

MORPHOLOGY, ECOLOGY AND DNA-BARCODING DISTINGUISH *PUPILLA PRATENSIS* (CLESSIN, 1871) FROM *PUPILLA MUSCORUM* (LINNAEUS, 1758) (PULMONATA: PUPILLIDAE)

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ABSTRACT

The taxonomic identity of *Pupilla pratensis* (Clessin, 1871), normally considered to be an ecophenotype of *Pupilla muscorum* (Linnaeus, 1758), was investigated using morphological, ecological and molecular data (cytochrome *c* oxidase subunit I and cytochrome *b*). We conclude that these two forms represent distinct species. This underused combined approach proves to be a powerful tool in distinguishing among closely related species within the genus *Pupilla*, and we suggest that it should be more widely adopted when sorting out complexes in other groups.

INTRODUCTION

The genus *Pupilla* s. s. (Fleming, 1828) comprises some 20–30 species and is widely distributed throughout the northern hemisphere – Europe, northernmost Africa, N. Asia and North America (Zilch, 1959). The most common and widespread species is *Pupilla muscorum* (L., 1758), which inhabits nearly the entire range of the genus (Ehrmann, 1933; Pilsbry, 1948) and has proved to be important indicator species in ecological studies (e.g. Chappell *et al.*, 1971; Juricková & Kucera, 2005), especially in the study of climatic change (e.g. Rousseau, 1985, 1987; Rossignol, Moine & Rousseau, 2004; Moine, Rousseau & Antoine, 2005; Chlachula *et al.*, 2007). *Pupilla muscorum* is also the type species (*Pupa marginata* Draparnaud, 1801 = *P. muscorum*) of the genus *Pupilla*.

Pupilla muscorum is rich in forms and highly variable, as is the genus as a whole. Several authors have pointed out the need for the taxonomic revision of *Pupilla* species and forms (e.g. Lozek, 1964). *Pupilla muscorum* var. *pratensis* was first described by Clessin (1871) based on material from Dinkelscherben in Bavaria (Germany), where it occurred in wet peat meadows. Since then, however, the form has attracted little attention and there are only scattered and brief references to it in the literature. Mostly, it has been referred to as an ecophenotype (e.g. Ehrmann, 1933; Lohmander, 1955, 1956; Jaeckel, 1962). Lohmander (1956) also mentioned specimens which he interpreted as intermediate between *pratensis* and typical *P. muscorum*. The rank of *pratensis* has been variously given as ‘variety’, ‘form’ or ‘mutation’. In the latest comprehensive field guide of the terrestrial molluscs from North and Central Europe (Kerney, Cameron & Jungbluth, 1983) *P. pratensis* was not mentioned. It was not until the publication of the study by Jueg (1997) on *Pupilla* material from the province of Mecklenburg-Vorpommern in north-eastern Germany, that the form attracted serious attention in recent times. As the results of Jueg (1997) coincided with observations on morphology and ecology of the forms in Scandinavia, we decided to study them more closely, using a combined approach involving morphology, ecology and molecular data.

We have therefore compared populations of *pratensis* with typical *P. muscorum*, both morphologically in terms of shell characters, and genetically using sequences of the mitochondrial genes cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (CytB). Here we offer a redescription of the shell of *pratensis* in comparison with related species of *Pupilla*, details of its ecology and distribution, and an account of the molecular basis for the separation of *pratensis* from *muscorum*.

MATERIAL AND METHODS

Specimens of *Pupilla* were collected from various locations and transported live to the laboratory where they were measured to nearest 0.1 mm, determined to taxonomic group on the basis of shell morphology, and then fixed in 96% alcohol for DNA analysis. Voucher specimens from each locality and for each taxon were preserved dry in the collection of the Göteborg Natural History Museum (GNM).

The material collected for sequencing is listed in Table 1. Additionally, more than 100 samples of *Pupilla* from Scandinavia were inspected by eye in order to confirm the stability of shell characters, and to obtain a broad picture of the geographical and ecological distribution of the two forms. A syntype of *Pupilla pratensis* from Dinkelscherben, Bavaria, Germany (Fig. 1A) and a specimen from Kobylno, Upper Silesia, Poland (Fig. 1B) (both coll. Westerlund no. 2306, GNM) were also studied. The studied samples of *Pupilla alpicola* were collected from two localities in the Austrian Alps: Franz-Josef Höhe (Carinthia) and Krahberg bei Landeck (Tyrol).

DNA from specimens to be sequenced was extracted using the DNeasy Kit (QIAGEN), following the manufacturer’s protocol for animal tissue, by crushing the entire animal in an ependorph tube. The best results were obtained when ethanol in the tissue was allowed to evaporate prior to extraction, using RNase, and when the DNA was eluted using the elution buffer provided with the kit. Extracted DNA was diluted 10× in ddH₂O, and stored at –20°C prior to amplification.

All PCRs were carried out in a final volume of 50 µl with 4 µl of DNA template, 1 µl of each primer, 4 µl dNTP, 4 µl 10× PCR buffer, 36.8 µl ddH₂O and 0.2 µl *Taq* DNA polymerase

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Table 1. Material of *Pupilla muscorum* and *Pupilla pratensis* used in the molecular genetic studies.

Locality	Species	Haplotypes of COI	Haplotypes of CytB	Number of specimens
Borgunda, Västergötland, Sweden	<i>muscorum</i>	1, 2, 3	1, 2, 3	30
Klädner Plage, Mecklenburg-Vorpommern, Germany	<i>muscorum</i>	4, 5		3
Heiterbachtal, Baden-Württemberg, Germany	<i>muscorum</i>	6	4	2
Klädner Plage, Mecklenburg-Vorpommern, Germany	<i>pratensis</i>	7	5	13
Lagmansro, Östergötland, Sweden	<i>pratensis</i>	7, 8, 9	6, 7	15
Ombergsliden, Omberg, Östergötland, Sweden	<i>pratensis</i>	10	8	1

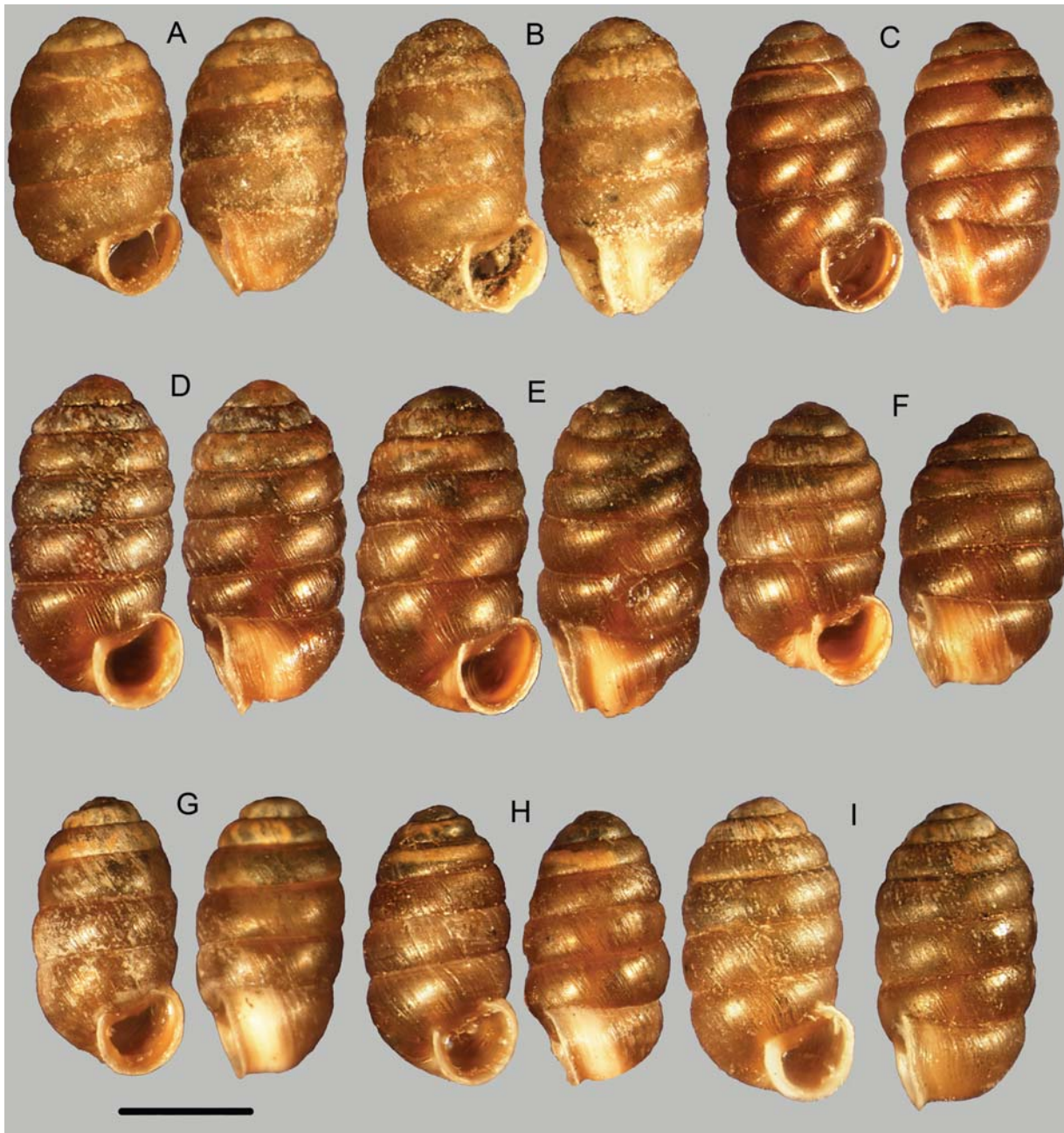


Figure 1. **A.** *Pupa (Pupilla) muscorum* v. *pratensis* Clessin = *Pupilla pratensis* (Clessin) Dinkelscherben, Bavaria, Germany. Syntype. (coll. Westerlund 2306, GNM). **B.** *Pupilla pratensis*, Kobylno, Upper Silesia, Poland. (coll. Westerlund 2306, GNM). **C.** *Pupilla pratensis*, Ombergsliden, Östergötland, Sweden. **D–F.** *Pupilla pratensis*, Klädner Plage, Mecklenburg-Vorpommern, Germany. **G.** *Pupilla muscorum*, Borgunda church, Västergötland, Sweden. **H.** *Pupilla muscorum*, Klädner Plage, Mecklenburg-Vorpommern, Germany. **I.** *Pupilla alpicola*, Franz-Josef Höhe, Carinthia, Austria. Scale bar = 1 mm.

Table 2. Number of specimens of each haplotype for COI and CytB and GenBank accession numbers.

Haplotype no.	Number of specimens	GenBank no.	<i>Pupilla</i> species
COI 1	6	EF457915	<i>muscorum</i>
COI 2	23	EF457916	<i>muscorum</i>
COI 3	1	EF457917	<i>muscorum</i>
COI 4	2	EF457918	<i>muscorum</i>
COI 5	1	EF457919	<i>muscorum</i>
COI 6	2	EF457920	<i>muscorum</i>
COI 7	14	EF457921	<i>pratensis</i>
COI 8	7	EF457922	<i>pratensis</i>
COI 9	7	EF457923	<i>pratensis</i>
COI 10	1	EF457924	<i>pratensis</i>
CytB 1	16	EF457907	<i>muscorum</i>
CytB 2	4	EF457908	<i>muscorum</i>
CytB 3	1	EF457909	<i>muscorum</i>
CytB 4	2	EF457910	<i>muscorum</i>
CytB 5	12	EF457911	<i>pratensis</i>
CytB 6	6	EF457912	<i>pratensis</i>
CytB 7	5	EF457913	<i>pratensis</i>
CytB 8	1	EF457914	<i>pratensis</i>

(Amersham Biosciences). We used the following temperature profile: 96°C 2 min (93°C 30 s, 47°C 30 s and 72°C 1 min) × 40; 72°C 7 min; 4°C hold. The primers used for COI were LCO 1490 (GGTCAACAAATCATAAAGATATTGG) and HCO 2198 (TAAACTTCAGGGTGACCAAAAATCA) (Folmer *et al.*, 1994). For comparison we also sequenced CytB for a smaller number of specimens. For the CytB gene, the primers used were CytB 811R (GCRWAYARAAARTAYCAYT CWGG) and CytB 397F (YWYTRCCTTGGRGG RCARATATC) (Dahlgren, Weinberg & Halanych, 2000), with an annealing temperature of 54°C. Amplification products were purified with QIAquick (QIAGEN), protocol for microcentrifuge. Purified PCR products were sequenced using BigDye 3.1™ with a final volume of 10 µl: 1 µl BigDye, 1 µl sequencing buffer, 0.5 µl primer, 6.5 µl ddH₂O and 1 µl purified PCR product. The temperature profile for the sequencing PCR was as follows: 95°C 3 min (96°C 10 s, 50°C 5 s and 60°C 4 min) × 25; 4°C hold. Sequence data were collected using an ABI 3700 at the SARS Centre, University of Bergen. All fragments were sequenced in both directions. For COI a 654 bp, and for CytB a 396 bp, fragment was used in the analysis.

Obtained sequences were assembled using the DNASTAR Lasergene (DNASTAR Inc.) package, and aligned using the Clustal W algorithm implemented in the module MegAlign. Further adjustments were made by hand using the MacClade software. All sequences were checked against the curves from the ABI sequencer and the accuracy was checked by performing a BLAST search against the NIH Blast database. Neighbour-joining analyses were carried out using the tree generator in MegAlign and bootstrap analysis was done in the PAUP* program on a Macintosh PowerBook G4. As outgroup for the COI analysis, we used the GenBank sequence AY148557 (*Laminella sanguinea*) because this was the closest hit generated for a BLAST search of the *Pupilla muscorum* COI sequence. Similarly the sequence AF350896 (*Partula suturalis*) was used as outgroup for the CytB gene. The number of specimens, the different haplotypes of COI and CytB, and their GenBank numbers are presented in Table 2.

SYSTEMATIC DESCRIPTION

Pupilla pratensis (Clessin, 1871)

(Fig. 1A–F)

Pupa (*Pupilla*) *muscorum* var. *pratensis* Clessin, 1871: 101 (Dinkelscherben, Bavaria, Germany). Clessin, 1884: 246. Clessin, 1887: 256, fig. 155. Westerlund, 1871: 243–244. Westerlund, 1887: 121.

Pupa muscorum var. *pratensis*—Goldfuss, 1883: 37.

Pupilla muscorum f. *pratensis*—Steenberg, 1911: 174. Ehrmann, 1933: 46. Jaeckel, 1962: 98 [with authority Reinhardt, in error]. Jueg, 1997: 277–285. von Proschwitz, 1998: 40, 52 [as *pratense*]. von Proschwitz, 2001: 56. von Proschwitz, 2002: 16. Zettler *et al.*, 2006: 133.

Pupilla muscorum mut. *pratensis*—Pilsbry, 1921: 178, pl. 20: fig. 9.

Pupilla muscorum pratensis—Lohmander, 1955: 53–54.

Lohmander, 1956: 47.

Pupa (*Pupilla*) *muscorum* var. *madida* Gredler [in part] Clessin, 1876: 201–202.

Types examined: One syntype, Dinkelscherben, Bavaria, Germany (leg. S. Clessin) (Fig. 1A) (coll. Westerlund no. 2306, GNM)

Material examined: Kobyllno, Upper Silesia, Poland (leg. O. Goldfuss) (Fig. 1B) (coll. Westerlund no. 2306, GNM); Klädner Plage, Meckenburg-Vorpommern, Germany (leg. U. Jueg); Heiterbachtal, Baden-Württemberg, Germany (leg. H.-J. Niederhöfer); Borgunda, Västergötland, Sweden (leg. T. von Proschwitz). *Pupilla alpicola*: Franz Josef-Höhe, Carinthia, Austria (leg. H. W. Waldén); Krahberg bei Landeck, Tyrol, Austria (leg. V. Wiese, ex coll. G. Falkner). Other material: A further more than 100 *Pupilla* samples from different localities in Scandinavia (Denmark, Norway and Sweden).

In addition, the following material of other species was examined for comparative purposes: *P. muscorum*: Klädner Plage, Meckenburg-Vorpommern, Germany (leg. U. Jueg); Heiterbachtal, Baden-Württemberg, Germany (leg. H.-J. Niederhöfer); Borgunda, Västergötland, Sweden (leg. T. von Proschwitz). *Pupilla alpicola*: Franz Josef-Höhe, Carinthia, Austria (leg. H. W. Waldén); Krahberg bei Landeck, Tyrol, Austria (leg. V. Wiese, ex coll. G. Falkner). Other material: A further more than 100 *Pupilla* samples from different localities in Scandinavia (Denmark, Norway and Sweden).

Shell description (Fig. 1A–F): Height: 3.48–4.54 mm; breadth: 1.86–2.06 mm. Number of whorls: 6.0–7.5. Cylindrical to somewhat ovoid, outline rather elongate and broad in profile, narrows somewhat more abruptly at apex, which is rather distinct. Colour dark to chestnut brown. Shell thin compared to other *Pupilla* species, translucent, with fine, somewhat pronounced growth-lines. Whorls strongly vaulted, each separate whorl comparatively high and separated by a deep suture. Aperture rounded with rather weakly developed lip; usually toothless, but often with weak parietal tooth, and sometimes also with more or less developed palatal tooth. A weak callus sometimes present.

Comparison with other species: The variety *pratensis* was described by Clessin (1871) as differing from typical *muscorum* as follows: “großer und dicker als die typische Form, mit dünnerer dunklerer Schale und schwächerer Mundlippe; im ganzen von weniger kalkigen Ansehen; bis 4 mm lang” [larger and thicker (in profile) than the typical form, with darker shell and weaker lip, on the whole of less chalky appearance, up to 4 mm high]. This is in accordance with characteristics given by Jueg (1997) for *pratensis* from Meckenburg-Vorpommern and is also consistent with Scandinavian material studied.

The shell of *pratensis* is larger (especially in breadth, but often also in height) and the number of whorls is usually greater than that of *muscorum* (Fig. 1G, H). The shell form is cylindrical in both species, but in *muscorum* the last whorls

Table 3. Shell morphological characters of *Pupilla muscorum* ('typical form'), *P. pratensis* and *P. alpicola*.

Character	<i>P. muscorum</i>	<i>P. pratensis</i>	<i>P. alpicola</i>
Height (mm)	3.0–3.5*; 2.98–4.06 [†]	3.5–3.9*; 3.48–4.54 [†]	3.0–3.3*; 2.8–3.3 [‡]
Breadth (mm)	1.75*; 1.65–1.74 [†]	2.00*; 1.86–2.06 [†]	1.80*; 1.80 [‡]
Number of whorls	6.0–6.5*; 5.5–7.0 [†]	6.0–6.5*; 6.0–7.5 [†]	6.0*
Shell form	Cylindrical – ovoid, outline rather elongate, narrows smoothly at apex	Cylindrical – somewhat ovoid, outline elongate and broad, narrows somewhat more abruptly at apex	Pronounced cylindrical, outline short and broad, narrows markedly abrupt at apex
Apex	Pronounced	Pronounced	Flat
Shell sculpture	Almost smooth, growth-lines fine	Growth-lines fine but somewhat more pronounced	Growth-lines marked, sometimes with periostracal lamella
Shell colour	Reddish brown – horn grey	Dark chestnut brown	Dark brown
Shell thickness	Rather thick	Rather thin, somewhat translucent	Rather thin, somewhat translucent
Form of whorls	Medium-strongly vaulted, each separate whorl rather high	Strongly vaulted, each separate whorl rather high	Strongly vaulted, each separate whorl low
Suture	Rather deep	Deep	Deep
Apertural lip	Distinct	Rather weak	Rather weak – weak
Apertural teeth	Rather strongly developed (parietal and sometimes palatal)	Weakly developed (parietal weak, palatal indicated) rather often toothless	Weakly developed (parietal weak, no palatal) often toothless
Form of aperture	Somewhat oval, high	Rounded	Rounded, but somewhat depressed
External rib close to aperture on body whorl	Pronounced	Rather weak or absent	Very weak or absent
Narrow, gutter-like depression on surface of body whorl	Absent	Absent	Present, visible as delicate ridge through the aperture

The measurements and the number of whorls for each species are taken from: *Ehrmann (1933); [†]Jueg (1997); [‡]Kerney *et al.* (1983).

converge rather smoothly towards the apex; in *pratensis* the convergence is somewhat more abrupt, giving the apex a blunt appearance. The whorls are usually more vaulted in *pratensis*, and the suture is deeper. The colour of the shell varies from brown to brown-grey in both species, but *pratensis* is often more of a darkish chestnut brown. In *muscorum* the colour is more variable, ranging from reddish brown to horn grey. The shell surface is nearly smooth in both species. It appears thinner in *pratensis*, and therefore sometimes slightly translucent. The growth lines are very fine in *muscorum* and somewhat coarser in *pratensis*. The apertural lip is often rather weakly developed in *pratensis* in contrast to *muscorum*, in which it is thicker and more pronounced. The apertural teeth are more weakly developed in *pratensis*, and always arise directly from the apertural walls, never from a callus (as is sometimes the case in *muscorum*). A weakly developed parietal tooth is rather frequently present, often together with a very weak, simply indicated palatal, but the mouth is often completely toothless (at least in Scandinavian material). The characters mentioned above may be weakly indicative of systematic affiliation when evaluated separately, but some of them are highly variable within species, as is often the case in *Pupilla*. For example, very high specimens with many whorls ('form *elongata*') or specimens completely lacking teeth ('form *edentula*') are found in both *muscorum* and *pratensis*, while other characters such as breadth, colour and shell thickness (cf. Jueg, 1997) seem to be stable. In other characters, there is geographical variation. For example the presence of an apertural rib (callus) in *pratensis* seems to be more common in northern German specimens than in those from Scandinavia. Apart from the series from Mecklenburg-Vorpommern examined by Jueg (1997) there are no detailed studies on the morphological variation in *pratensis*. Variation also exists within *P. pratensis*, but comparison of Scandinavian and northern German specimens with topotypical material from Dinkelscherben (in the Westerlund Collection in GNM) shows that they are very close.

There is a resemblance in some shell characters between *P. pratensis* and the rare high-alpine *P. alpicola* (Charpentier, 1837; Fig. 11). In fact Clessin (1876) synonymized his var. *pratensis* with *Pupa muscorum* var. *madida* Gredler, 1856, a junior synonym of *P. alpicola*. However, in the second edition of this handbook (Clessin, 1884) and in his *Fauna of Austria, Hungary and Switzerland* (Clessin, 1887), he returned to his original point of view, and regarded *pratensis* as an ecophenotype of *muscorum*. These two species resemble each other mainly in that the shells of both are markedly broad. They differ, however, in other general morphological characters: *P. alpicola*, compared to *P. pratensis*, is shorter and more cylindrical, and narrows abruptly at the apex, which is flatter than in *pratensis*. Also characteristic of *P. alpicola* is the presence of a shallow but distinct gutter-like depression in the external surface of the body whorl, at about one-quarter of the distance from its base. In oblique light it is visible in the aperture as a narrow, delicate ridge. The shell characters for all three species are summarized in Table 3.

Ecology: Ehrmann (1933) characterized var. *pratensis* as a "Standortform sehr feuchter Wiesen" [ecophenotype of very wet meadows], and Jaekel (1962) listed it as a form occurring in "nasse Standorte" [wet habitats], and indeed *P. pratensis*, unlike the predominantly xerophilous *P. muscorum* s. s., is a pronounced hygrophilous species; its association with wetland habitats had already been indicated in the original description (Clessin, 1871). As the species has been ignored, details of its ecological preferences are poorly known. The only detailed study is that of Jueg (1997) from Mecklenburg-Vorpommern, where the species inhabits open, richer, often calcareous moist and wetland habitats (cf. also Zettler *et al.*, 2006). There also exists scattered information from Scandinavia where this

variety has attracted some attention as an ecophenotype found in calcareous fens (Lohmander, 1955, 1956). Throughout Sweden it occurs as a typical species of open calcareous fens or wet, calcareous meadows (Lohmander, 1956; von Proschwitz, 1998). According to Waldén (1965) the lower limit of its pH tolerance is 7.5, when occurring in rich fens in the eastern part of central Sweden. This is an indication of the species' fastidious character, although in other parts of Sweden it is also found on soils with a lower pH. Further examples and descriptions of localities can be found in von Proschwitz (2001, 2002).

Distribution: Due to the fact that *P. pratensis* has been largely overlooked or ignored, our knowledge of its recent distribution is fragmentary. However, the species occurs in Germany and in Scandinavia (Denmark, Norway and Sweden), where it extends to the northernmost parts. Clessin (1887) additionally recorded it from Moravia (Czech Republic). Goldfuss (1883) published an occurrence at Kobyllno in the adjacent Upper Silesia (Poland), and a specimen (Fig. 1B) from this site is present in the Westerlund Collection. Boycott's (1934) observation that *P. muscorum* has very occasionally been found in marshes in Britain indicates possible occurrences of *P. pratensis* in that country as well. A wide, but scattered distribution, mainly in calcareous areas in central, north (and possibly western) Europe is thus to be expected.

Pupilla pratensis has also largely been ignored in the Quaternary literature, although Turner *et al.* (1998) referred to a Post-Glacial record in Switzerland. Evans (1971) reported that in some Weichselian deposits in Britain *P. muscorum* is large, and he figured a specimen that is very similar to *P. pratensis*. He also stated that this large form is associated with hygrophilous species. These facts indicate that revisions of fossil material of *Pupilla* may reveal Quaternary occurrences of *P. pratensis* (see Discussion).

MOLECULAR ANALYSIS

Both COI and CytB sequences clearly separate *Pupilla pratensis* and *P. muscorum* into two distinct, well-supported clades (Figs 2, 3). Bootstrap analysis of the COI sequences gives values of 95 for the *muscorum* clade and 100 for *pratensis*, and the corresponding values for CytB are 99 and 100 (Fig. 2). The bootstrap support for the internal topology of each clade

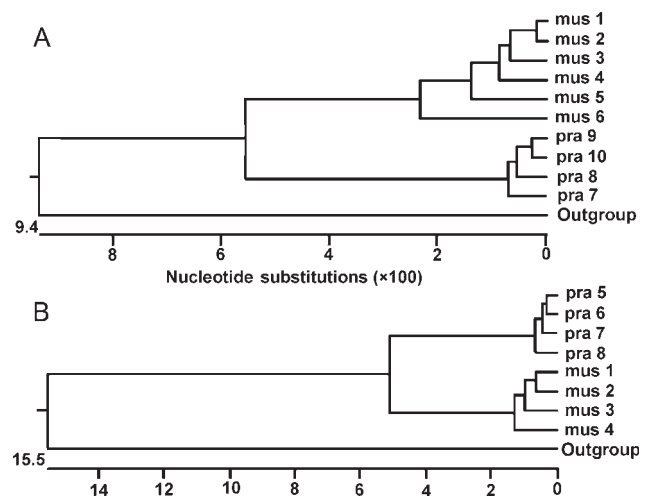


Figure 2. **A.** Neighbour-joining (NJ) tree of all haplotypes of the COI fragment. **B.** NJ tree of all haplotypes of the CytB fragment. Numbers denote haplotype number (Table 2).

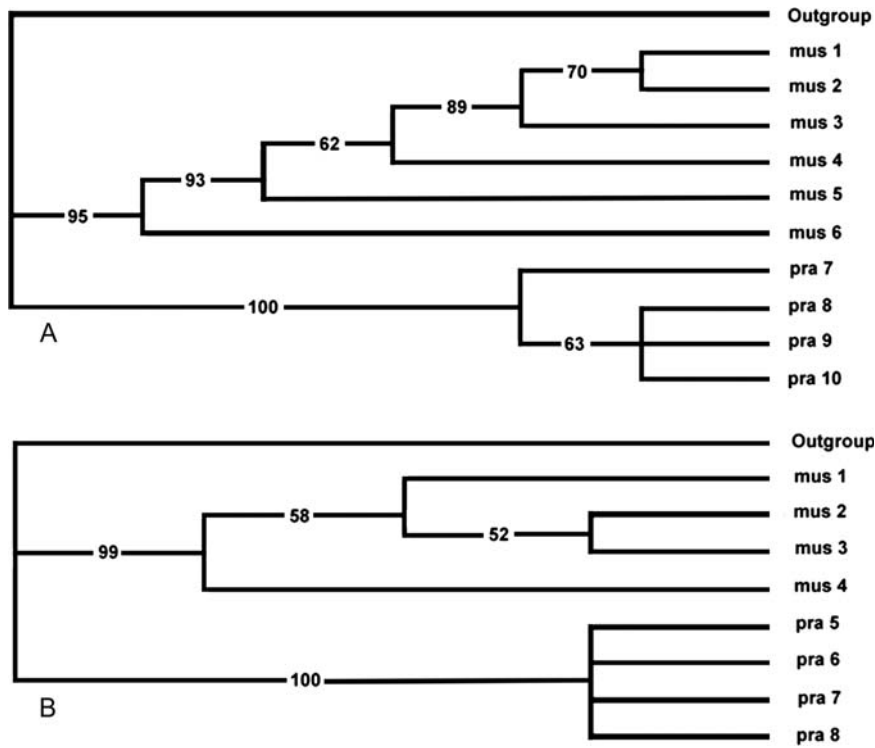


Figure 3. Bootstrap 50% majority-rule consensus trees (1000 replicates) of all haplotypes of *Pupilla muscorum* and *P. pratensis*. **A.** COI fragment. **B.** CytB fragment.

Table 4. Uncorrected *P*-values for the COI haplotypes in the study.

Haplotype	1	2	3	4	5	6	7	8	9	10	11
1	-										
2	0.01376	-									
3	0.00306	0.0107	-								
4	0.01376	0.01835	0.01376	-							
5	0.02599	0.03058	0.02599	0.02446	-						
6	0.04128	0.04587	0.04128	0.03976	0.04587	-					
7	0.0948	0.09633	0.0948	0.09021	0.1055	0.09786	-				
8	0.10092	0.10245	0.10092	0.09633	0.11162	0.10398	0.0107	-			
9	0.10092	0.10245	0.10092	0.09633	0.11162	0.10398	0.00765	0.01223	-		
10	0.09633	0.09786	0.09633	0.09174	0.10703	0.09939	0.00306	0.00765	0.00459	-	
11											-
AY148557	0.15749	0.15749	0.15749	0.15596	0.15902	0.14985	0.17431	0.17431	0.17431	0.17431	-

Note that the values between *Pupilla muscorum* and *P. pratensis* (boxed) are almost an order of magnitude larger than within the species.

is low, indicating that these genes are not useful for population genetics in this group. Using PAUP* to calculate the uncorrected p-value for the COI fragment shows that variation within each species is almost one order of magnitude lower than that between species, indicating that the taxa are well separated (Table 4).

DISCUSSION

The separation of *Pupilla pratensis* from *P. muscorum* is a first step in the revision of this form-rich genus, which is in great need of taxonomic reevaluation. Concerning the European forms, at least *P. triplicata* (Studer, 1820) is certainly a species complex. The relationships among and status of the North American *Pupilla* species and their relationship to their European relatives should be studied further. The uncertain relationships of some Quaternary forms to recent species also need to be clarified. For example, the affinities of *P. pratensis* with the Quaternary subspecies *P. muscorum densegyrata* Lozek, 1954, should be investigated. *Pupilla muscorum densegyrata*, reported from many localities in Central Europe, has been described as a large form that is toothless and has a weak callus (Lozek, 1954, 1964). The specimens illustrated in these papers show close resemblance to *P. pratensis*. Lozek (1954) pointed to the possible affinity of *P. m. densegyrata* with smaller specimens of *P. m. pratensis*, and Lozek (1964) characterized its status as: "Vom alpicola-Kreis kaum zu trennen, jedoch mit muscorum durch Übergangsformen verbunden." [impossible to separate from the *alpicola*-group, however united to *muscorum* by transitional forms]. It seems possible that *P. m. densegyrata*, although rather small, might fall within the range of variation considered to be *P. pratensis*, and hence be a synonym of the latter. There is a great need for revision of *P. m. densegyrata* as well as other Quaternary species of *Pupilla*. It seems probable that, in studies of Quaternary material, specimens of *P. pratensis* have frequently been wrongly attributed to either *P. muscorum* or *Pupilla alpicola* (e.g. Rousseau, 1985, 1987).

In this study we show that using morphology, ecology and DNA sequences ('barcodes') in combination provides strong evidence for the separation of the two species investigated and that these methods complement each other. The philosophy of DNA barcoding has been criticized on various grounds, and obvious problems have been discussed (e.g. Will & Rubinoff, 2004). Schander & Willassen (2005) point out that DNA barcoding and 'traditional' methods are not mutually exclusive, but should be used as complements of each other. We also believe that much of the criticism of DNA barcoding is based on confusion with the ideas of DNA-taxonomy (Tautz *et al.*, 2003). We are highly sceptical of the DNA taxonomy approach and see DNA barcoding as one of many tools useful in species identification, not as a fundamental criterion upon which we should base taxonomic decisions.

The COI gene fragment has been suggested for use as a universal DNA barcode for species identification, and for uncovering hidden biodiversity (Hebert *et al.*, 2003). It has recently been applied in several taxonomic and biodiversity studies of various groups (e.g. Meyer & Paulay, 2005; Hajibabael *et al.*, 2006). A recent study of the marine bivalve genus *Acesta* H. & A. Adams (Järnegren *et al.*, 2007) showed that additional mitochondrial genes may also prove useful in separating closely related species, as confirmed in the present study.

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