Phenotypic plasticity can explain evolution of sympatric polymorphism in the hairy snail *Trochulus hispidus* (Linnaeus, 1758)

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Abstract

Morphological variation of snails from the genus *Trochulus* is so huge that their taxonomy is unclear. The greatest variability concerns forms *hispidus* and *sericeus/plebeius*, which are often considered as separate species. To evidence the species barriers, we carried out crossbreeding experiments between these two sympatric morphs. Moreover, we compared the shell morphology of laboratory-bred offspring with their wild parents to test if the variation can be explained by the phenotypic plasticity model. We found that the two *Trochulus* morphs show no reproductive barriers. The fecundity rates, the mean clutch size, and F\(_1\) viability observed for all crosses were not significantly different. In hybrid crosses (in F\(_2\) generation), we also recorded reproduction compatibility, similar fecundity, and hatching success as in their parents. Accordingly, phylogenetic analyses revealed the significant grouping of sequences from these different morphs and supported no constrains in reproduction between them. Comparison of shell morphology between wild and laboratory samples showed that various characters appeared highly plastic. The average shell shape of the *hispidus* morph changed significantly from flat with wide umbilicus to elevated with narrower umbilicus such as in the *sericeus/plebeius* morph. All these findings indicate that the examined morphs do not represent separate biological species and the evolutionary process is not advanced enough to separate their genetic pool. Therefore, phenotypic plasticity has played a significant role in the evolution of *Trochulus* shell polymorphism. The two morphs can evolve independently in separate phylogenetic lineages under the influence of local environmental conditions.

Key words: crossbreeding, cytochrome c oxidase subunit I, molecular phylogeny, phenotypic variation, principal component analysis, shell morphology.

Understanding the process of speciation is crucial for explaining the diversity of life and is fundamental for evolutionary biology. An essential insight in speciation is provided by examining contemporary patterns of reproductive isolation among partially isolated populations (e.g., Berlocher and Feder 2002; Drés and Mallet 2002; Via and West 2008). Although characteristics of reproductive isolation to delimit species is still hotly debated, it is obvious that mechanisms acting to prevent or restrict gene flow are important in the preservation of evolutionarily independent units and in speciation. Most species concepts define a species as a group of individuals that are potentially able to interbreed and thereby produce fertile offspring (Otte and Endler 1989). However, biologists identify and name...
species using various available features and very seldom have possibility to assess interbreeding potential of studied organisms (Futuyma 1998). Despite severe criticism and opposition (Donoghue 1985; Gornall 1997), the biological species concept (BSC) still remains popular—at least among zoological evolutionists—and plays a key role in evolutionary theory. Among others, it provides the basis for the theory of speciation. The evolution of new species is most often regarded as a result of the development of reproductive isolating mechanisms (Coyne 1993; Niklas 1997; Futuyma 1998). Many geneticists who work on the process of species formation favor the BSC because it emphasizes the role of barriers in reproduction of species (Coyne and Orr 2004).

The BSC subdivides the mechanisms responsible for the development of barriers into premating and postmating isolation mechanisms (Mayr 1963). The first one can be studied by careful observations of behavioral differences during controlled mate-choice tests, whereas the second is preferentially studied in appropriately designed breeding experiments in the laboratory, which are known as no-choice tests (Coyne and Orr 2004).

Research on the mechanisms of reproductive isolation helps establish standards for the species delimitation in taxonomy. In pulmonates, such mechanisms have been elucidated between some taxa of freshwater Physa (Dillon et al. 2002, 2007, 2011; Dillon 2009) and in land snails Arianta arbustorum and Helix aspersa (Baur and Baur 1997; Gomot-DeVaulx andef Borg 2001). The estimation of gene flow and/or hybrid viability have been studied for a few syntopic Albinaria species (Schilthuizen 1994; Giokas et al. 2000) and subspecies (e.g., Schilthuizen and Lombaerts 1995; Schilthuizen et al. 1999) to define their relationships.

Simultaneously, separating phenotypic plasticity from the genetically determined variation is important for assessing the status of taxa as biological species. Models describing the early stages of speciation suggest that the inherited differences in phenotype are not essential precursors of evolutionary divergence within a single gene pool (Adams and Huntingford 2004), and show how divergence may arise prior to any genetic segregation as a result of environmentally induced phenotypic plasticity at the individual level. A key element of the model proposed by Skalsen et al. (1999) is that environmental influence precedes genetic control of phenotypic variation. Thus, two preconditions should occur to this, mechanism of evolutionary diversification could work. Firstly, the potential species must be phenotypically plastic and thus able to express more than one variant of a phenotypic trait within a single gene pool. Secondly, diversifying selection must act on the different phenotypic modes (West-Eberhard 1989). Strong candidates for this mechanism of evolution would be species that exhibit a discrete polymorphism in their phenotype.

A potential species that fulfills these conditions is Trochulus hispidus (Linnaeus, 1758), in which a conspicuous variability in shell morphology has been widely known (Naggs 1985; Procków 1997, 2009; Duda et al. 2014). There are disputes about its status and relationship to T. sericeus (Draparnaud, 1801), often referred to as T. plebeius (Draparnaud, 1805) (Forcart 1965; Perrin et al. 1984; Naggs 1985). According to Falkner (1982, 1990), the names T. plebeius and T. sericeus refer to two species that differ in shell size but their globose shape is similar. Trochulus plebeius is larger than T. sericeus, with more prominent growth lines, less and shorter hairs, with last whorl weakly keeled, and a strong lip in aperture. Trochulus sericeus is characterized by long curved hairs, a weak lip, and a narrow umbilicus, as well as by convex whorls, with coarse growth lines. These taxa also prefer different habitats. Trochulus plebeius lives in dry and warm forests and shrubs in the Swiss and French Jura while T. sericeus inhabits the herb layer in damp forests and shrubs. In contrast to that, T. hispidus has a broad geographic and altitudinal range and inhabits a large variety of habitats from anthropogenic to rocky alpine sites (Procków 2009; Duda et al. 2011). Genital morphological analyses showed no constant traits that could distinguish taxa in question (Procków et al. 2013b; Duda et al. 2014). Moreover, molecular data revealed high similarity between populations that morphologically correspond to T. hispidus (flattened shells with wide umbilicus) and T. plebeius (elevated shells with narrow umbilicus) (Procków et al. 2013b). On the contrary, there was no clear morphological differentiation among the distinct genetic clades of the T. hispidus complex (i.e., individuals resembling T. hispidus and T. sericeus) and geographic populations could not be distinguished based only on their morphology (Dépraz et al. 2009; Duda et al. 2014). Thus, the final number of species morphologically resembling T. hispidus is far from consensus (Anderson 2005; Welter-Schultes 2012; Duda et al. 2014; Kruckenhaus et al. 2014). The examination of reproductive isolation between the morphospecies could undoubtedly provide valuable information on the taxonomic status of these forms and species complex. Hence, the purpose of our study was to investigate postmating isolation in two distinct morphs, regarded sometimes as separate species (Wiktor 1964, 2004; Kerney et al. 1983). Breeding experiments were carried out to determine whether heterotypic crosses have a reduced fertility. We also checked if F1 and F2 hybrids are viable and partially or completely sterile. To assess fitness, we measured reproductive traits such as: (1) fecundity, the total number of eggs laid per pair; (2) clutch size; (3) F1 and F2 viability (hatching success); (4) the number of hatchinglings per pair; and (5) offspring survival. Additionally, shell measurements were performed to estimate the shell variation of the wild populations and the experimental offspring. We also compared shell morphology of individuals that were maintained in the laboratory for two generations to that of wild caught individuals of the same origin. Finally, we carried out phylogenetic analysis to assess genetic similarity and relationships between the Trochulus taxa.

Materials and Methods

Three and two populations of snails morphologically similar to T. hispidus and T. sericeus/plebeius were collected in Lower Silesia of SW Poland (Table 1). The populations were assigned by initials of their localities: W, L, S and M, Z, respectively. The sites are situated 20–125 km away from each other. The two samples of T. plebeius were given by Wiktor (1964, 1972). Due to a lack of a definite distinction between T. sericeus and T. plebeius, we use name T. sericeus/plebeius for globose and narrow-umbilicated morphs. Characteristics of sampling localities are shown in Table 1. All the snails used in the experiments were collected as juveniles (3.4–4.5 whorls) to avoid prior mating experience and thus interference through stored sperm. Then they were paired and crossed in the following combinations and the number of pairs: 19 W × L, 19 L × Z, 15 L × M, 11 W × M, 6 W × Z, 7 S × Z, 13 Z × M. It resulted in 90 pairs of crosses. Additionally, 28 pairs of control samples were established: 5 W, 5 L, 8 M, and 10 Z. Subsequently, altogether 134 pairs of F1 hybrids and control pairs were mated as follows: 17 F1 (LW × LW), 3 F1 (LZ × LZ), 23 F1 (LM × LM), 2 F1 (WM × WM), 12 F1 (W/SZ × W/SZ), 2 F1 (ZM × ZM), 10 F1 (W × W), 4 F1 (L × L). Because of an insufficient number of F1 offspring of M and Z, it was impossible to use them in the subsequent experiment. To control a possible self-fertilization, six individuals from each population were raised alone.
Pairs were kept in plastic containers measuring 7 × 6 × 5 cm and 12 × 7 × 5 cm whose bottoms were covered with tissue paper and moist soil to encourage egg-laying. Additionally, litter brought from the habitat were used as substratum. Dolomite tablets were served to maintain pH 7.8 and to maintain the carbonate-C02-C ratio close to 0.86. The humidity of the soil was maintained at 80% relative humidity. Food (e.g., lettuce, carrot) was provided depending on the needs.

Since the heavy mortality of juvenile *T. hispidus* in laboratory conditions is a serious problem (Procków et al. 2013a), the containers were cleaned carefully and checked weekly for eggs. The eggs were counted and placed in separate Petri dishes lined with damp tissue paper and moist soil to avoid desiccation until their hatch, then checked for hatching success. The survivorship of juveniles was assessed by counting them every two or three weeks.

The breeding experiment was established in May of 2012 and run until March 2016. The morphological and reproductive data of control and experimental crosses were compared using Kruskal–Wallis non-parametric analysis of variance (ANOVA) or Mann–Whitney U-test with Statistica, version 10 (StatSoft, Inc. 1984–2011). The Benjamini–Hochberg method for *p*-value correction was applied to control the false discovery rate in the case of multiple testing in R package (R Core Team, R: A Language and Environment for Statistical Computing, Vienna, Austria, 2015). Some snails died during this experiment before they reached maturity and therefore were excluded from analyses. The final number of pairs taken to the experiment is shown in Table 2. Due to the low sample size, two populations from closer localities Wrocław and Szczyma were pooled in one case of cross in statistical analyses (Table 2).

### Morphometric analyses

For each shell from the side perspective, we measured height (H), width (W), body whorl height (bwH), aperture height (h), and aperture width (w). From the underneath, umbilicus major diameter (U) (i.e., the longest diameter parallel to the shell diameter, D), umbilicus minor diameter (u) (i.e., perpendicular to umbilicus major diameter) and shell diameter (D) were taken. Finally, the number of whors (whl) were counted according to Ehrmann’s (1933) method. Besides, the following coefficients of shell proportions were calculated: height/width ratio (H/W), relative height of body whorl = body whorl height/shell height ratio (bwHH), umbilicus relative diameter = umbilicus major diameter/shell diameter ratio (U/D), and ratio of umbilicus minor to its major diameter (u/U). Altogether 471 specimens (150 wild caught and 321 laboratory-bred) were measured in standardized views (Procków 2009) by the same person (MP) with a graduated eyepiece in a stereomicroscope and with the accuracy of 0.1 mm. Then statistical parameters were calculated and principal component analyses (PCA) performed (using a correlation matrix). Only shells from Wrocław and Lubawka were appropriate to compare their morphology between laboratory and wild samples of the same origin.

### Genetic analysis and molecular phylogeny

To estimate genetic divergence and phylogenetic relationships of considered taxa, we used all available sequences of mitochondrial cytochrome *c* oxidase subunit I (COI) gene assigned to *T. hispidus*, *plebeius*, or *sericeus*, obtained from GenBank database (http://www.ncbi.nlm.nih.gov/) and our previous studies (Procków et al. 2013b, 2014). In total, we gathered 229 sequences of *Trochulus*. The final alignment consisted of 176 sequences with the length 561 bp after reduction of identical sequences and exclusion of sites that were represented by a gap in at least one taxon. Sequences of three representatives from Hygromiidae: *Candidula unifasciata*, *Lindholmiola girva*, and *Kovacsia kovacsi* were used as an outgroup. To infer phylogenetic trees based on the alignment, we applied four approaches: two Bayesian analyses in MrBayes 3.2.3 (Ronquist et al. 2012) and PhyloBayes MPI 1.5 (Lartillot et al. 2013), as well as two maximum likelihood analyses in TreeFinder (Jubb et al. 2004) and RAxML 8.2.3 (Stamatakis 2014).

The best-fit partitioned nucleotide substitution model was selected according to PartitionFinder 1.1.1 based on BIC criterion (Lanfear et al. 2012); see Table 3. We searched all 56 models and also those available for RaxML. The program proposed three separate models for particular codon positions in COI gene in the two cases. In MrBayes analysis, we used the PartitionFinder scheme and information about heterogeneity rate but applied mixed nucleotide models to specify appropriate substitution models across the larger space in the Bayesian MCMC analysis (Huelsenbeck et al. 2004). Similarly, in TreeFinder, we also applied separate substitution models for three codon positions as suggested by TreeFinder Propose Model module. When gamma-distributed rate variation across sites was implemented, we approximated it by five discrete rate categories.

In PhyloBayes, we used CAT-GTR model with rate variation across sites modeled by five discrete rate categories of gamma distribution. The number of components, weights, and profiles of the model were inferred from the data. Two independent Markov chains were run for 50,000 generations in each of these analyses. The last 35,000 trees from each chain were collected to compute

### Table 1. Characteristics of sampling localities

<table>
<thead>
<tr>
<th>Acronym-locality</th>
<th>Coordinates</th>
<th>Altitude a.s.l.</th>
<th>Habitat description</th>
<th>Annual mean temperature</th>
<th>Annual precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T. hispidus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W-Wrocław</td>
<td>51°07’16.9°N 16°50’38.7°E</td>
<td>130 m</td>
<td>Open, human affected nettle (<em>Urtica dioica</em>) patch</td>
<td>8.4°C</td>
<td>554 mm</td>
</tr>
<tr>
<td>L-Lubawka</td>
<td>50°42’19.2°N 16°00’09.8°E</td>
<td>420 m</td>
<td>Open, human affected nettle (<em>Urtica dioica</em>) patch</td>
<td>5.7°C</td>
<td>730 mm</td>
</tr>
<tr>
<td>S-Szczynia</td>
<td>50°24.514’N 16°24.884’E</td>
<td>502 m</td>
<td>Alder forest in the Bystrzyca Dusznicka valley</td>
<td>6.4°C</td>
<td>659 mm</td>
</tr>
<tr>
<td><strong>T. hispidus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W-Wrocław</td>
<td>50°38.458’N 16°57.050’E</td>
<td>219 m</td>
<td>Natural ash forest close to the border of the nature reserve Muszkowicki Las Bukowy</td>
<td>8.0°C</td>
<td>579 mm</td>
</tr>
<tr>
<td>M-Muszkowice</td>
<td>50°20’07.7°N 16°24’34.6°E</td>
<td>686 m</td>
<td>Natural ash forest mainly with <em>Petasites</em> sp. div. in the Bystrzyca Dusznicka valley</td>
<td>5.2°C</td>
<td>745 mm</td>
</tr>
</tbody>
</table>

*Data from ClimWorld (Global Climate Data, http://www.worldclim.org, Hijmans et al. 2005).*
Table 2. Results of no-choice experiments for F1 generation

<table>
<thead>
<tr>
<th>Cross type</th>
<th>Number of pairs</th>
<th>Number and % of ovipositing pairs</th>
<th>Mean number and range of fecundity (= eggs/pair)</th>
<th>Mean clutch size and its range</th>
<th>F1 viability (=hatching success)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W × W</td>
<td>5</td>
<td>5 (100%)</td>
<td>68.8 (30–164)</td>
<td>12.29 (1–47)</td>
<td>95.35%</td>
</tr>
<tr>
<td>L × L</td>
<td>5</td>
<td>5 (100%)</td>
<td>67.4 (8–128)</td>
<td>12.48 (1–22)</td>
<td>85.46%</td>
</tr>
<tr>
<td>M × M</td>
<td>5</td>
<td>4 (80%)</td>
<td>55 (15–159)</td>
<td>16.92 (2–31)</td>
<td>91.82%</td>
</tr>
<tr>
<td>Z × Z</td>
<td>7</td>
<td>3 (42.9%)</td>
<td>55.67 (11–91)</td>
<td>13.92 (1–37)</td>
<td>86.83%</td>
</tr>
<tr>
<td>W × L</td>
<td>14</td>
<td>9 (64.3%)</td>
<td>52.78 (4–148)</td>
<td>15.32 (2–42)</td>
<td>82.11%</td>
</tr>
<tr>
<td>L × Z</td>
<td>9</td>
<td>1 (11.1%)</td>
<td>63</td>
<td>12.6 (6–17)</td>
<td>90.48%</td>
</tr>
<tr>
<td>L × M</td>
<td>10</td>
<td>5 (50%)</td>
<td>44.6 (16–102)</td>
<td>14.87 (2–26)</td>
<td>81.61%</td>
</tr>
<tr>
<td>W × M</td>
<td>5</td>
<td>2 (40%)</td>
<td>41 (31–51)</td>
<td>8.2 (3–19)</td>
<td>76.83%</td>
</tr>
<tr>
<td>W/S × Z</td>
<td>9</td>
<td>5 (55.6%)</td>
<td>61.6 (8–202)</td>
<td>11 (4–29)</td>
<td>67.53%</td>
</tr>
<tr>
<td>Z × M</td>
<td>7</td>
<td>3 (42.9%)</td>
<td>9.33 (3–19)</td>
<td>7 (1–19)</td>
<td>82.14%</td>
</tr>
</tbody>
</table>

Notes: Differences between all cross types are not significant.

Table 3. Partitioning schemes and nucleotide substitution models applied in phylogenetic reconstruction

<table>
<thead>
<tr>
<th>Partition</th>
<th>MrBayes</th>
<th>TreeFinder</th>
<th>RAxML</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. codon position of COI</td>
<td>mixed+Γ</td>
<td>GTR+I+Γ</td>
<td>GTR+Γ</td>
</tr>
<tr>
<td>2. codon position of COI</td>
<td>mixed+I</td>
<td>HKY(Ts = 3, Tv = 1)+I+Γ</td>
<td>GTR+Γ</td>
</tr>
<tr>
<td>3. codon position of COI</td>
<td>mixed+Γ</td>
<td>TVM+Γ</td>
<td>GTR+Γ</td>
</tr>
</tbody>
</table>

Results

Crossing experiments

In 43 of the 118 pairings (36%), one or both snails died before reaching sexual maturity. None of the snails kept alone reproduced. Therefore, autogamy or parthenogenesis can be excluded. In total, eggs and offspring were produced for 17 control pairs (77%) and for 25 experimental pairs (64%), which is not statistically different (P = 0.3701) (Table 2). Moreover, there were no statistically significant differences in total reproduction compatibility, that is, fecundity (χ² = 9.67; P = 0.378) and F1 viability (χ² = 13.06; P = 0.1599) between heterotypic and parental crosses. The clutch size was not different either (χ² = 18.89; P = 0.058) between heterotypic and parental crosses, although all pairs laid the different number of eggs, with the control pairs lying more than experimental pairs (Table 2). There was a different number of ovipositing pairs (Table 2), although the ratio of reproductive versus non-reproductive pairs did not differ significantly between the homo- and heterotypic crosses (P = 1.000).

In F1 crosses, 14 control pairs (26%) and 59 hybrids (49%) laid eggs and produced offspring, which is not statistically different (P = 0.1196) (Table 4). The fecundity rates were not significantly different either among all F1 hybrids (χ² = 4.12; P = 0.766). However, the clutch size and F1 viability did differ in a few cases (Table 4). A number of ovipositing pairs varied widely (Table 4) but the differences between the homo- and heterotypic crosses were not significant considering the ratio of reproductive versus non-reproductive pairs (P = 1.000).

Figure 1A–F shows the number of hatchlings per pair and per successful pair that emerged from all crosses. Although the number of offspring produced per pair varied greatly between each cross, showing 2- to 53-fold differences, it did not differ significantly among the three treatments [i.e., two controls and the outcross] of each experimental setup. There was, however, one exception, that is, L versus L × Z (P = 0.0133; Figure 1E) but only in the case when all pairs were considered.

Figure 2A demonstrates that the survivorship of the F1 offspring was roughly comparable in all crosses. The greatest mortality was recorded during the first 150 days, when only ca. 32% of the juveniles survived. At this time, the offspring survival ranged from 14% to 52%. The differences were not statistically significant for all crosses except L versus W × M (P = 0.013).

The survival of F2 offspring during the first 150 days was approximately 51% (range 18.75–100%; Figure 2B), when all available types of crosses were considered. The statistically significant differences were recorded between L × Z versus W, W × L, L × M (P = 0.017, P = 0.025, and P = 0.004, respectively). The F2 offspring survival was also significantly different from that of the F1 generation (P < 0.001). Similarly, when only heterotypic crosses were included in the analysis, the survivorship of the F1 hybrids did differ from that observed for F2 (app. 36% and 50%, respectively; P < 0.001).

Morphometric analysis

The shell shapes of 150 individuals collected from the field were analyzed. PCA extracted two meaningful axes, representing 63.7% of the total morphometric variance. The first axis ordinated the shells in a gradient from high shape with erect whorls (negative scores) to a flattened shell with barely extruded whorls (positive scores). The second axis opposed shells with wide (U and u) and relatively wide umbilicus (U/D) on the negative side, and shells with narrower umbilici on the positive side (Figure 3A). On the plot, two large separated morphospace areas, only slightly overlapping, could
be distinguished. The one space consists of individuals collected from populations in Wrocław and Lubawa (W and L). Their shells are predominantly flat with wide umbilicus. The opposite features are characteristic of snails from Muszkowice and Zieleniec populations (M and Z), which occupy the second morphospace.

In contrast to that, no such differentiation into distinct shell morphospecies was observed for F₁ and F₂ offspring of heterotypic crosses (n = 191 individuals) (Figure 3B). All crosses occupied one large area in PCA plot and their morphospaces clearly overlapped.

An additional PCA ordination between two morphs collected in the field, that is, *T. hispidus* (W/L) and *T. sericeus*/*plebeius* (M/Z) with their experimental offspring showed that all experimental progeny occupied a large area of the morphospace overlapping with the field populations (Figure 4). The sequential chi-square test showed that the first (χ² = 20.28; P = 0.000) and the second (χ² = 89.92; P = 0.000) components accounted for most of the variance in the observed variables. Significant differences were found between all tested groups regarding both PC1 and PC2 scores (P < 0.001) with an exception between M/Z and experimental offspring in PC1 scores (P = 1.000).

Considering the shell traits u, U, H/W, and U/D, we observed significant differences (P < 0.001) between all groups. These measures also had the highest loadings of the PC2. The features W, D, and whl appeared to be not significant (P = 1.000) between all three groups. Whereas H, bwH, h, and w were significantly different (P < 0.001) for the comparison of W/L field forms with M/Z field forms and offspring. Furthermore, bwH/H and h/w were significantly different (P < 0.001) for offspring compared with both field groups. Finally, M/Z field group differed significantly (P < 0.000001) from W/L field forms and experimental offspring by u/U.

**Phenotypic plasticity**

The shell shape of laboratory snails derived from two populations of *T. hispidus* morph (W and L) revealed a big sensitivity to the environmental conditions for two generations. In both populations the average shell shape (expressed as PC2) changed significantly from flat with wide umbilicus to more globular or even elevated with narrower umbilicus (χ² = 63.375; P < 0.001, Figure 5). Similar, significant tendencies but toward larger shells were also observed along the first axis (PC1, data not shown).

We further found that the morphological differences seen in the wild populations actually disappeared in the laboratory environment. Laboratory-bred morphs of *T. hispidus* from Wrocław and Lubawa became similar to wild populations from Muszkowice and Zieleniec representing *T. plebeius* morph. They occupied an overlapping morphospace on the PCA plot (Figure 6).

**Molecular phylogeny**

In order to determine phylogenetic relationships of the studied *Trochulus* taxa, we carried out phylogenetic analyses by four approaches. We analyzed all available 176 different sequences of mitochondrial COI gene fragments assigned to *T. hispidus, plebeius, or sericeus*, including samples from populations, which were used in crossbreeding experiments. Trees obtained by all four approaches showed very similar topology with the same strongly supported clades. Only internal deep branches did not obtain the significant support and were not recovered by all methods.

Sequences representing populations whose individuals were subjected to crossbreeding experiments occupy different positions in the tree (Figure 7). Three sequences from specimens collected in Zieleniec (*T. plebeius/sericeus*), Muszkowice (*T. sericeus*), and Wrocław (*T. hispidus*) were grouped together with very high support values. They created also very significant clade (clade 2 in Figure 7) together with a very-well supported group including the second *T. hispidus* sequence from Wrocław and *T. hispidus* from Sweden. The second sequence from Zieleniec (*T. plebeius/sericeus*) branched off in the subsequent node, which obtained a significant support value only in MrBayes. However, this position was inferred in 3 of 4 applied methods.

One specimen from Lubawa (*T. hispidus*) was the most closely related to other *T. hispidus* sequences from Austria as well as T. sericeus from Germany (Figure 7). The placement of the Lubawa sequences was inferred by three methods without significant support values. However, all these sequences were firmly located within the bigger clade 3, which was significantly supported by at least Bayesian methods and included other European *T. hispidus* and German *T. sericeus*. The second sequence from Lubawa and the third sequence from Wrocław were very significantly placed among the clade 7 including other *T. hispidus* from the Netherlands, Sweden, and also Canada. Very short genetic distances between European and American specimens indicate their common origin.

It should be noted that besides two above-mentioned cases, there were also at least three additional ones in which specimens assigned to *T. plebeius or sericeus* were very significantly grouped with *T. hispidus* (Figure 7). It concerns *T. sericeus* from Germany and *T. hispidus* from Austria (clade 1) and *T. plebeius/sericeus* and *T. hispidus* from UK. Moreover, in clade 8, the basal position was occupied by...
several lineages assigned to *T. plebeius* or *T. sericeus* from France, which clustered with the group including *T. hispidus* from Germany and Switzerland as well as *T. sericeus* from Switzerland.

**Discussion**

The aim of this study was to evidence the species barriers between *T. hispidus* and *T. sericeus/plebeius* in crossbreeding experiments as well as by their morphological and phylogenetic analyses. These different morphological forms are regarded as separate species (Wiktor 1964, 2004; Paul 1967; Kerney et al. 1983) but our no-choice tests for postzygotic reproductive isolation revealed that their representative specimens could breed in laboratory conditions without constraints. None of our isolated individuals produced eggs, which indicates that they are obligate outcrossers, or at least that uniparental reproduction is very uncommon. However, to confirm this finding parent–offspring genetic analyzes are required. Our results

**Figure 1(A-F)** Number of hatchlings (mean ± SE) in homo- and heterotypic crosses derived from all pairs (white bars) and successful pairs (gray bars). Different letters (a, b) indicate statistically significant differences.
indicate that the morphological forms examined here belong to the same species and their distinction as separate biological species is not justified. These findings corroborate our previous results (Procków et al. 2013b) that showed no significant anatomical and genetic differences between *T. hispidus* and *T. sericeus/plebeius* morphspecies coming from the same geographical region and thus providing evidence for a lack of reproductive barriers. Differences in the structure of the genitalia are often used to designate species of land snails, and they have been assumed to be the basis of mate recognition (Webb 1961). Nevertheless, similar genitalia in the different populations as well as the co-presence of globular, intermediate, and flat shells were detected in single lineages of the Sicilian helicid *Marmorana* (Fiorentino et al. 2008). Similarly, homogenous genital morphologies among the *Trochulus* species seem to be more common (Procków et al. 2013b, 2014) than it is usually found in other pulmonates.

Some snail pairs did not reproduce successfully in the present experiment. It most probably results from reasons other than cross type, as the ratio of reproductive versus non-reproductive pairs did not differ significantly between the homo- and heterotypic crosses. It is possible that unknown laboratory effects prevented some snails from copulating and reproducing. It was also reported that in captivity, land snails copulate less readily than freshwater snails (Duncan 1975) or those from the field (Chen 1993). The difficulties

Figure 2. Temporal trend in survivorship of (A) F1 and (B) F2 offspring emerged from all crosses.
Figure 3. Principal component analysis of shell morphology in wild populations (A) and experimental offspring (B).

Figure 4. Principal component analysis of shell morphology in wild populations (W/L and M/Z) and their experimental offspring.
with breeding colonies of other *Trochulus* species in captivity (Cain 1959; Falkner 1973; Procków et al. 2013a) may suggest some specific requirements for these hygromiid snails that are not easy to maintain in standard laboratory conditions.

The lack of reproductive barriers between *T. hispidus* and *T. sericeus*/*plebeius* is also supported by our phylogenetic studies. In five from nine well-defined and supported clades in *Trochulus* phylogeny, samples from *T. hispidus* and *T. sericeus*/*plebeius* were grouped together, which indicates rather unrestricted gene flow between these taxa. As a result, all sequences from *T. hispidus*, *T. plebeius*, and *T. sericus* did not create clear monophyletic groups in the obtained phylogenetic trees. It implies that different morphs can evolve independently in separate genetic lineages, most probably under various local environmental conditions. The representatives of populations which in crossbreeding experiments produced offspring were closely related in phylogenetic trees (clade 2 with Muszkowice, Wroclaw, and Zieleniec sequences; clade 7 with Lubawka and Wroclaw sequences) as well as clearly separated (Lubawka sequences vs. Zieleniec and Muszkowice sequences). It suggests that successful crossbreeding is possible even between individuals, which seem to be distantly related in phylogenetic trees. They may represent one biological species.

However, some globose and narrow-umbilicated *Trochulus* forms named as *montanus* and present in the Jura Mountains might constitute a separate species (Procków et al. 2014). They according to Falkner (1982, 1990) should be named *T. plebeius*. In agreement with that, the forms from French and Swiss Jura (Figure 8A–E) described under these names and showing similarity in shell morphology are significantly grouped in phylogenetic trees (Procków et al. 2014). Other snails from Jura described as *T. sericus* (Figure 8F–G) with similar shell morphology to *T. plebeius* also clustered together (Procków et al. 2014, clade 8 in Figure 7). However, they also mixed with *T. hispidus* morphs from Germany and other localities in Switzerland. In turn, flat and wide-umbilicated *T. hispidus* also originating from Jura (clade 4, Figures 7 and 9A) created a separate clade with no significant clustering also with other morphologically similar or different snails (Figure 9B–G) from UK (clade 5 in Figure 7) and Poland (sequences scattered in clades 2, 3, and 7 in Figure 7).

As it could be expected, the wild populations from Wroclaw and Lubawka (W/L) assigned to *T. hispidus* differ clearly in shell shape from Muszkowice and Zieleniec (M/Z) populations classified to *T. sericus*/plebeius* (Figures 3A and 9C–F). However, the morphological differences seen in the wild disappeared in a laboratory environment (Figure 4). Therefore, the phenotypic difference found in the wild can be explained by phenotypic plasticity. The wild environment of these populations is much more diversified than the laboratory conditions, which were the same for the cultured snails. The sites from which the snails were collected differ in some climatic variables (i.e., annual mean temperatures and total precipitation) (Table 1), most probably related with the altitude of these sites. W/L and M/Z populations are also subjected to other factors in their natural microhabitats. For example, both W and L sites are typical human affected, open environments whereas M and Z represent natural ash forests (Table 1). As shown in other studies many traits in shell shape are susceptible to substantial environmental variation (Cotton et al. 2004; Chiba 2009). Generally, organisms which inhabit divergent environments but having intensive gene flow between those environments are at a selective advantage to display
Figure 7. MrBayes tree of Trochulus COI gene sequences. Numbers at nodes, in the order shown, correspond to: posterior probabilities estimated in MrBayes (MB) and PhyloBayes (PB) as well as bootstrap support values obtained in TreeFinder (TF) and in RAXML (RM) by maximal likelihood method. Values of the posterior probabilities and bootstrap percentages lower than 0.50% and 50%, respectively, were omitted or indicated by a dash “–”. Samples representing populations whose individuals were subjected to crossbreeding experiments are bolded.

plastic phenotypes (Sultan and Spencer 2002; DeWitt and Scheiner 2004). It is also possible that specific interactions between genotype and environment may dictate that morphological differences seen in the wild are not manifested in the laboratory environment.

Multiple independent adaptations to local selection pressures were demonstrated for several land gastropods (Pfenninger and Magnin 2001; Chiba 2009). However, in our study, the variation in shell shape of the laboratory generations did not follow their ancestral lineages. Moreover, the transitions from flattened shells with wider umbilicus toward more elevated shells with narrower umbilicus happened under constant laboratory conditions very fast within one generation (Figure 5). It indicates that rather phenotypic plasticity of the shell during its growth is responsible for this change. The phenotypic changes in shell morphology also persisted in the F2 generation. The induced changes may become canalized after the interaction is over (Agrawal 2001). A similar change in shell morphology was observed in freshwater (Wullschleger and Jokela 2002; Pfenninger et al. 2006; Kistner and Dybdahl 2013) and land snails (Pokryszko 1990; Kuźnik-Kowalska 2008). Shell morphology may indeed depend on several environmental factors, for example, habitat type (Cameron and Pannett 1985; Chiba 2002), the duration of growth season (Pfenninger and Magnin 2001; Procków et al. 2012), predation (Pascoal et al. 2012), or even pollution (Mulvey et al. 1996). Moreover, our recent study revealed that the ultimate size of T. striolatus was mostly a response to prevailing local environmental and/or climate variables, thus, showing no sufficient justification for its infraspecific classification (Procków et al. in review). Since the characteristic shell morph of two T. hispidus populations (W and L) studied here changed in laboratory conditions, it is probable that constant high humidity and temperature which changed in a constant way may play an important role in determining shell shape. Specimens with very much elevated spire, narrow umbilicus, and occasionally—elevated shells with descending body whorl were obtained in the first generation of laboratory-bred snails of Discus rotundatus and D. ruderatus kept in high-humidity conditions (Boettger 1929, 1931; Kuźnik-Kowalska 2008). Similar forms of D. rotundatus were described from greenhouses in Berlin (Boettger 1930), caves in Belgium (Boettger 1939), and D. ruderatus from the Karkonosze Mts (Umiński 1962). Unlike Boettger (1929, 1930), Umiński (1962) explained such shell modifications not by humidity and temperature conditions (not lower than 0°C) but by the hibernation period. Hibernating snails build flattened shells, and those not forced to hibernate—elevated shells with descending body whorl. Additionally, Umiński (1962) argued that if it was the influence of high humidity, that is, a factor acting continuously and not too strongly, one should expect gradual and more slowly progressing changes. Such a suggestion concerning hibernation, however, does not justify the presence of high-spired and narrow-umbilicated though hibernating snails from natural populations of Zieleniec and Muszkowice in this study examined. In fact, we observed the strongest significant negative correlations of shell width and height as well as umbilicus major and minor diameter with precipitation variables in T. striolatus (Procków et al. in review). Nevertheless, to confirm Umiński’s (1962) assumption further experiments are needed.

In conclusion, our findings indicate that the identification of snails resembling T. hispidus and T. sericus/plebeius based only on shell morphology may be prone to error due to the environmental influence. Laboratory crossbreeding experiments and genetic similarity indicate that the phenotypic plasticity seems to be the best explanation for the evolution of the sympatric polymorphism in Trochulus taxa. The discrepancies between morphological and molecular data may result from that the different polymorphisms are at different stages of divergence and the shell differentiation may proceed faster than the sequence of mitochondrial genes (Haase et al. 2003). Therefore, our results confirm a suggestion that in Trochulus species, some morphological differences may reflect an incipient stage of speciation (Procków et al. in review). Moreover, if we regard high levels of shell plasticity in size and shape as ancestral traits in this genus, it may be assumed that shells in the common ancestor were similarly plastic. The presence of spatially separated metapopulation lineages in Trochulus were detected (Procków et al. 2013b). Thus, future studies should focus on the genetic structure of metapopulations, and comparing genetic variation within and among populations. introgression of haplotypes by hybridization between populations with globular and flat shells has been hypothesized as a secondary source of shell variability (Dawson 2002; Teshima et al. 2003).

Supplementary material
Supplementary material can be found at http://www.cz.oxfordjournals.org/.

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References


Donoghue MJ, 1985. A critique of the biological species concept and recom-


