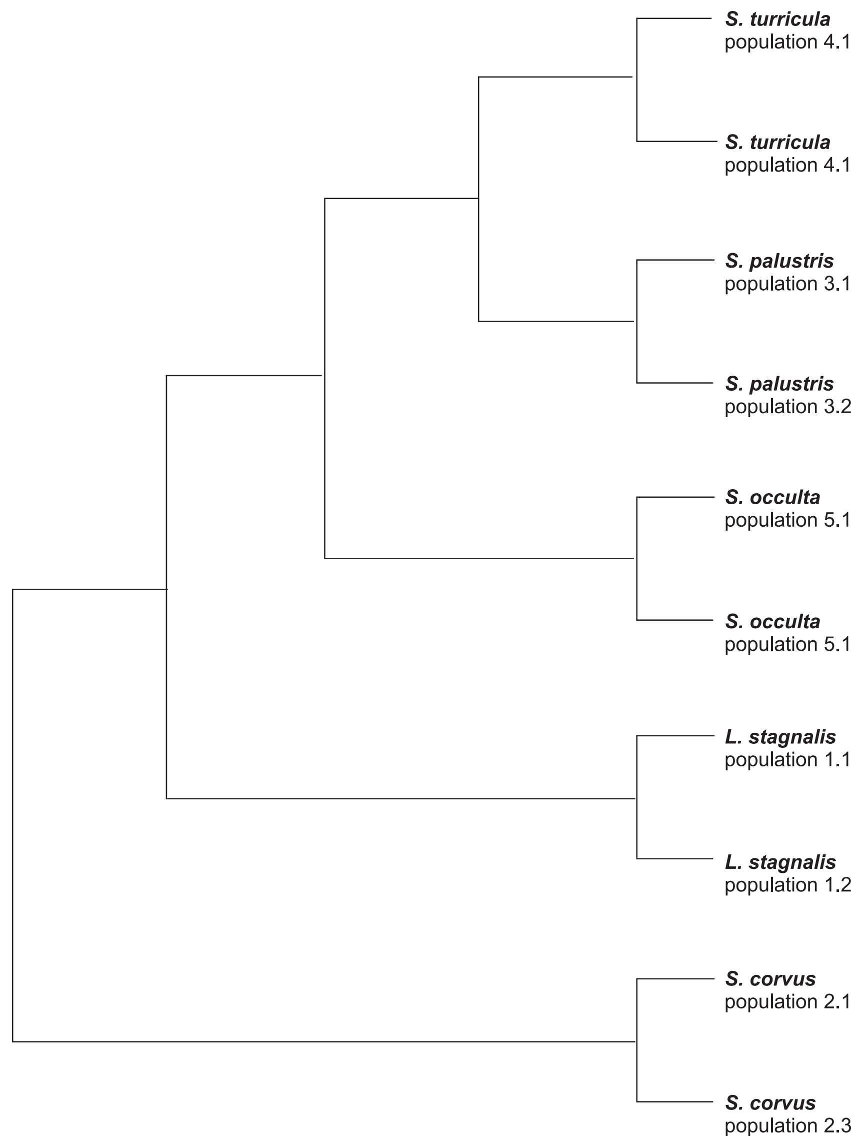

Fig. 7. Dendrogram for the studied species of *Lymnaea* and *Stagnicola*, based on an analysis of RAPD results by the Dollo parsimony method (PHYLIP 3.6 software). Each species is represented by two individuals originating from the same or from two different populations.

DISCUSSION

Comparison of individuals respect of the presence or absence of molecular characters based on RAPD, i.e. presence or absence of DNA fragments (obtained by this method) with the same electrophoretic mobility, revealed variation within populations, as well as among populations and species. The genetic distances were clearly the smallest (0.0008–0.0188) between individuals originating from the same population. The smallest distances (Tables 1–6) were found within *L. stagnalis* (range 0.0045–0.0150; mean 0.0098) and *S. corvus* (range 0.0049–0.0165; mean 0.0115). A slightly higher variation was observed in populations of other species: *S. palustris* (range 0.0060–0.0169; mean 0.0123), *S. turricula* (range 0.0008–0.0188; mean 0.0129) and *S. occulta* (range 0.0056–0.0186; mean 0.0124).

A greater variation was observed between individuals from different conspecific populations, but the distances varied within a relatively narrow range for each species. The smallest pairwise distances were found between individuals from two populations of *S. corvus* (0.0177–0.0289), slightly larger – for *L. stagnalis* (0.0253–0.0357), *S. palustris* (0.0340–0.0441), and *S. turricula* (0.0392–0.0589). For *S. occulta*, individuals from only one population were available. According to THROP (1982), if genetic distances exceed 0.15 between two populations, then the studied individuals probably belong to distinct species. All the calculated pairwise distances between the studied populations within the distinguished species were much lower than the threshold set by THROP (1982), so according to his hypothesis, they belong to the same species.

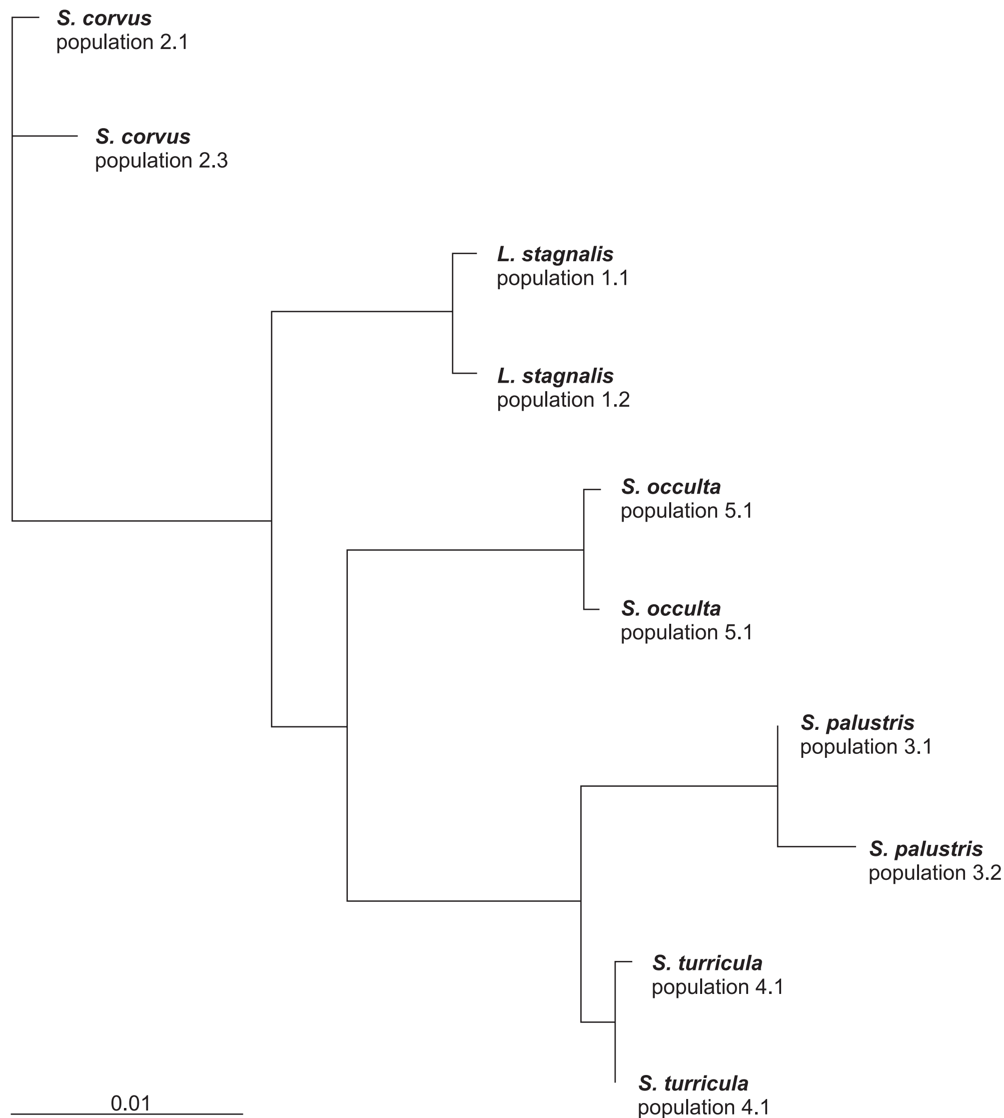

Fig. 8. Dendrogram for the studied species of *Lymnaea* and *Stagnicola*, based on an analysis of RAPD results by the neighbour-joining method (PHYLIP 3.6). Each species is represented by two individuals originating from the same or from two different populations

Thus pairwise distances attest to a relatively low genetic variation within populations and a slightly higher, but still low variation among conspecific populations. In both cases, values of distances were lower than 0.10. Similar values within species of the genus *Pellia*, also based on RAPD analysis, were reported by PACAK et al. (1998). In their study, pairwise distances within and between conspecific populations ranged from 0.00788 to 0.15083. Values of Nei's genetic distances between different populations of *Tridacna maxima*, ranged from 0 to 0.065 (BENZIE & WILLIAMS 1992).

Pairwise distances calculated on the basis of RAPD analysis of individuals from all five species (Table 6) were always much higher between species than between and within populations (Tables 1–5). Even the smallest distances between individuals of *S. palustris* and *S. turricula*, ranging from 0.1257 to 0.1845, are by one order of magnitude larger than the distances within populations of those species (Tables 3 and 4). For all other pairs of species, interspecific distances were even larger (Table 6), ranging from 0.40 to 0.65, but the most distant from one another were *L. stagnalis* vs. *S. palustris* as well as *S. occulta* vs. *S. palustris*. The above pairwise distances between the studied species do not deviate from the values recorded by other researchers. For example, in liverworts of the genus *Pellia* (PACAK et al. 1998), the distances between species varied from 0.04 to 0.80. SCHMIDT & WESTHEIDE (1999), who on the basis of RAPD compared two polychaete species, *Hesionides gohari* and *H. riegerorum*, reported distances of 0.56 to 0.87. In research on three species of marine snails of the genus *Haliotis* (abalones), interspecific distances ranged from 0.0411 (for closely related species) to 0.2898 (TAIWU et al. 2004). For five oyster species from Thailand, the distances varied from 0.105 to 0.811 (KLINBUNGA et al. 2001). The distances be-

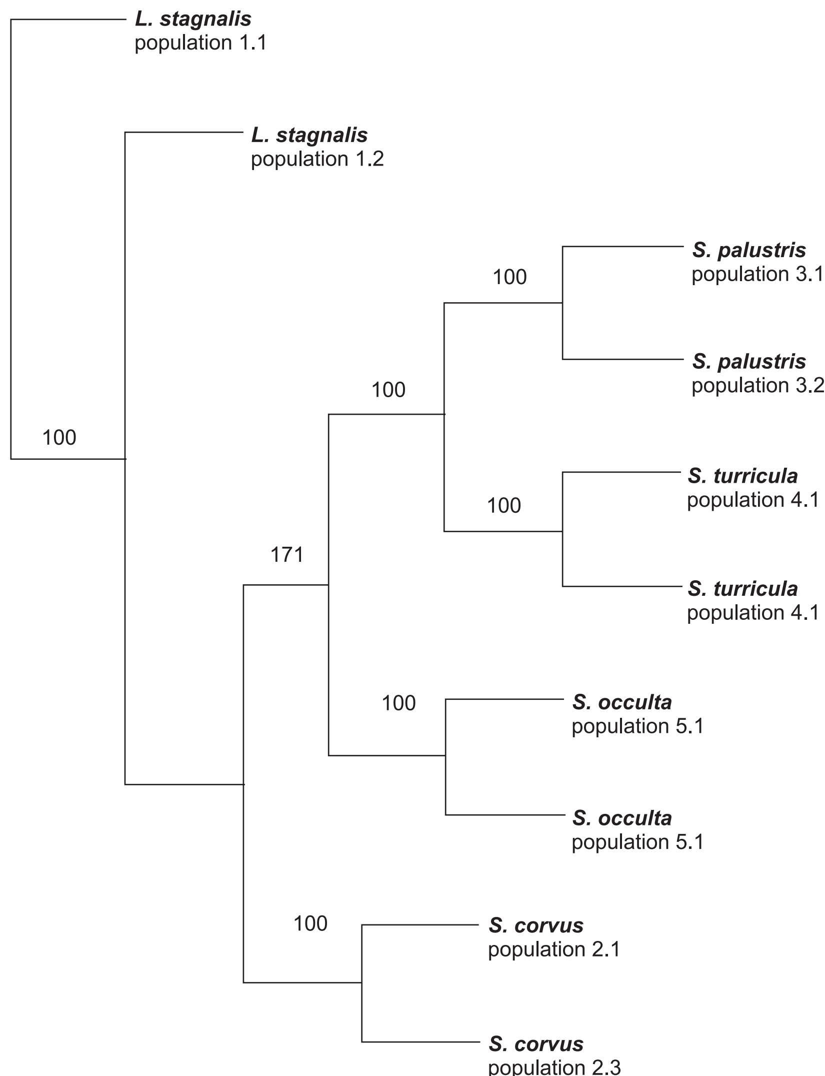

Fig. 9. Dendrogram for the studied species of *Lymnaea* and *Stagnicola*, based on an analysis of RAPD results by program Neighbour, option UPGMA (PHYLIP 3.6 software), bootstrap analysis. Bootstrap values for 100 replicates are presented as percentages above the tree nodes. Each species is represented by two individuals originating from the same or from two different populations

tween lymnaeid species in this study fit within the above ranges. This applies also to the values presented earlier (RYBSKA et al. 2000).

The RAPD-based pairwise distances confirm that *L. stagnalis*, *S. corvus*, *S. palustris*, *S. turricula* and *S. occulta* are clearly distinct. A similar conclusion can be drawn from all the dendrograms (Figs. 7–9). As mentioned above, caution should be applied when drawing taxonomic conclusions based on the analysis of RAPD molecular characters (BACKELJAU et al. 1995, 1996). Moreover, values of genetic distances do not resolve clearly what rank should be given to the distinguished taxa. However, it can be definitely concluded that the differences in ranges of pairwise distances within the populations in this study (intrapopulation variation), among individuals from different conspecific populations (interpopulation variation), and among individuals identified anatomically as different species (interspecific variation), attest to the usefulness of RAPD for taxonomic studies of lymnaeids. The variation in RAPD characters confirms the conclusions drawn by other researchers on the basis of morphological and anatomical characters (JACKIEWICZ 1959, 1993, 1998, 2000, FALKNER 1995, FALKNER et al. 2001) about the specific status of *L. stagnalis, S. corvus* and *S. occulta*. Undoubtedly, *S. palustris* and *S. turricula* are clearly separate from these three species. However, differences between *S. palustris* and *S. turricula* in RAPD characters are much smaller than those between each of them and any of the other species. This is indicated by the analysis of pairwise distances (Table 6) and of the characters common to those species (RYBSKA et al. 2000). These results indicate a close relationship between *S. palustris* and *S. turricula*. However, it must be emphasised that differences between them are greater than those found among conspecific populations (Tables 3, 4, 6). The analysis of RAPD characters does not enable an unequivocal decision whether it is justifiable to treat *S. palustris* and *S. turricula* as two separate species (JACKIEWICZ 1993, 1998, 2000, FALKNER 1995, FALKNER et al. 2001), or as two subspecies: *S. palustris palustris* and *S. palustris turricula* (BARGUES et al. 2001, 2003).

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