# INTRASPECIFIC MORPHOLOGICAL AND GENETIC VARIABILITY IN *RADIX BALTHICA* (LINNAEUS 1758) (GASTROPODA: BASOMMATOPHORA: LYMNAEIDAE) WITH MOPHOLOGICAL COMPARISON TO OTHER EUROPEAN *RADIX* SPECIES

KATRIN SCHNIEBS[1], PETER GLÖER[2], MAXIM V. VINARSKI[3] & ANNA K. HUNDSDOERFER[1]

[1]Senckenberg Natural History Collections Dresden, Museum of Zoology, Königsbrücker Landstraße 159, D-01109 Dresden, Germany
[2]Biodiversity Research Laboratory, Schulstraße 3, D-25491 Hetlingen, Germany
[3]Museum of Siberian Aquatic Molluscs, Omsk State Pedagogical University, Tukhachevskogo Emb. 14, 6440099 Omsk, Russian Federation

*Abstract* Radix balthica *is a morphologically very variable species that is often very difficult to determine on the basis of the shell characters. Since DNA-taxonomy is still expensive and requires much more time than anatomical determination, and the methodology is hardly available for most colleagues that work faunistically, this work aims to broaden knowledge of the intraspecific variability in the more important systematic characters used for determination of* Radix balthica. *To find reliable distinguishing characters from other* Radix*-species, an integrative approach was sought and data from three different sources were acquired for analysis. Molecular sequence data of the gene fragments ITS-2 and/or cyt–b was obtained for 58 individuals in order to study the species identity of* Radix balthica *(Linnaeus 1758). Of the subsample of 24 individuals for which both gene fragments were available, the variability of several characters that are commonly used for determination was documented. These include shell morphology, mantle pigmentation, shape and position of the bursa copulatrix, length and position of the bursa duct, and length ratio of praeputium to penis sheath. Morphological distinguishing characters from* R. auricularia, R. labiata, R. lagotis *and* R. ampla *are discussed and summarised in a table. Analysis of the network of haplotypes (cyt–b) shows that there is no distinct correlation to the geographic distribution pattern.*

*Key words* Radix balthica, R. auricularia, R. labiata, R. lagotis, R. ampla*, morphology, molecular genetics, variation*

## INTRODUCTION

The common pond snail *Radix balthica* (Linnaeus 1758) is a Palaearctic species widely distributed from Iceland (Mandahl-Barth, 1938) and Norway (Økland, 1990) in the north, Ireland and Great Britain (Kerney, 1999) as well as Spain in the west, up to Southern Siberia in the east (Kruglov, 2005), and N-Africa in the south (Brown, 1994; van Damme 1984).

This euryoecious species prefers low-altitude running and standing freshwater bodies such as lakes, ponds, drainage ditches and lentic zones of rivers, rich in nutrients and submerged vegetation (Glöer & Diercking, 2010). In the brackish water areas of the Baltic Sea it can tolerate a salinity of 10–15 psu (Zettler *et al*., 2006).

Reliable identification of this species is not only important for malacologists working on faunistics. The exact determination of *Radix* species is important because some act as vectors of human fascioliasis (e.g. Bargues & Mas-Coma, 2005). On the base of his laboratory studies, Kruglov (2005) reported *R. balthica* as a possible vector of fascioliasis. Under artificial conditions the species is susceptible to *Fasciola hepatica* invasion. It is also essential to have accurate identification when using them in climate modelling predictions (Cordellier & Pfenninger, 2009).

The shells of *Radix* species show an enormous variation in Europe that may be influenced by ecological (Pfenninger *et al*., 2006) and other conditions. For instance, the growth of *Radix* shells (including *R. balthica*) can increase by 10% (Ward *et al*., 1997) through infection of the snail by trematodes. Lakowitz *et al.* (2008) identified a predator-induced phenotypic plasticity in *R. balthica* shells. These facts make the traditional determination based only on shell morphology very difficult. Also Pfenninger *et al.* (2006) concluded that taxonomic distinction of species in

Contact author : katrin.schniebs@senckenberg.de

this genus cannot be based on shell morphology and proposed DNA-taxonomy for species identification.

In the past years it has become common practice for determination to take additional anatomical characters into account, as well as mantle pigmentation (e.g. Jackiewicz, 1993, 1998, 2000; Gittenberger *et al*., 1998; Glöer, 2002; Glöer & Meier-Brook, 2003; Stadnichenko, 2004; Kruglov, 2005). Nevertheless, determination often remained unreliable, since the variability of each of the characters had not been studied and documented sufficiently. Furthermore, the different species concepts of authors can lead to difficulties and confusion.

To analyse the variability of the most important distinguishing characters of *Radix baltica* (shell morphology, mantle pigmentation, shape and position of the bursa copulatrix, length and position of the bursa duct, and length ratio of praeputium to penis sheath), 58 individuals of several European localities, as well as from Kazakhstan and Siberia were studied anatomically. Additionally, we attempted to obtain sequence data of the complete nuclear ITS-2 spacer and a 370 bp fragment of the cyt–b gene as mitochondrial markers for these individuals. The variability of several characters that are commonly used for determination was documented in the 24 individuals for which both gene fragments were available.

## MATERIAL AND METHODS

Since anatomical material that could be used for genetic analyses of the type specimen of *Radix balthica* is not available, our investigations are based on "topotypes" from Øland, which lies geographically close to the original type locality i.e. the beach of Gotland. Since the neotype of *R. balthica* defined by Kruglov & Starobogatov (1983) was collected in the vicinity of Stockholm, the validity of that can be questioned because of its relative distance from the type locality (ICZN 75.3.6). All Swedish *R. balthica* used in the present study was collected in Øland (Fig. 8. 1, 2). Nevertheless, the shell is almost identical with that of the neotype of Kruglov & Starobogatov 1983 and the anatomy of specimens from Øland is identical with that of *R. balthica* sensu Kruglov & Starobogatov.

For outgroup comparison in the molecular genetic analyses we used Palaearctic specimens of the species *Planorbarius corneus* (Linnaeus 1758) and *Aplexa hypnorum* (Linnaeus 1758). As the ingroup we used *Lymnaea stagnalis* (Linnaeus 1758), *Radix labiata* (Rossmässler 1835), *R. ampla* (Hartmann 1821), *R. auricularia* (Linnaeus 1758) and *R. lagotis* (Schrank 1803).

*Morphology* Snails were fixed in 70–80% ethanol. Shell morphology, mantle pigmentation and anatomy were documented from the specimens studied. The dissections and measurements of the genital organs and shells were carried out using stereo microscopes (Zeiss and Olympus). Photographs were taken using a digital camera system (Leica R8).

All specimens used for molecular examination are listed in Table 1. They were collected or donated for this study and stored in the Molluscan Collection of the Senckenberg Natural History Collections Dresden, Museum of Zoology (SNSD) (see under http://sesam.senckenberg.de/ for information additional to that in Table 1).

*Molecular techniques – DNA extraction* Tissue samples were taken under a microscope from the soles of the snails and fixed in 100% ethanol. The samples were registered in the tissue collection of the SNSD with a new collection number (additional to the collection number of the specimen in the molluscan collection) of SNSD and stored at – 80°C.

DNA was extracted using DTAB (dodecyl trimethyl ammonium bromide) buffer (Gustincich *et al*., 1991). The tissue samples were washed with 100 µl TE (Tris-EDTA) buffer and subsequently incubated with 500 µl of preheated DTAB for 30 min at 65°C. The incubation was continued after adding 10 µl Proteinase K (50mg/ml) for 20–24 hours, followed by a short incubation with 10 µl RNase (10 mg/ml) for 30 min at 37°C. Remaining tissue fragments disintegrated after vortexing. For cleaning, 550 µl chloroform/isoamyl alcohol (24:1) was used. The samples were vortexed for 20 s and the phases subsequently separated again at 12,000 g for 3 min. With the upper aqueous phase the procedure was repeated. 100 µl 4M LiCl and 400 µl isopropanol were added to the aqueous phase for precipitation. The samples were cooled at –20°C for 30 min and subsequently the DNA was pelleted

**Table 1** Material used in the molecular genetic studies.

| Code | Collection No. SNSD | Locality | Genbank No. CytB | ITS-2 |
|---|---|---|---|---|
| *Planorbarius corneus* (Linnaeus, 1758) | | | | |
| Linz-1 | Moll 52556 | Germany, Saxony, Linz, pond Goldgrubenteich, 13°43′09″E 51°19′45″N | ENA FR797880 | ENA FR797830 |
| Linz-2 | Moll 52557 | Germany, Saxony, Linz, pond Goldgrubenteich, 13°43′09″E 51°19′45″N | ENA FR797881 | ENA FR797831 |
| *Aplexa hypnorum* (Linnaeus, 1758) | | | | |
| Ne-1 | Moll S348 | Germany, Mecklenburg-Vorpommern, lake Nebel, 12°42′02″E 53°15′32″N | ENA FR797882 | ENA FR797832 |
| Ne-2 | Moll S350 | Germany, Mecklenburg-Vorpommern, lake Nebel, 12°42′02″E 53°15′32″N | ENA FR797883 | ENA FR797833 |
| *Lymnaea stagnalis* (Linnaeus, 1758) | | | | |
| Kon-1 | Moll 53108 | Germany, Baden-Württemberg, Konstanz-Egg, ditch Hockgraben, 9°11′34.2″E 47°40′57.3″N | ENA FR797894 | ENA FR797834 |
| Kon-2 | Moll 53109 | Germany, Baden-Württemberg, Konstanz-Egg, ditch Hockgraben, 9°11′34.2″E 47°40′57.3″ N | ENA FR797895 | ENA FR797835 |
| DD | Moll 49239 | Germany, Saxony, Dresden-Zschieren, old branch of river Elbe, 13°52′28″E 50°59′50″N | ENA HE573102 | ENA HE573064 |
| NS | Moll 49835 | Germany, Saxony, Niederspree, small pond, 14°54′03″E 51°24′28″N | ENA HE573103 | ENA HE573065 |
| *Radix auricularia*(Linnaeus, 1758) | | | | |
| Wei-1 | Moll 53071 | Germany, Bavaria, Weichering near Ingolstadt, pond in the riverside forest of Danube 11°19′23.6″E 48°43′34.1″N | ENA FR797903 | ENA FR797843 |
| Wei-2 | Moll 53072 | Germany, Bavaria, Weichering near Ingolstadt, pond in the riverside forest of Danube 11°19′23.6″E 48°43′34.1″N | ENA FR797904 | ENA FR797844 |
| Nie | Moll 50005 | Germany, Saxony, Niederspree, pond Neuwiesenteich, 14°52′57″E 51°24′19″ | ENA HE573104 | ENA HE573066 |
| Frei | Moll 50079 | Germany, Saxony, pond Vierteich near Freitelsdorf, 13°41′57″E 51°15′43″ | ENA HE573105 | ENA HE573067 |
| *Radix labiata* (Rossmässler, 1835) | | | | |
| Lan-1 | Moll 51275 | Germany, Saxony, pond near Langenberg, 12°51′21″E 50°33′09″N | ENA HE573106 | ENA HE573068 |
| Lan-2 | Moll 51276 | Germany, Saxony, pond near Langenberg, 12°51′21″E 50°33′09″N | ENA HE573107 | ENA HE573069 |
| Wach-1 | Moll 51697 | Germany, Brandenburg, small lake near Wachow, 12°43′05″E 52°32′05″N | ENA HE573108 | ENA HE573070 |
| Wach-2 | Moll 51698 | Germany, Brandenburg, small lake near Wachow, 12°43′05″E 52°32′05″N | ENA HE573109 | ENA HE573071 |
| *Radix ampla* (Hartmann, 1821) | | | | |
| Am-1 | Moll 53098 | Germany, Bavaria, lake Ammersee, Stegen, 11°08′07″E 48°04′32″N | ENA HE573110 | ENA HE573072 |

| Code | Collection No. SNSD | Locality | Genbank No. CytB | ITS-2 |
|---|---|---|---|---|
| Am-2 | Moll 53099 | Germany, Bavaria, lake Ammersee, Stegen, 11°08′07″E 48°04′32″N | ENA HE573111 | ENA HE573073 |
| Lueb | Moll S2193 | Mecklenburg-Vorpommern, lake Luebkowsee 2 km east of Schwichtenberg, 13°44.567′E 53°40.967′N | ENA HE573112 | ENA HE573074 |
| *Radix lagotis* (Schrank, 1803) | | | | |
| AJ319638 | | Czech Republic, Kadov, Vasi and Podkadovsky pond | – | GenBank AJ319638 |
| AJ319639 | | Austria, Schoenau, southeast of Vienna | – | GenBank AJ319639 |
| Doe | Moll 53239 | Saxony, dam Doellnitzsee near Mutzschen, 12°55′18″E 51°15′45″N | ENA HE573113 | ENA HE573075 |
| Fr | Moll 49868 | Saxony, pond Vierteich near Freitelsdorf, 13°41′59″E 51°15′39″N | ENA HE573114 | ENA HE573076 |
| Linz | Moll 52563 | Saxony, pond Goldgrubenteich near Linz, 13°43′09″E 51°19′46″N | ENA HE573115 | ENA HE573077 |
| *Radix balthica* (Linnaeus, 1758) | | | | |
| Kon-1 | Moll 53111 | Germany, Baden-Württemberg, Konstanz-Egg, pond near University, 09°11′29″E 47°41′09″ N | ENA HE573116 | ENA HE573078 |
| Kon-2 | Moll 53112 | Germany, Baden-Württemberg, Konstanz-Egg, pond near University, 09°11′29″E 47°41′09″ N | ENA HE573117 | – |
| DD-1 | Moll 51833 | Germany, Saxony, Dresden-Kleizschachwitz, river Elbe, 13°52′21″E 51°00′03″N | ENA HE573118 | – |
| DD-2 | Moll 51834 | Germany, Saxony, Dresden-Kleizschachwitz, river Elbe, 13°52′21″E 51°00′03″N | ENA HE573119 | ENA HE573079 |
| Sig-1 | Moll 52663 | Germany, Baden-Württemberg, river Danube near Sigmaringendorf, 09°15′49.36″E 48°03′45.54″ N | ENA HE573120 | ENA HE573080 |
| Sig-2 | Moll 52665 | Germany, Baden-Württemberg, river Danube near Sigmaringendorf, 09°15′49.36″E 48°03′45.54″ N | ENA HE573121 | – |
| Wald | Moll 52685 | Germany, Baden-Württemberg, Waldbeuren, small creek in a meadow, 09°21′02.39″E 47°54′34.32″ N | ENA HE573122 | – |
| Phil-1 | Moll S2202 | Germany, Baden-Württemberg, Philippsburg, 49°12′16.39″ N ditch Geißböckelgraben, 08°27′06.12″E | ENA HE573123 | – |
| Phil-2 | Moll S2203 | Germany, Baden-Württemberg, Philippsburg, ditch Geißböckelgraben, 08°27′06.12″E 49°12′16.39″ N | ENA HE573124 | – |
| Ihl | Moll S2198 | Germany, Schleswig-Holstein, lake Ihlsee North of Bad Segeberg, 10°17.533′E 53°57.633′N | ENA HE573125 | – |
| Zsch-1 | Moll S369 | Germany, Saxony, Dresden, Zschoner Grund, pond Mühlteich, 13°38′26″E 51°03′30″E | ENA HE573126 | – |

| Code | Collection No. SNSD | Locality | Genbank No. CytB | ITS-2 |
|---|---|---|---|---|
| Zsch-2 | Moll S370 | Germany, Saxony, Dresden, Zschoner Grund pond Mühlteich, 13°38′26″E 51°03′30″E | ENA HE573127 | – |
| Zsch-3 | Moll S371 | Germany, Saxony, Dresden, Zschoner Grund pond Mühlteich, 13°38′26″E 51°03′30″E | ENA HE573128 | – |
| Zsch-4 | Moll S374 | Germany, Saxony, Dresden, Zschoner Grund pond Mühlteich, 13°38′26″E 51°03′30″E | ENA HE573129 | – |
| Th-1 | Moll S133 | France, Region Centre, Thenay, small creek 01°17′31″E 47°23′22″N | ENA HE573130 | – |
| Th-2 | Moll S135 | France, Region Centre, Thenay, small creek 01°17′31″E 47°23′22″N | ENA HE573131 | – |
| Lie-1 | Moll 51282 | Switzerland, canton Basel-Landschaft, Liestal, Orishof, 07°43′03″E 47°28′22″N | ENA HE573132 | ENA HE573081 |
| Lie-2 | Moll 51283 | Switzerland, canton Basel-Landschaft, Liestal, Orishof, 07°43′03″E 47°28′22″N | ENA HE573133 | ENA HE573082 |
| Rie-1 | Moll 51292 | Switzerland, canton Basel City, Riehen, Wiesengriener, 07°38′32″E 47°35′21″N | ENA HE573134 | ENA HE573083 |
| Rie-2 | Moll 51293 | Switzerland, canton Basel City, Riehen, Wiesengriener, 07°38′32″E 47°35′21″N | ENA HE573135 | ENA HE573084 |
| Mu-1 | Moll 52736 | Germany, Saxony, Mutzschen, river Mutzschener Wasser, 12°53′24″E 51°15′32″N | ENA HE573136 | ENA HE573085 |
| Mu-2 | Moll 52737 | Germany, Saxony, Mutzschen, river Mutzschener Wasser, 12°53′24″E 51°15′32″N | ENA HE573137 | ENA HE573086 |
| Ko-1 | Moll 51118 | Kazakhstan, Akmolinsk region, lake Kopa near Kokshetau, 69°22′14″E 53°17′22″N | – | ENA HE573087 |
| Ko-2 | Moll 51119 | Kazakhstan, Akmolinsk region, lake Kopa near Kokshetau, 69°22′14″E 53°17′22″N | ENA HE573138 | ENA HE573088 |
| Mi-1 | Moll 52412 | Croatia, lake Milanovac near Plitvica, 15°36′34″E 44°53′45″N | ENA HE573139 | ENA HE573089 |
| Mi-2 | Moll 52411 | Croatia, lake Milanovac near Plitvica, 15°36′34″E 44°53′45″N | ENA HE573140 | – |
| Oe | Moll 51860 | Sweden, Øland, east shore near Lille Seby, 16.565°E 56.345°N | | ENA HE573141 |
| Hi-1 | Moll 51894 | Germany, North Rhine-Westphalia, Hillegossen, creek Meyerbach, 08°36′04″E 51°59′11″N | ENA HE573142 | ENA HE573091 |
| Hi-2 | Moll 51895 | Germany, North Rhine-Westphalia, Hillegossen, creek Meyerbach, 08°36′04″E 51°59′11″N | ENA HE573143 | – |
| Wil | Moll 51865 | Germany, North Rhine-Westphalia, Wilkenhoeher, clay pit, 08°34′15.87″E 52°05′42.59″N | ENA HE573144 | – |

| Code | Collection No. SNSD | Locality | Genbank No. CytB | ITS-2 |
|---|---|---|---|---|
| Del-1 | Moll 51897 | Germany, North Rhine-Westphalia, Joellenbeck, Deliussiek, small pond, 08°31′22.16″E 52°05′20.80″N | ENA HE573145 | – |
| Del-2 | Moll 51898 | Germany, North Rhine-Westphalia, Joellenbeck, Deliussiek, small pond, 08°31′22.16″E 52°05′20.80″N | ENA HE573146 | – |
| Bu | Moll 53279 | Germany, Brandenburg, river Havel near Buetzer, 12.3051°E 52.5375°N | ENA HE573147 | ENA HE573092 |
| Kau | Moll 51024 | Germany, Saxony, creek Geberbach near dam Kauscha, 13°46′42″E 50°59′26″N | ENA HE573148 | ENA HE573093 |
| Am-1 | Moll 53105 | Germany, Bavaria, river Amper near lake Ammersee, 11°07′43″E 48°04′43″N | | ENA HE573149 |
| Am-2 | Moll 53106 | Germany, Bavaria, river Amper near lake Ammersee, 11°07′43″E 48°04′43″N | | ENA HE573150 |
| Am-3 | Moll 53107 | Germany, Bavaria, river Amper near lake Ammersee, 11°07′43″E 48°04′43″N | | ENA HE573151 |
| Schw-1 | Moll 52467 | Germany, Bavaria, Schwillach-Quelle between Hörlkofen and Erding, 11°55′E 48°15′N | ENA HE573152 | – |
| Schw-2 | Moll 52468 | Germany, Bavaria, Schwillach-Quelle between Hörlkofen and Erding, 11°55′E 48°15′N | ENA HE573153 | – |
| Toll | Moll 53293 | Germany, Mecklenburg-Vorpommern, lake Tollensesee near Klein Nemerow, 13.2146° E 53.4909°N | ENA HE573154 | – |
| Flee | Moll S334 | Germany, Mecklenburg-Vorpommern, Oberbek south of Fleeth, 12°50′59.76″E 53°12′42.41″N | ENA HE573155 | – |
| Rue-1 | Moll S1299 | Germany, Saxony, Ruesdorf, ditch, 12°40′56″E 50°46′59″N | ENA HE573156 | – |
| Rue-2 | Moll S1300 | Germany, Saxony, Ruesdorf, ditch, 12°40′56″E 50°46′59″N | ENA HE573157 | – |
| Tor-1 | Moll S2150 | Germany, Mecklenburg-Vorpommern, lake Torgelower See, 12°46.622′E 53°34.252′N | ENA HE573158 | ENA HE573095 |
| Tor-2 | Moll S2151 | Germany, Mecklenburg-Vorpommern, lake Torgelower See, 12°46.622′E 53°34.252′N | ENA HE573159 | ENA HE573096 |
| Tie-1 | Moll S2174 | Germany, Mecklenburg-Vorpommern, lake Tiefwarensee, 12°41.258′E 53°32.332′N | ENA HE573160 | ENA HE573097 |
| Tie-2 | Moll S2175 | Germany, Mecklenburg-Vorpommern, lake Tiefwarensee, 12°41.258′E 53°32.332′N | ENA HE573161 | ENA HE573098 |
| Ma | Moll S1743 | Spain, Mallorca, Tramuntana Mountains, La Granja Manor near Esporles, spring streams, 02°33′33″E 39°40′08″N | ENA HE573162 | ENA HE573099 |

| Code | Collection No. SNSD | Locality | Genbank No. CytB | ITS-2 |
|---|---|---|---|---|
| Gre-1 | Moll S2099 | Germany, Saxony, nature reserve "Alte See Grethen", muddy ditch, 12°40'19"E 51°13'44"N | ENA HE573163 | ENA HE573100 |
| Gre-2 | Moll S2100 | Germany, Saxony, nature reserve "Alte See Grethen", muddy ditch, 12°40'19"E 51°13'44"N | ENA HE573164 | – |
| HH-1 | Moll S965 | Germany, Hamburg, Ruschort, temporary ditch, 10°03'32.27"E 53°30'04.46"N | ENA HE573165 | – |
| HH-2 | Moll S966 | Germany, Hamburg, Ruschort, temporary ditch, 10°03'32.27"E 53°30'04.46"N | ENA HE573166 | – |
| Mer-1 | Moll S144 | France, Mérignac near Bordeaux, 01°10'04.79"E 45°47'20.96"N | ENA HE573167 | – |
| Mer-2 | Moll S147 | France, Mérignac near Bordeaux, 01°10'04.79"E 45°47'20.96"N | ENA HE573168 | – |
| Gro | Moll 48597 | Germany, Saxony, Großbardau, creek Springbach, 12°40'08"E 51°12'32"N | ENA HE573169 | – |
| Moos-1 | Moll S251 | Switzerland, Basel, Mooswäldchen, 07°39'43"E 47°34'55"N | ENA HE573170 | – |
| Moos-2 | Moll S252 | Switzerland, Basel, Mooswäldchen, 07°39'43"E 47°34'55"N | ENA HE573171 | – |
| Mot | Moll 51139 | Russia, lake Motshishtshe near Tomsk, 84.91333°E 56.219496°N | ENA HE573172 | – |

by centrifugation at 11,200 g for 20 min at 4°C. The liquid was disposed of and the pellets were dried by inverting the tubes on a paper towel. The pellets were cleaned twice with 200 µl ice-cold 70% ethanol. The DNA pellets were dried 10 min at 50°C and subsequently redissolved in 50 µl of TE buffer.

*Molecular techniques – Polymerase Chain Reaction (PCR) and purification of PCR products* The PCRs were carried out in a final volume of 20 µl with quantities of DNA from 0.5–5.0 µl depending on the concentration estimated by gel electrophoresis, 2 µl 10× PCR buffer (Bioron, incomplete), 1 µl $MgCl_2$ (Bioron, 0.055 µS/cm), 1 µl of each primer (10 pmol/µl), 0.5 µl dNTP (10 mM), 0.2 µl Taq DNA polymerase (DFS-Taq, Bioron) and the corresponding volume of sterile water.

From the cyt–b gene a region of about 370 bp was amplified with the primers UCyt*b*151F and UCyt*b*270R (Merritt *et al*., 1998) and a temperature profile of 94°C 4 min (94°C 40 s, 48°C 40 s, 72°C 1.15 min)×40, 72°C 6 min, 8°C hold.

The primers used for ITS-2 were LT1 (Bargues *et al*., 2001) and ITS2-Rixo (Almeyda-Artigas *et al*., 2000). The temperature profile used was the following: 94°C 4 min (94°C 30 s, 50°C 30 s, 72°C 1 min)×40, 72°C 7 min, 8°C hold.

PCR products were purified with 0.1 µl Exo Sap-It plus 4 µl double-distilled water and incubation for 30 min at 37°C, and deactivation for 15 min at 80°C.

*Molecular techniques – DNA sequencing* The primers used for the cycle sequencing were UCyt*b*151F for cyt–b and LT1 for ITS-2. Samples were sequenced in both directions if necessary. The quantity of PCR product used for cycle-sequencing ranged from 0.5–5.0 µl depending on the concentration estimated by gel electrophoresis. The following were added were added to make a final volume of 10 µl in sterile water: 0.5 µl BigDye T-Mix (ABI, Applied Biosystems); 2.25 µl BigDye buffer (5x); 0.5 µl primer (10 pmol). The following temperature profile was used: (96°C 10 s, 50°C 5 s, 60°C 4 min)×25, 8°C hold. The products were purified by adding

1 µl 3M NaAc (pH 4.6) and 25 µl EtOH (100%), centrifuging at 13 000 g for 15 min., inverting the tubes on a paper towel and washing with 200 µl 70% EtOH. After removing the EtOH the pellets were dried for 10 min at 50°C. Samples were sequenced on an ABI 3130 xl (Applied Biosystems).

*Molecular techniques – sequence alignments* Alignment was performed by eye using BioEdit Sequence Alignment Editor (Hall, 1999). It was demanding for ITS-2 sequences, which is why we repeated it three times independently. Since the results were the same in all three trials, we accepted the alignment for analyses.

*Phylogenetic analyses of sequences* For maximum-likelihood analyses, including bootstrap support, we used raxmlGUI 0.9 beta 2 (RAxML) (Silvestro & Michalak, 2010; Stamatakis *et al*., 2005). The settings were "ML+thorough bootstrap" with 100 (replicate) runs and 1000 (bootstrap) repetitions.

Maximum-parsimony (MP) trees were reconstructed using PAUP (version 4.0b10; Swofford, 2002; settings: gapmode=NewState, addseq=closest, maxtree=10000). For presentation of the MP results, one of the best trees was chosen to be able to illustrate branch lengths (one showing the same overall topology as the majority rule consensus tree was chosen).

Genetic distances of the cyt–b were calculated using MEGA version 4 (Tamura *et al*., 2007). This program was also used to produce the dataset of cyt–b sequences without missing data for network analyses (median joining; Bandelt *et al*., 1999) with the program "Network" (www.fluxus-engeneering.com). This dataset contained 308 bp of 58 individuals.

## RESULTS

### Molecular genetics

*Distance analyses* Genetic distances from pairwise comparisons of cyt–b sequences (fragment of about 370 bp) are shown in Table 2. Differences between species of different families (outgroup comparison) ranged between 31.2% and 25.8%. Distances between *Lymnaea stagnalis* and the *Radix* species ranged from 26.0% to 20.3%. Among the five *Radix* species analysed, the highest values are between *R. auricularia* and the other species (between 19.2% and 15.6%). Lower values (from 13.5% to 9.0%) are between *R. labiata*, *R. balthica*, *R. ampla* and *R. lagotis*.

*Molecular phylogeny* The maximum-parsimony (MP) tree of the cyt–b sequences is illustrated in Fig. 1 (tree length = 380, consistency index = 0.7474, retention index = 0.9091). Although basal branches have less than 80% bootstrap support, the clades of the species themselves have high, often full support. The result of the RAxML (not shown) calculation is very similar. In both trees *L. stagnalis* groups are sister to the *Radix*-species. Although a branching pattern between the *Radix*-species is proposed in both trees, very low support speaks in favour of interpretation as a polytomy. With both methods, the specimens of *R. balthica* form a distinct clade that is well separated from those of the other *Radix* species.

The maximum-parsimony (MP) tree of the nuclear marker ITS-2 (tree length = 1414, consistency index = 0.7560, retention index = 0.9290) (Fig. 2) is well-supported within the Lymnaeidae (*Lymnaea* and *Radix*). It shows *R. auricularia* as a well-separated sister group to the other *Radix*-species analysed. Within these, *R. labiata* forms a

**Table 2** Evolutionary distances of the cyt–b gene fragment (about 370 bp) calculated using MEGA version 4 (Tamura *et al*., 2007).

| | *P. corneus* | *A. hypnorum* | *L. stagnalis* | *R. auricularia* | *R. labiata* | *R. ampla* | *R. balthica* | *R. lagotis* |
|---|---|---|---|---|---|---|---|---|
| *Planorbarius corneus* | – | – | – | – | – | – | – | – |
| *Aplexa hypnorum* | 0.312 | – | – | – | – | – | – | – |
| *Lymnaea stagnalis* | 0.282 | 0.290 | – | – | – | – | – | – |
| *Radix auricularia* | 0.303 | 0.268 | 0.260 | – | – | – | – | – |
| *Radix labiata* | 0.285 | 0.265 | 0.236 | 0.177 | – | – | – | – |
| *Radix ampla* | 0.285 | 0.260 | 0.213 | 0.192 | 0.104 | – | – | – |
| *Radix balthica* | 0.310 | 0.258 | 0.224 | 0.156 | 0.135 | 0.120 | – | – |
| *Radix lagotis* | 0.280 | 0.262 | 0.203 | 0.163 | 0.129 | 0.092 | 0.090 | – |

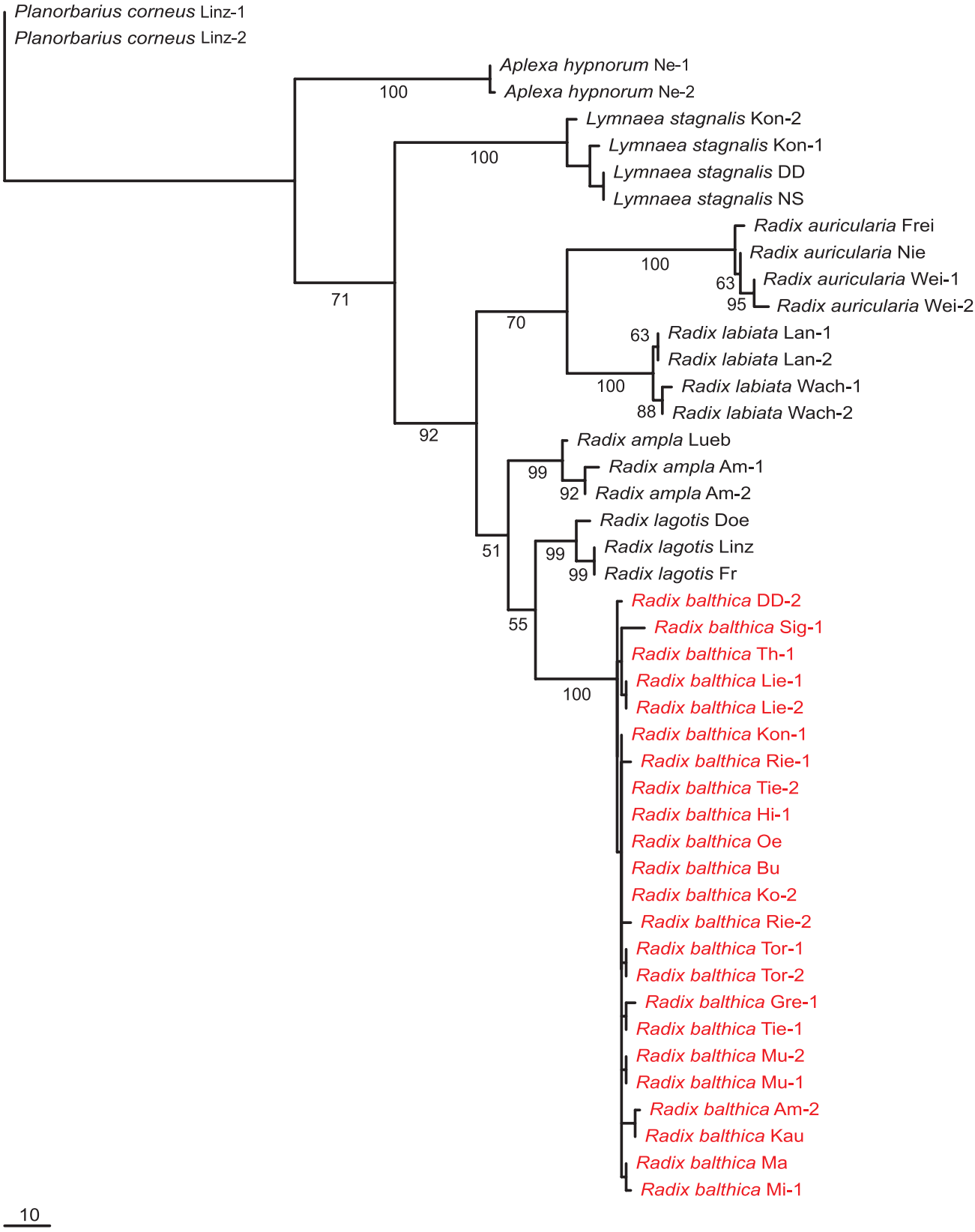


**Figure 1** Hypothesis for the phylogenetic relationships of *R. baltica* based on one of the 10000 best maximum-parsimony trees of the sequenced fragment of the mitochondrial marker cyt–b (about 370 bp; tree length = 380, consistency index = 0.7474, retention index = 0.9091). Branch lengths are proportional to the number of substitutions and the overall topology corresponds to that of the strict consensus tree. Bootstrap support values above 50% are reported below nodes.

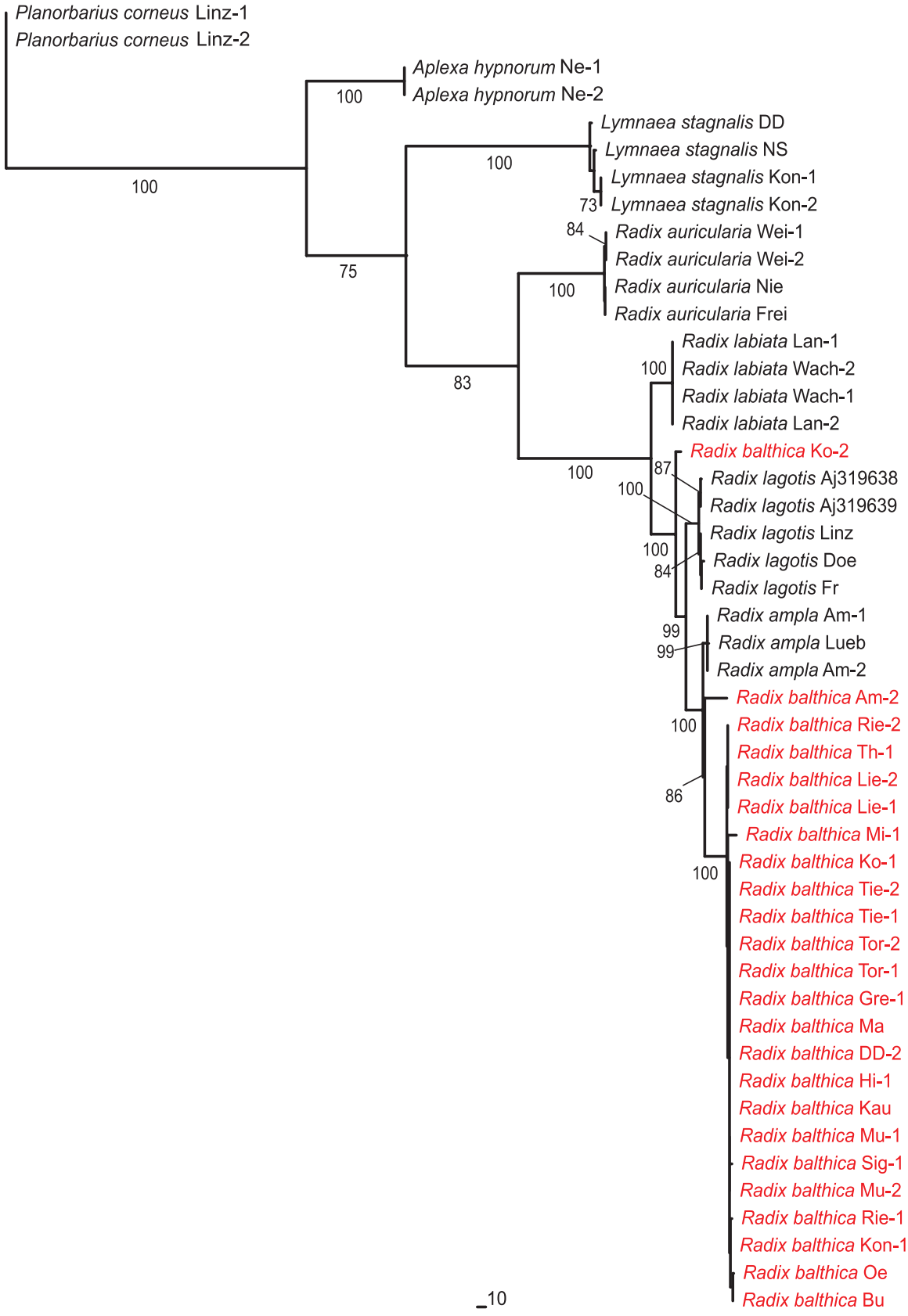


**Figure 2** Hypothesis for the phylogenetic relationships of *R. baltica* based on one of the best 118 maximum-parsimony trees of the nuclear marker ITS-2 (tree length = 1414, consistency index = 0.7560, retention index = 0.9290). Branch lengths are proportional to the number of substitutions and the overall topology corresponds to that of the strict consensus tree. Bootstrap support values above 50% are reported below nodes.

distinct sister group to *R. lagotis*, *R. ampla* and *R. balthica*, this sister group relation has full bootstrap support. *Radix lagotis* forms a distinct sister group to *R. ampla* plus *R. balthica* (nearly full support). The Genbank ITS-2-sequences for *R. lagotis* specimens published by Bargues *et al*. (2001) are used in this analysis to show that the species definition of *R. lagotis* is the same as in that study. In contrast to the two trees of the mitochondrial marker, not all individuals of *R. balthica* fall into one distinct cluster. Two specimens (*R. balthica* Am-1 and *R. balthica* Ko-2) lie clearly outside of the main *R. balthica* cluster. While they each form a separate branch within *Radix* in the MP tree they form a poorly supported clade in the RAxML tree (not shown), which forms the sister group to *R. ampla*. A further difference of the RAxML tree (not shown), to the MP tree (Fig. 2) is that there is hardly any differentiation between *R. balthica* and *R. lagotis*. In both reconstructions these two species, together with *R. ampla*, form the sister group to *R. labiata*. *Radix auricularia* forms a clearly differentiated sister group to these four species in both reconstructions.

*Network analyses* The network representation of *R. balthica* mitochondrial sequences (cyt–b, about 370 bp; Fig. 3) shows hardly reticulated differentiation of up to about 13 mutations between individuals. The greatest differentiation can be observed between individuals from geographically close localities, for example within the federal state of Baden-Württemberg in Germany (light yellow). The same haplotype can occur in individuals from localities situated far from one another, for example in 14 individuals from different federal states of Germany, from Kazakhstan and from the island of Øland in Sweden. Sequences of specimens from Saxony in Germany, from France, Switzerland and Siberia (Russia) only differ by one substitution each.

## Morphology

*Shell* (Fig. 4) The shells of examined specimens vary from conical egg-shaped to egg-shaped to nearly spherical and ear-shaped. They vary from thin-walled and fragile to solid and are of a light horn to reddish-brown in colour. The height of the shells ranges from 9.9 to 28.0 mm and the number of whorls between 3 and 4.

*Mantle pigmentation* (Fig. 5) Mantle pigmentation of the sequenced specimens of *R. balthica* shows a broad polymorphism. It is however possible to distinguish three types:

- mantle and mantle collar of a deep blue-black with a bluish grey mantle edge (Fig. 5: 8)
- mantle grey-black with roundish patches of lighter grey-black mantle collar bluish grey with numerous irregular patches of black; mantle edge light (Fig. 5: 7)
- mantle black, grey-black, or grey-yellow with few or numerous roundish distinct spots of white or rarely grey-green or grey-yellow, that are very variable in size; mantle collar white or bluish white with numerous irregular small patches of black; mantle edge white or rarely yellowish (Fig. 5.1–6, 9–12).

The third type of mantle pigmentation occurs most frequently and is the one usually described in the literature (Jackiewicz, 1993, 1998, 2000; Gittenberger *et al*., 1998; Glöer, 2002; Stadnichenko, 2004).

*Male genitalia* (Fig. 6) The ratio of the length of the praeputium to that of the penis sheath varies from 0.7–1.3 in the specimens examined. The preputium is pigmented grey mostly over the entire surface, although in some individuals the distal part (about one third of the total length) is not pigmented.

*Bursa copulatrix* (Fig. 7) The following three characters were analysed:

Position of bursa copulatrix. The bursa is positioned dorsally to the vagina and rarely (if of a certain size) also dorsally to the provaginal duct.

Shape of the bursa. The shape of the bursa varies from nearly spherical over pear-shaped up to elongate-tubular.

Length of the bursa duct. The length of the bursa duct varies between nearly not visible up to half of the length of the bursa (if bursa is filled).

## DISCUSSION

*Molecular phylogeny* One aim of the molecular genetic analysis was to show that the individuals described morphologically and anatomically belong to the species *R. balthica*. In both the MP (Fig. 1) and the RAxML tree (not shown) based

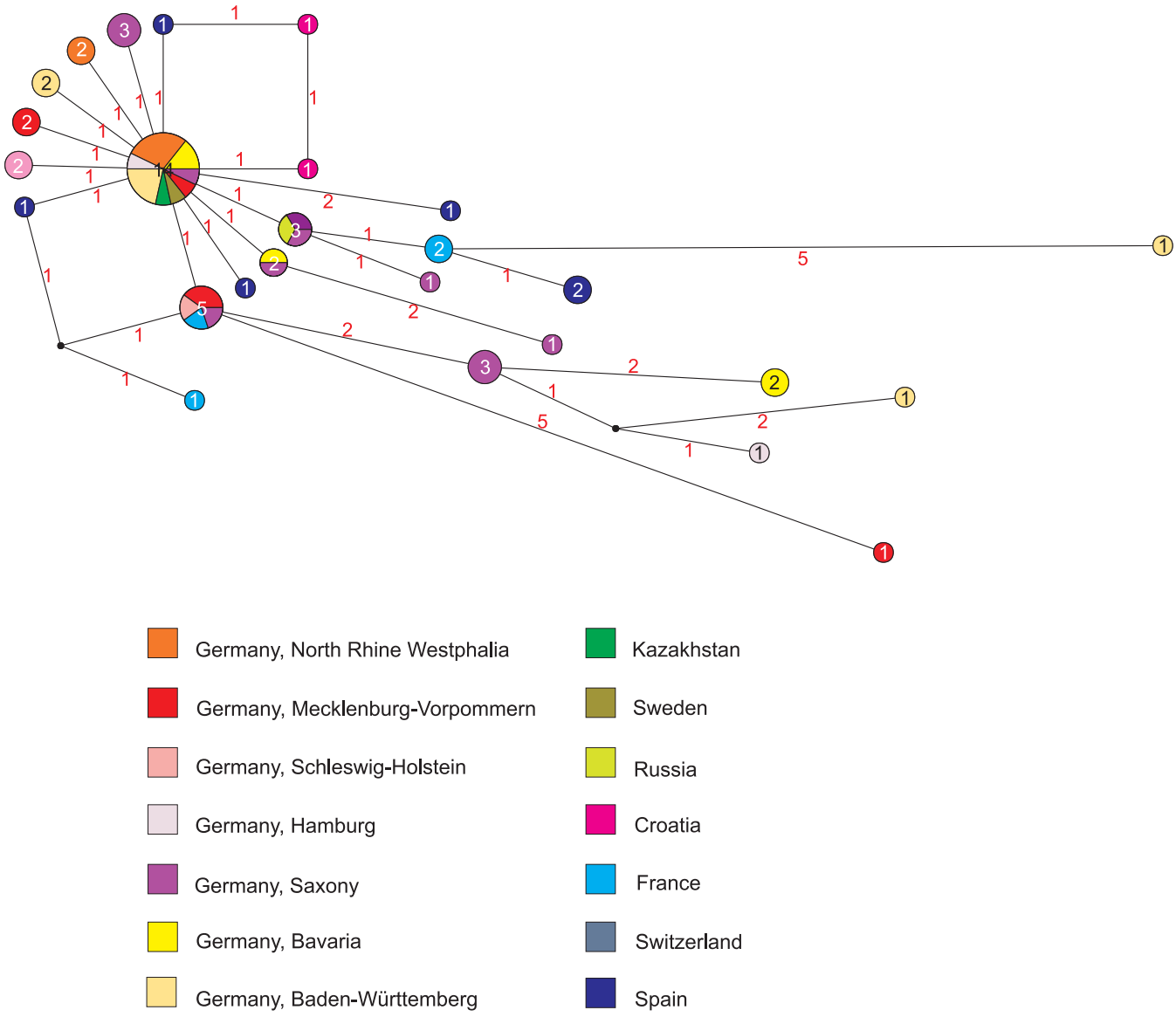


**Figure 3** Haplotype network of cyt–b sequences of 58 *Radix balthica* specimens from different locations in Europe, Kazakhstan and Russia. The size of the balloons is proportional to the number of individuals having each haplotype, which corresponds to the black number inside the balloon. The small black dots represent internal haplotypes not represented by the dataset. The length of the connecting lines is proportional to the number of substitutions between haplotypes, corresponding to the red number on the line.

on the cyt–b gene fragment (about 370 bp) these individuals form a clade including a "topotype" of *Radix balthica* from Øland (*R. balthica* Oe). This allows the conclusion that in spite of the high morphological variability observed, they all appear to belong to one species – *Radix balthica* – that clearly differs from the other *Radix*-species analysed. A different picture appears with the tree result of the nuclear marker ITS-2, based on both MP (Fig. 2) and RAxML (not shown). The two trees differ slightly, probably due to the fact that the alignment contains many indels and the RAxML-analysis does not interpret these as a fifth character state. The RAxML-analysis of this ITS-2 dataset (not shown) appears not to be able to distinguish closely related species: there is hardly any differentiation between *R. balthica* and *R. lagotis* and the support for differentiation to the cluster containing *R. ampla* is not very high either (70). In our opinion the three species *R. ampla*, *R. lagotis* and *R. balthica* do have species status, supported by sufficient large genetic distances based on the cyt–b fragment (Tab. 2). Further support comes from the MP tree of the ITS-2 dataset (Fig.

2), in which these three closely related species appear as well differentiated clades (with the exceptions discussed below). In our opinion the overall topology of this tree reflects the morphological and anatomical specialties and differences between the representatives of the genus *Radix* well.

However, in both the MP and RAxML trees based on ITS-2, individuals *R. balthica* Ko-2 and *R. balthica* Am-1 are clearly different from the remaining individuals of *R. balthica*, and appear to belong to other species. Since their mitochondrial haplotype based on the cyt–b fragment is clearly part of the *R. balthica* cluster (Fig. 1), these individuals may represent hybrids with other species of *Radix*. However, since they do not appear to group as part of the clusters formed by the other species included in this analysis, the hybridisation appears to have been with *Radix* species that have not been sampled. Alternatively, the results could reflect incomplete lineage sorting of the nuclear ITS-2 marker since the time when *R. balthica*, *R. labiata*, *R. ampla* and possibly even R. *lagotis* separated. In our molecular studies we have detected several such cases in which the mitochondrial genes (cyt–B, COI) indicate different species affiliation than the nuclear marker ITS-2 (e.g. also in the genus *Stagnicola*, unpublished results).

The analysis of the network relationship of the mitochondrial haplotypes illustrated with respect to their geographic origin (Fig. 3) shows that there is no distinct correlation of genetic variability to the geographic distribution pattern.

*Morphology* The anatomy of *Radix balthica* is much more plastic than usually believed. Glöer & Beckmann (2007) described *Radix lilli* from Majorca, mainly separated by the bursa duct which was longer than in typical *Radix balthica*, as depicted in Glöer (2002: 212, fig. 240b). But the DNA sequences indicate to us that this species (*R. balthica* Ma) belongs to the same clade as *R. balthica*. On the other hand the sequenced species had a sitting bursa copulatrix, which might be explained in a single population if the bursa duct varies or if two species occur at a particular sampling site.

The shells of the examined specimens of *R. balthica* vary considerably, not only in size and colour, but also in form and the number of whorls, which partly also leads to strong deviation from the egg-shaped shell of the neotype which has a relatively low spire (Kruglov & Starobogatov, 1983). The heights of the shells lie above that of the neotype, at 9.8 mm (Kruglov & Starobogatov, 1983). Usually the shell has a weak columellar fold and a regular convex last whorl.

The differentiation of the shells of amploid forms of *R. balthica* (Fig. 4.10) from shells of *R. ampla* can be difficult. *R. ampla* usually has no columellar fold (Fig. 9.3), however in rare cases this can also be missing in *R. balthica* f. *subampla*. In these cases the anatomy may also not provide certainty, since the anatomical character states of *R. balthica* and *R. ampla* overlap. Juvenile specimens may possibly be of help, since the shells of juvenile *R. ampla* already show the typical amploid shape, which is not the case in other *Radix*-species, such as *R. balthica* and *R. auricularia*. Mantle pigmentation can however be useful as a source of morphological differentiation: the white spots on dark background are larger in *R. ampla* (Fig. 9.3) than in *R. balthica*.

In addition, the shell shape of *R. balthica* (Fig. 4.11) can be similar to that of *R. auricularia* (see Fig. 9.2). In this case the anatomical differentiation is relatively easy, as *R. auricularia* has a very long bursa duct with a spherical bursa (see Fig. 9.2), in contrast to *R. balthica* (Fig. 7). Furthermore, *R. auricularia* is the only European species with a pigmentation of "freckles" on the tentacles, head and foot (see Fig. 9.2).

Whether a clear differentiation from *R. lagotis* is possible with characters of the shell only, has not been studied sufficiently.

A typical morphology for *R. balthica* is a shell with a regular, convex last whorl and a weak columellar fold (see shell of the neotype Fig. 8.3).

The mantle pigmentation of specimens analysed shows more variation than commonly described in the literature (Jackiewicz, 1993, 1998, 2000; Glöer, 2002). Only Hubendick (1945) and Falniowski (1980a, b) mentioned specimens with a completely black mantle pigmentation in *Radix peregra*. Unfortunately, it is unclear from Falniowski (1980a,b) which *Radix*-species is referred to, since *R. lagotis* and *R. labiata*, which were interpreted as forms of *R. peregra* by the author, can also have completely black mantle pigmentation. Hubendick (1945) mentions that mantle pigmentation may also be absent.

As a distinguishing characteristic to other *Radix* species only the typical mantle pigmentation can


**Figure 4** Variability in *Radix balthica* shells: **1** Germany, Baden-Württemberg, river Danube near Sigmaringendorf (Sig-1); **2** France, Region Centre, Thenay (Th-1); **3** Kazakhstan, lake Kopa (Ko-2); **4** Germany, Saxony, creek Geberbach near Kauscha (Kau); **5** Germany, Bavaria, river Amper near lake Ammersee (Am-1); **6** Germany, Saxony, Dresden-Kleizschachwitz, river Elbe (DD-2); **7** Switzerland, canton Basel City, Riehen, Wiesengriener (Rie-1); **8** Switzerland, canton Basel City, Riehen, Wiesengriener (Rie-2); **9** Switzerland, canton Basel-Landschaft, Liestal, Orishof (Lie-1); **10** Germany, Mecklenburg-Vorpommern, lake Torgelower See (Tor-2); **11** Germany, Mecklenburg-Vorpommern, lake Tiefwarensee (Tie-1); **12** Germany, North Rhine-Westphalia, Hillegossen, creek Meyerbach (Hi-1).

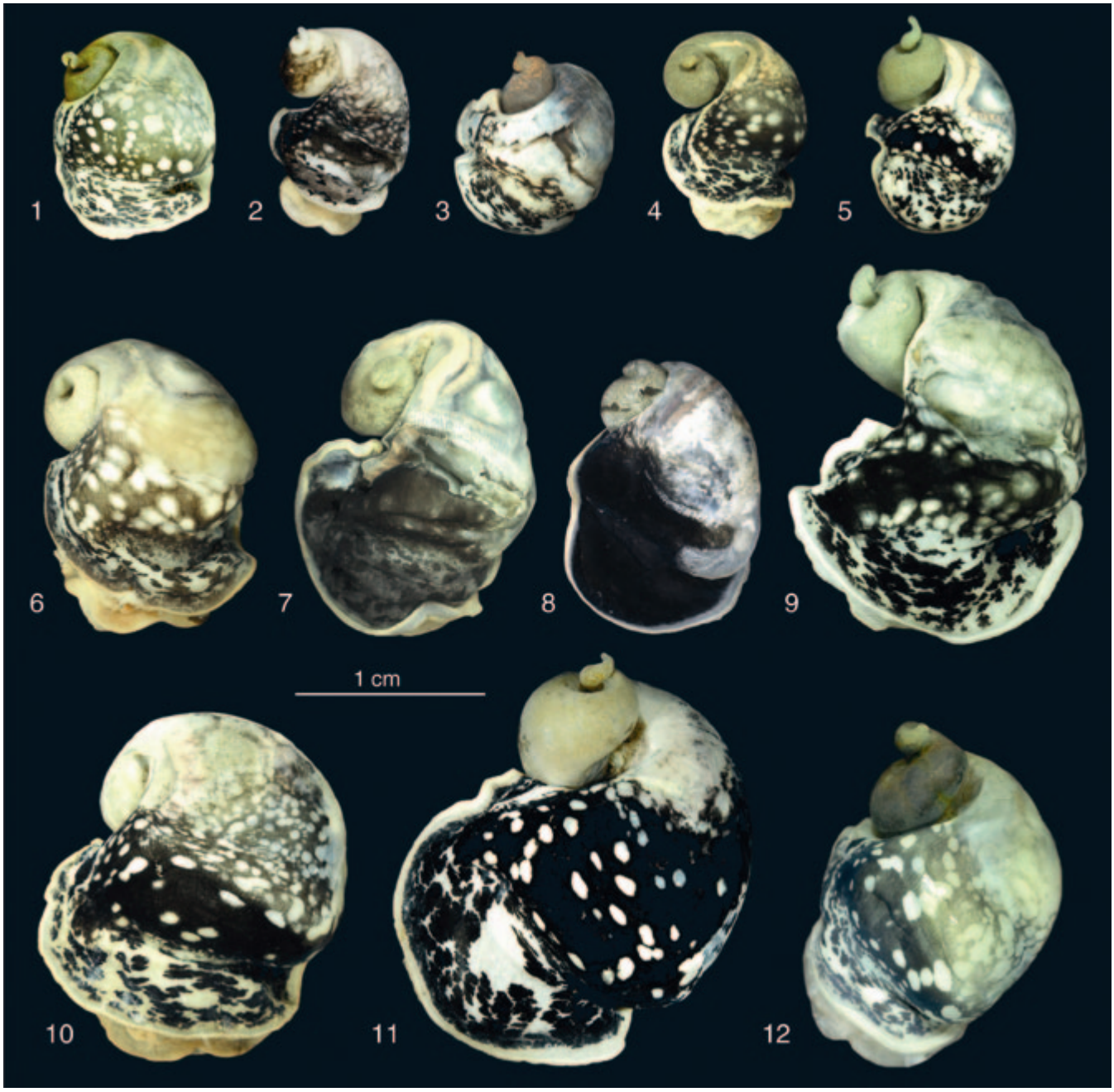


**Figure 5** Variability in *Radix balthica* mantle pigmentation: **1** Germany, Baden-Württemberg, river Danube near Sigmaringendorf (Sig-1); **2** France, Region Centre, Thenay (Th-1); **3** Kazakhstan, lake Kopa (Ko-2); **4** Germany, Saxony, creek Geberbach near Kauscha (Kau); **5** Germany, Bavaria, river Amper near lake Ammersee (Am-1); **6** Germany, Saxony, Dresden-Kleizschachwitz, river Elbe (DD-2); **7** Switzerland, canton Basel City, Riehen, Wiesengriener (Rie-1); **8** Switzerland, canton Basel City, Riehen, Wiesengriener (Rie-2); **9** Switzerland, canton Basel-Landschaft, Liestal, Orishof (Lie-1); **10** Germany, Mecklenburg-Vorpommern, lake Torgelower See (Tor-2); **11** Germany, Mecklenburg-Vorpommern, lake Tiefwarensee (Tie-1); **12** Germany, North Rhine-Westphalia, Hillegossen, creek Meyerbach (Hi-1

be used (see Fig. 5.1, 5, 10, 11). However, this character should best be used in combination with anatomical criteria for determinations.

A typical morphology for *R. balthica* is a black, grey-black, or grey-yellow mantle with many medium-sized distinct lighter spots on a dark background.

In the specimens of *R. balthica* studied, the shape of the bursa copulatrix as well as the length of the bursa duct show a similar high variation


**Figure 6** Variability in *Radix balthica* male genitalia: **1** Germany, Baden-Württemberg, river Danube near Sigmaringendorf (Sig-1); **2** France, Region Centre, Thenay (Th-1); **3** Germany, Bavaria, river Amper near lake Ammersee (Am-1); **4** Germany, Saxony, Dresden-Kleizschachwitz, river Elbe (DD-2); **5** Switzerland, canton Basel-Landschaft, Liestal, Orishof (Lie-1); **6** Germany, Mecklenburg-Vorpommern, lake Torgelower See (Tor-2).


**Figure 7** Variability of the form of the bursa copulatrix and the length of the bursa duct in *Radix balthica:* **1** France, Region Centre, Thenay (Th-1); **2** Germany, Saxony, creek Geberbach near Kauscha (Kau); **3** Germany, Bavaria, river Amper near lake Ammersee (Am-1); **4** Germany, Saxony, Dresden-Kleizschachwitz, river Elbe (DD-2); **5** Germany, Mecklenburg-Vorpommern, lake Torgelower See (Tor-2); **6** Germany, Mecklenburg-Vorpommern, lake Tiefwarensee (Tie-1); **7** Switzerland, canton Basel City, Riehen, Wiesengriener (Rie-2).


**Figure 8** Examined topotypes of *Radix balthica:* **1** Öland, seashore; **2** Öland, moor; **3** Neotype of *Radix balthica* of Kruglov & Starobogatov. The Russian inscriptions and abbreviations on the table say that this shell was collected by Westerlund and determined by Starobogatov. The word underlined red is "Неотип" = neotype. **1a-2a** mantle pigmentation, **1b-2b** female sex tract, **1c-2c** male copulatory organ.

(Fig. 7) as observed by Hubendick (1945, 1953) and Falniowski (1980a,b) for this species. Since the length of the bursa duct varies so strongly and can reach nearly half of the length of the bursa (if bursa is filled), we do not find this a good differentiating character with respect to *R. labiata*. However, the position of the bursa dorsally to the vagina and (depending on the size) to the provaginal duct is a distinct character for differentiating *R. balthica* from *R. labiata*, in which the bursa is positioned ventrally to the vagina (see also Stadnichenko, 2004: 197; Kruglov, 2005: 309).

No differential character states of *R. balthica* could be found with respect to *R. ampla*.

*Radix lagotis* can clearly be differentiated by the distinctly longer bursa duct, the length reaching half to about 2/3 of the length of the bursa (if the bursa is filled) (Fig. 9.4).


**Figure 9** **1** *Radix labiata* (Germany, Bavaria, Loiperding), **2** *R. auricularia* (Germany, Hamburg), **3** *R. ampla* (Germany, Mecklenburg-Vorpommern, Tollensesee), **4** *R. lagotis* (Germany, Brandenburg Havelland).

A typical morphology for *R. balthica* is a sitting bursa or a bursa with a very short duct. The colour of the filled bursa is orange in most cases, rarely white.

In the specimens from Europe in which we examined the male genitalia (n=10), the measured length ratio of praeputium to penis sheath varies from 0.7–1.3, confirming the interval provided by Meier-Brook in Glöer (2002), who reported that this characteristic can vary between 0.4:1 and 1.6:1 in *R. balthica*. In Western Siberia this ratio varies from 1.16:1 to 1.59:1 (n = 10) but falls within

**Table 3** Distinguishing characters for *Radix balthica*, *R. labiata*, *R. ampla*, *R. auricularia* and *R. lagotis*.

| Character | *Radix balthica* | *Radix labiata* | *Radix ampla* | *Radix auricularia* | *Radix lagotis* |
|---|---|---|---|---|---|
| shape of the line tangential to the whorls in adult shells | usually convex, rarely concave | usually straight | always concave | always concave | always convex |
| upper edge of aperture towering above whorls in adult shells | no, or hardly | no | yes | no | no |
| columella fold in adult shells | weak | weak | absent | distinct | weak |
| typical mantle pigmentation above lung cavity | many medium-sized light, distinct spots on dark background | many small light blurred dots on dark background | a few large white spots on dark background | a few large white spots on dark background | a few light distinct or blurred medium-sized spots on dark background |
| presence of pigmentation reminding of "freckles" on tentacles, head and foot | no | no | no | yes | no |
| length of the bursa duct (if bursa is filled) | between nearly not visible and half of the length of the bursa | from short to about nearly one third of the length of the bursa | between nearly not visible and half of the length of the bursa | very long, usually as long as the provaginal duct | from half to about 2/3 of the length of the bursa |
| position of bursa and bursa duct | above vagina and provaginal duct | behind vagina and above provaginal duct | above vagina and provaginal duct | near pericardium | above vagina, provaginal duct, uterus and prostata |
| colour of praeputium | usually uniform dark grey or dark bluish grey | uniform grey greenish or dark grey | light and without pigmentation | with dorsal pigmentation reminding of "freckles" | light to dark bluish grey |

Meier-Brook's data. In comparison, Jackiewicz (1998, 2000) quotes the ratio of praeputium to the penis sheath length as 1:1, and Stadnichenko (2004) as 1.2:1 and Kruglov (2005) 1.20:1.

According to Meier-Brook in Glöer (2002) the ratio of praeputium length to penis sheath in *R. labiata* can vary from 0.7:1 to 2.3:1. Thus this character can not always be used for differentiation between *R. balthica* and *R. labiata*. It is also not useful for differentiation between *R. balthica*, *R. auricularia* and *R. ampla*. In *R. auricularia* the ratio of praeputium to the penis sheath length is 1:1 (Jackiewicz, 1998, 2000; Glöer, 2002), 1.10:1 (Stadnichenko, 2004; Kruglov, 2005), or 0.6–1.3 according to our own measurements (n=6 from different locations). Vinarski & Glöer (2009) reported values of this index in *R. auricularia* between 0.96:1 and 1.41:1 (n = 65).

Falniowski (1980a) mentioned the existence of a dorsal pigmentation of the praeputium in *R.*

*auricularia*, which is confirmed by our own observations. This pigmentation consists of single very small, dark pigmentation marks ("freckles").

The praeputium of the specimens of *R. balthica* analysed by molecular genetics was regularly dark grey or dark grey-blue over the entire surface. In one case the colouration was regular (without clearly distinguishable single dots of pigmentation) only to about two thirds. The praeputium of the individual from Øland was light yellow. The specimens of *R. balthica* with *ampla*-shaped shell we studied, clearly differ from *R. ampla* by a praeputium with dark pigmentation. It appears to be light and without pigmentation in *R. ampla* of seven different sampling sites. In *R. labiata* the praeputium is grey-greenish or dark grey in colour, in *R. lagotis* it varies from light to dark blue-grey.

## Conclusion: Differentiating Features from other European *Radix*-Species

The most important morphological characters for differentiation of the species *R. balthica*, *R. labiata*, *R. ampla*, *R. lagotis* and *R. auricularia* are summarised in Table 3 taking account of our present knowledge on the morphological variability of these species.

Specimens of *R. balthica* with shells similar to those of *R. auricularia* are differentiated by the length of the bursa duct, which is often nearly as long as the provaginal duct and ends in a rounded or pyriform bursa (see Fig. 9.2). The mantle pigmentation can be used for differentiation in addition. In most cases, the mantle of *R. auricularia* has distinctly larger white spots than that of *R. balthica* (see Fig. 9.2). The tentacles, the head, the surface of the foot and often also the sole of the foot show a pigmentation reminding of "freckles" in *R. auricularia* (see Fig. 9.2), which is absent in all the other European *Radix* species, but is apparent in many Asian *Radix* spp. A reliable differentiation and determination based on shell morphology alone is very difficult.

Specimens of *R. balthica* with amploid shells mostly differ from *R. ampla* in mantle pigmentation, which often shows a few large white spots on dark background in *R. ampla* (Fig. 9.3), that sometimes fuse with one another. A reliable differentiation and determination based on shell morphology alone is very difficult.

*Radix balthica* differs most clearly from *R. labiata* by the position of the bursa and the bursa duct. They lie dorsal to the vagina and the provaginal duct in *R. balthica*, whereas in *R. labiata* bursa and bursa duct lie ventrally to the vagina and the provaginal duct. *R. labiata* differs from specimens of *R. balthica* in most cases in that the typical mantle pigmentation has many more small light diffuse dots on a dark background (Fig. 9.1).

The best character for distinguishing *Radix lagotis*, which is clearly differentiated from *R. balthica*, *R. labiata*, *R. ampla* und *R. auricularia* based on molecular genetics (see also Bargues *et al*., 2001), is the length of the bursa duct, which is distinctly longer in *R. lagotis*, reaching half to about 2/3 of the length of the bursa (if filled) (Fig. 9.4).

## Acknowledgements

We would like to express our thanks to Prof. Dr. Uwe Fritz (SNSD) for financial support of the greater part of the molecular analyses, Anke Müller (SNSD) for some sequences and the instruction of K.S. in lab work, as well as André Reimann (SNSD), Dr. Michael L. Zettler (Leibnitz Institute for Baltic Sea Research Warnemuende), Michael Korn (University of Konstanz, Limnological Institute), Dr. Nicole Schröder-Rogalla (Munich), Susanne Thiel (Munich), Robert Haldemann (Strausberg), Christoph Oberer (Natural History Museum Basel), Eric Gallerne (Leguevin), Dr. Daniel Rondelaud (Faculté de Médecine Limoges Cedex), Holger Menzel-Harloff (Wismar), Uwe Jueg (Ludwigslust), Alfried V. Karimov (Omsk State Pedagogical University), Gerhard Falkner (State Museum of Natural History Stuttgart), Dr. Ira Richling (Kronshagen), Armin Deutsch (Bielefeld), Reinhard Diercking (Hamburg), Hajo Kobialka (Hoexter), Andrea Pohl (Dresden), Gudrun Rutsch (Dresden) and Christa Schniebs (Oelsnitz) for the material collected and provided. Last but not least we also thank two anonymous reviewers for improvements to the manuscript.

## References

Almeyda-Artigas RJ, Bargues MD & Mas-Coma S 2000 ITS-2 rDNA sequencing of *Gnathostoma* species (Nematoda) and elucidation of the species causing human human gnathostomiasis in the Americas. *Journal of Parasitology* **6** (3): 537–544.

BANDELT H–J, FORSTER P & RÖHL A 1999 Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37–48.

BARGUES MD & MAS-COMA S 2005 Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. *Journal of Helminthology* **79**: 257–267.

BARGUES MD, VIGO M, HORAK P, DVORAK J, PATZNER RA, POINTIER JP, JACKIEWICZ M, MEIER-BROOK C & MAS-COMA S 2001 European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. *Infection, Genetics and Evolution* **1**(2): 85–107.

BROWN DS 1994 *Freshwater snails of Africa and their medical importance*. Second edition. Taylor & Francis, London. 608 pp.

CORDELLIER M & PFENNINGER M 2009 Inferring the past to predict the future: climate modelling predictions and phylogeography for the freshwater gastropod *Radix balthica* (Pulmonata, Basommatophora). *Molecular Ecology* **18**: 534–544.

DAMME D VAN 1984 *The freshwater mollusca of Northern Africa*. Dr. W Junk Publishers, Dordrecht. 164 pp.

FALNIOWSKI A 1980a Podrodzaj *Radix* s. str. (Gastropoda, Basommatophora) w Polsce. I Pigmentacja i anatomia. Opis *Lymnaea peregra roszkowskiana* subsp. nov. *Zeszyty Naukowe Uniwersytetu Jagiellońskiego, Prace Zoologiczne* **26**: 67–108.

FALNIOWSKI A 1980b Pigmentation of the mantle border in Polish representatives of the subgenus *Radix* (Lymnaeidae, Basommatophora, Gastropoda). *Basteria* **44**(1/4): 3–8.

GITTENBERGER E, JANSSEN AW, KUIJPER WJ, KUIPER JGJ, MEIJER T, VAN DER VELDE G & DE VRIES JN 1998 De Nederlandse Zoetwatermollusken. Recente en fossiele Weedieren uit Zoet en Brak Water. *In* GITTENBERGER E & JANSSEN AW (eds) *Nederlandse Fauna 2*. National Natuurhistorisch Museum Naturalis, Utrecht. 288 pp.

GLÖER P 2002 *Die Süßwassergastropoden Nord- und Mitteleuropas. Die Tierwelt Deutschlands. 73*. Conchbooks, Hackenheim. 327 pp.

GLÖER P & BECKMANN K–H 2007 *Radix lilli* n. sp. und drei neue *Bithynia*-Arten von den Balearen (Gastropoda: Bithyniidae, Lymnaeidae). *In* BECKMANN K–H (ed.) *Die Land- und Süßwassermollusken der Balearischen Inseln*. *CLECOM-Projekt*: 163–170. ConchBooks, Hackenheim.

GLÖER P & DIERCKING R 2010 *Atlas und Rote Liste der Süßwassermollusken in Hamburg*. Behörde für Stadtentwicklung und Umwelt, Freie und Hansestadt Hamburg. 180 pp.

GLÖER P & MEIER-BROOK C 2003 *Süßwassermollusken. Ein Bestimmungsschlüssel für die Bundesrepublik Deutschland*. 13th edition. Deutscher Jugendbund für Naturbeobachtung, Hamburg. 134 pp.

GUSTINCICH S, MANFIOLETTI G, DEL SAL G, SCHNEIDER C & CARNINCI C 1991 A fast method for high-quality genomic DNA extraction from whole human blood. *Bio Techniques* **11**: 298–302.

HALL TA 1999 BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* No. 41: 95–98.

HUBENDICK B 1945 Die Artabgrenzung bei den schwedischen Lymnaeiden der *Radix*-Gruppe. *Arkiv för zoologi* **37A** (10): 1–57 + 1 pl.

HUBENDICK B 1953 Recent Lymnaeidae. Their variation, morphology, taxonomy, nomenclature, and distribution. *Kungliga Svenska Vetenskapsakademiens Handlingar* Ser. 4, **3**(1): 1–223.

JACKIEWICZ M 1993 Die Mantelpigmentation als Diagnosemerkmal bei Schlammschnecken (Gastropoda, Pulmonata: Lymnaeidae). *Malakologische Abhandlungen des Staatlichen Museums für Tierkunde Dresden* **16** (2): 165–172.

JACKIEWICZ M 1998 European species of the family Lymnaeidae (Gastropoda: Pulmonata: Basommatophora). *Genus* **9**(1): 1–93.

JACKIEWICZ M 2000 *Błotniarki Europy (Gastropoda: Pulmonata: Lymnaeidae)*. Poznań, Wydawnictwo Kontekst. 115 pp.

KERNEY MP 1999 *Atlas of the land and freshwater molluscs of Britain and Ireland*. Harley Books, Colchester. 264 pp.

KRUGLOV ND 2005 *Lymnaeid snails (Lymnaeidae Gastropoda Pulmonata) of Europe and Northern Asia.* Smolensk State Pedagogical University Press, Smolensk. 507pp.

KRUGLOV ND & STAROBOGATOV YI 1983 A contribution to the morphology and taxonomy of European representatives of the subgenus *Peregriana* (*Lymnaea*, Gastropoda, Pulmonata). *Zoologichesky Zhurnal* **62**(10): 1462–1473.

LAKOVITZ T, BRONMARK C & NYSTRÖM P 2008 Tuning in to multiple predators: conflicting demands for shell morphology in a freshwater snail. *Freshwater Biology* **53**: 2184–2191.

MANDAHL-BARTH G 1938 Land and freshwater Mollusca. *The Zoology of Iceland* **4**(65): 1–31.

MERRITT TJS, SHI L, CHASE MC, REX MA, ETTER RJ & QUATTRO JM 1998 Universal cytochrome *b* primers facilitate intraspecific studies in molluscan taxa. *Molecular Marine Biology and Biotechnology* **7**(1): 7–11.

PFENNINGER M, CORDELLIER M & STREIT B 2006 Comparing the efficacy of morphologic and DNA-based taxonomy in the freshwater gastropod genus *Radix* (Basommatophora, Pulmonata). *BMC Evolutionary Biology* 6, 10.

ØKLAND J 1990 *Lakes and snails. Environment and Gastropoda in 1.500 Norwegian lakes, ponds and rivers*. Backhuys, Oegstgeest. 516 pp.

SILVESTRO D & MICHALAK I 2010 RAXMLGUI: a graphical front-end for RAxML. (Online at http://sourceforge.net/projects/raxmlgui/ accessed February 2011).

STADNICHENKO AP 2004 Lymnaeids and acroloxids (Lymnaeidae, Acroloxidae) of the Ukraine. Tsentr nauchnoy literatury Publisher. 327 pp.

STAMATAKIS A, LUDWIG T & MEIER H 2005 Raxml-iii: a fast program for maximum likelihood-based infer-

ence of large phylogenetic trees. *Bioinformatics* **21**(4): 456–463.

SWOFFORD DL 2002 *PAUP* - Phylogenetic analysis using parsimony (*and other methods)*. Version 4. Sinauer Associates Inc. Publishers, Sunderland.

TAMURA K, DUDLEY J, NEI M & KUMAR S 2007 MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.

VINARSKI VM & GLÖER P 2009 Taxonomic notes on Eurosiberian freshwater molluscs. 4. Re-examination of *Limnaea psilia* Bourguignat, 1862 with the description of *Lymnaea* (*Radix*) *parapsilia* n. sp. (Gastropoda: Pulmonata: Lymnaeidae). *Archiv für Molluskenkunde* **138**: 123–136.

WARD PI, GOATER CP & MIKOS M 1997 Shell variation in sympatric freshwater *Lymnaea peregra* and *L ovata* (Gastropoda: Lymnaeidae). *Biological Journal of the Linnean Society* **61**: 139–149.

ZETTLER ML, JUEG U, MENZEL-HARLOFF H, GÖLLNITZ U, PETRICK S, WEBER E & SEEMANN R 2006 *Die Land- und Süßwassermollusken Mecklenburg-Vorpommerns*. Schwerin, Obotritendruck. 318 pp.