Genetic diversity and evolutionary relationships of the troglodytic ‘living fossil’ Congeria kusceri (Bivalvia: Dreissenidae)

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Abstract
Population genetic theory predicts that long-term isolation of ‘living fossils’ in relic habitats might reduce genetic variability due to small population sizes and inbreeding. The recent description of a troglodytic ‘living fossil’ Congeria kusceri — the only known subterranean bivalve mollusc from a genus thought to be extinct since the Miocene, offers a unique opportunity to examine this hypothesis. Here, we use DNA sequences from two mitochondrial genes to compare levels of genetic variability and to test phylogenetic relationships of C. kusceri with surface-dwelling dreissenid relatives. Phylogenetic analyses of sequences from the cytochrome oxidase 1 (COI) and 16S rDNA genes reveal that Mytilopsis is the sister genus to Congeria and this clade forms the sister taxon to Dreissena. Relatively high levels of DNA diversity characterized the population of C. kusceri (haplotypic diversity = 0.50 for 16S rDNA and 0.66 in the COI gene), in contrast to no intraspecific variability in populations of Dreissena polymorpha, D. bugensis, Mytilopsis leucophaeta, and Corbicula fluminea. Maintenance of genetic variability in C. kusceri may result from long-term population size stability, which merits further investigation. This underground species apparently was buffered from the climatic changes and resultant population bottlenecks that affected its surface-dwelling relatives during the Pliocene and Pleistocene Ice Ages.

Keywords: 16S mtDNA, cave-dwelling, Congeria, COI mtDNA, Dreissenidae, genetic diversity

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Introduction
Subterranean caverns and rivers house unique aquatic faunas. They comprise long-term stable temperate freshwater environments, serving as refugia from the stochastic climatic fluctuations that have shaped surface-level communities. Subterranean habitats have no light and temperatures are relatively constant, but may experience some seasonal variations in water flow. Population genetic theory predicts that long-term isolation of ‘living fossils’ might reduce genetic variability due to relatively small population sizes and possible inbreeding (Avise 1994, 2000).

The bivalve mollusc Congeria kusceri, was recently discovered living exclusively in subterranean waters of the Dinaric karst of Slovenia, Croatia, and Bosnia-Herzegovina (Fig. 1) (Bole 1962; Morton et al. 1998). The Dinaric karst region of the northern Adriatic contains nearly 400 specialized stigobiont taxa in an area of only 153 400 km² (Sket 1999), suggesting long-term evolutionary stability. Until this discovery, the dreissenoid genus Congeria was believed to have become extinct about 5 million years ago (Ma; Nuttall 1990), coinciding with the break-up of the Paratethys Sea and the Messinian salinity crisis (Morton 1992). Congeria comprised a large number of species of common coastal zone burrowing mussels during the late Miocene (Morton 1992). For example, approximately 30 species have been described from Miocene deposits in the region of Croatia and Bosnia-Herzegovina (Kochansky-Devide &
Finally fertilized, and then brooded in the ctenidia — an important systematic discovery (Morton 1990; Morton 1992), the finding of living C. kusceri distinguishes from Mytilopsis and Dreissena — and free-swimming larvae (Mills et al. 1996). There is thus a fundamental difference in life history traits between the opportunistic r-selected Dreissena and Mytilopsis (Mills et al. 1996), which are its closest living relatives, and C. kusceri. C. kusceri has a tiny foot, rendering it incapable of significant locomotion (unlike juvenile D. polymorpha) and the byssal threads are short and thick — also indicating that C. kusceri moves little after attachment (Bole 1962; Morton et al. 1998).

**Hypothesized evolutionary relationships of Congeria**

C. kusceri is a relic of Pliocene lakes, which formed above fractured limestone and dolomite deposits of the western Paratethys. Today, C. kusceri lives in the caves and subterranean rivers of this karst, where it apparently followed the waters down as they eroded the porous rock (Morton et al. 1998). Another Paratethys species, the similarly troglobitic polychaete Marifugia caratica (Serpulidae) appears sympatrically with C. kusceri and encrusts the shells with its tubes (Morton et al. 1998).

Congeria belongs to the Dreissenoida, which contains a single family, the Dreissenidae and two other extant genera; the freshwater Dreissena and the marine Mytilopsis (Nuttall 1990). The Dreissenoida is believed to have arisen from an ancestor shared with the Corbiculoida during the late Mesozoic era (Morton 1993). The dreissenoids appear united by having an apical shell septum to which the anterior adductor and anterior byssal retractor muscles are attached (Nuttall 1990; Morton 1993). They also are characterized by the neotenous retention of the byssus (a larval character) and the foot has consequently ceased to be an organ of locomotion (except in juveniles), with corresponding enlargement of the larval byssal retractor muscles and diminution of the adult pedal ones. The three dreissenoid genera are believed to have diverged during the mid to late Miocene Epoch (Morton 1993).

Whether Congeria and Mytilopsis belong to two separate genera and how these groups are related has been debated (Nuttall 1990; Morton et al. 1998). A hypothesized sister relationship between the genera Congeria and Mytilopsis is supported by the shared character of attachment of the anterior byssal retractor muscle to a special lobe of the anterior shell septum called the apophysis (Morton 1993). In contrast, the anterior byssal retractor muscle is attached directly to the septum in Dreissena. Congeria is distinguished from Mytilopsis by its distinct shell form and microstructure, and different reproductive strategy (Morton 1993; Morton et al. 1998). It also has been disputed whether Mytilopsis (Nuttall 1990) or Congeria (Morton 1993) is more closely related to an ancestor shared with the sister superfamily Corbiculoidea.

The number of species comprising the euryhaline Mytilopsis are disputed; they are native to the New World,
but Old World fossils exist and some species have been introduced back into Europe (M. leucophaeta) (van der Velde et al. 1992) and Asia (M. sallier) (Morton 1980). The genus Dreissena includes the zebra mussel D. polymorpha and the quagga mussel D. bugensis, which were spread via ballast water introductions from Europe to the North American Great Lakes during the last 15 years and where they now form large established populations (Mills et al. 1996; Berkman et al. 2000).

**Objectives and hypotheses**

The first objective of the present study was to test the phylogenetic relationships of the Dreissenidae, comprising Congeria and the genera Dreissena and Mytilopsis, using sequence data sets from two mitochondrial DNA (mtDNA) genes. The relationship of the Dreissenoidae with the Corbiculidae (represented by the Asian clam Corbicula fluminea), which is their hypothesized sister superfamily, was also examined and the results compared with morphological hypotheses. The second objective was to compare the relative level of DNA genetic variability in a population of the troglobytic living fossil C. kusceri with its surface-dwelling close relatives (D. polymorpha, D. bugensis, and M. leucophaeta), in order to examine the possible influences of long-term isolation in a climatically stable environment (C. kusceri) vs. larger extant populations that were subjected to historic climatic changes (Dreissena spp. and M. leucophaeta).

**Materials and methods**

Ten individuals of Congeria kusceri were collected from the pit ‘Jama u Predolcu’, near Metkovic, Croatia in August 1998 (Fig. 1) and placed in 95% ethanol. The pit is about 10 m deep, with a subterranean lake at its bottom (Jalzic 1998). Genomic DNA was extracted from nine specimens using standard methods (Stepien et al. 1999). The remaining sample was kept as a whole voucher. Shells from the nine specimens sequenced were archived. The mitochondrial sample was kept as a whole voucher. Shells from the nine close relatives (D. polymorpha, D. bugensis, M. leucophaeta, and C. fluminea) (GenBank Accession nos AF0389989–92). The 16S RNA sequences were rooted to those published for M. edulis (Hoffman et al. 1992), which was used as the outgroup for both gene regions. The alignments for the 165 rDNA gene data are given in Stepien et al. (1999).

Both gene regions were analysed separately and the resulting phylogenetic trees compared for congruency of relationships among taxa. Genetic distances, including Kimura (1980) 2-parameter, Tamura & Nei (1993), and uncorrected pairwise distances (following Nei 1987), between each pair of taxa were calculated and clustered using the neighbour-joining (NJ) algorithm in PAUP* 4.0A4b (Swofford 2000). Maximum parsimony (MP) trees were determined using exhaustive searches in PAUP* (Swofford et al. 1996; Swofford 2000), with M. edulis designated as the outgroup. Taxa contained in the MP trees were compared using strict and 50% majority-rule consensus analyses (Margush & McMorris 1981). Support of the data sets for the nodes of the NJ and MP trees were determined from 1000 bootstrap replications (Kumar et al. 1993; Swofford et al. 1996). Measures of nucleotide variability were calculated according to Nei (1987) and included the number of polymorphic nucleotide sites per nucleotide position pm (eqn 10.1) ± its variance over the stochastic process (eqn 10.3), haplotypic diversity (eqn 8.5) ± its variance (eqn 8.8), and the average number of nucleotide differences among haplotypes (nucleotide diversity) (eqn 10.5) ± its variance due to stochastic errors (eqn 10.9).

**Results**

**Interspecific divergences**

Our sequencing results from the mtDNA COI (reported in GenBank as Accession no. AF325444) and the 16S ribosomal gene (GenBank Accession no. AF320601) regions showed that Congeria, Mytilopsis, and Dreissena comprise a monophyletic lineage (see Figs 3 and 4), confirming their morphological classification together as the Dreissenidae (Nuttall 1990; Morton 1993). The NJ trees from both genes and the single MP tree from 16S rDNA indicated that Congeria and Mytilopsis are sister genera, and this clade then forms the sister group to Dreissena. Genetic distance estimates from Kimura (1980) 2-parameter, Tamura & Nei (1993), and uncorrected pairwise distances (following Nei 1987), were very similar (within 0.003), and the former was used in Table 1 and to construct the NJ tree (Fig. 2). The Dreissena and the Mytilopsis/Congeria clade appear to have diverged between 10 and 17 Ma, using a molecular clock calibrated to fossil record dates for this group given in Stepien et al. (1999). The genera Mytilopsis and Congeria then diverged from each other approximately 5–15 Ma and D. bugensis and D. polymorpha speciated about 10–13
Intraspecific variation

Our investigation revealed two haplotypes in the mtDNA 16S RNA gene region for C. kusceri. The haplotypes differ...
by a transitional substitution from cytosine (the apparent ancestral condition found in six individual *C. kusceri* and in all specimens of *Dreissena* and *Mytilopsis*) to thymine (in three individuals). Measures of DNA polymorphism (Nei 1987) for the 16S RNA gene in *C. kusceri* included the number of polymorphic nucleotide sites per nucleotide position \( p_n = 0.0024 \pm 0.0001 \), haplotype diversity \( h = 0.503 \pm 0.005 \), and the average number of nucleotide differences among haplotypes (nucleotide diversity) \( \pi = 0.00050 \pm 0.00017 \). The Kimura 2-parameter genetic distance between the haplotypes was \( 0.0022 \pm 0.0018 \). This difference indicates a possible divergence of approximately 189,000 ± 150,000 years, suggesting their differentiation during the Pleistocene Ice Ages.

The COI gene sequences revealed four different haplotypes of *C. kusceri*. The haplotypes differ by single substitutions, including two transitions and one transversion. None of these substitutions produces an amino acid change (Baldwin et al. 1996). Haplotype 1 characterized five individuals, haplotype 2 represented two individuals, and haplotypes 3 and 4 each characterized single individuals. Measures of DNA polymorphism (Nei 1987) for the COI gene included \( p_n = 0.00352 \pm 0.0010 \), \( h = 0.659 \pm 0.014 \), and \( \pi = 0.0006 \pm 0.0001 \). The Kimura 2-parameter genetic distance between the pairs of haplotypes was \( 0.0037 \pm 0.0010 \). This difference indicates a possible genetic divergence of 130,000 ± 77,000 years, which is congruent with the above estimate from the 16S RNA sequences.

Discussion

Systematic relationships of Congeria

Phylogenetic trees from mtDNA 16S RNA and COI sequences support monophyly of the Dreissenidae, consistent with morphological hypotheses (Nuttall 1990; Morton 1993). *Dreissena* is the sister group to a *Congeria/Mytilopsis* clade. The genera *Congeria* and *Mytilopsis* are sister taxa, which was hypothesized by Morton (1993) on the basis of anterior pedal retractor muscle attachment. The molecular clock calibration indicates that the genera diverged approximately 5–15 Ma, consistent with the mid to late Miocene separation hypothesized by Morton (1993).

Comparative genetic diversity of Congeria and its relatives

Our study discerned two haplotypes for 16S rDNA and four for the COI gene, revealing considerably higher genetic diversity in *C. kusceri* than for the same region sequences in populations of its surface-dwelling dreissenid relatives (Baldwin et al. 1996; Stepien et al. 1999). The latter taxa have considerably greater distributional ranges and larger population sizes today (Morton 1993; Stepien et al. 1999). A study of the same 16S rDNA region found no intraspecific variability in samples representing the ranges of *D. polymorpha* (n = 24 individuals) and *D. bugensis* (n = 20), yielding values of \( h = 0.00 \) (Stepien et al. 1999) vs. 0.50 for *C. kusceri* (Results). Similarly, no intraspecific variation was found in sequences for the same region of the 16S rDNA gene in three samples of *M. leucophaeta* and seven *Corbicula fluminea* (both from single sites; Stepien et al. 1999).

Likewise, a previous study of the same region of the COI gene by Baldwin et al. (1996) found no intraspecific sequence variability in *D. polymorpha*, *M. leucophaeta*, or *C. fluminea*. The sole intraspecific variation discerned by Baldwin et al. (1996) was two different haplotypes of *D. bugensis* alone, differentiating the deeper-water white-coloured ‘profundal’ ecomorph in Lake Erie from the ‘shallow’ and more widespread morph in the lower Great Lakes (\( p_n = 0.0065 \)). However, the profound *D. bugensis* was not genetically divergent from the shallow form using nuclear random amplified polymorphic DNA (RAPD) markers, which evolve more quickly than the mtDNA COI and 16S RNA genes examined in the present study (Stepien et al. submitted). The profound ecomorph of *D. bugensis* in Lake Erie is a close genetic match for specimens from the Ukraine. The lack of divergence between the two Great Lakes ecomorphs of *D. bugensis* suggests they are not biogeographically separated in their native range (Stepien et al. submitted), indicating that they are a naturally occurring single population (like the sample of *C. kusceri* in the present investigation). The profound and shallow water ecomorphs of *D. bugensis* in Lake Erie probably were introduced from similar founding source populations, and the divergence between them is thus not greater than that within a single population (Stepien et al. submitted).

A study of North American unionid bivalves by King et al. (1999) using 710 bp of the same region of the COI gene discerned three haplotypes (differing by a single transition and a single transversion) for *Lamigona subviridis* from nine locations and a total of 37 individuals (\( p_n = 0.0028 \)). The species ranges south of the area covered by the Pleistocene glaciations in North America and was presumed less affected by climatic changes than were more northerly species (King et al. 1999). The level of variation for *C. kusceri* in our study thus appears relatively high, in comparison with the same genes in other bivalves.

Why is a troglodytic species, which is restricted to the Dinaric karsts of the former Yugoslavia (Morton et al. 1998), more variable in these slowly evolving genes? Greater genetic diversity may be explained by the buffering of the population from climatic fluctuations in the unique subterranean habitat of *C. kusceri*. Insulation from the climatic changes during the Pleistocene Ice Ages apparently...
preserved its accumulated genetic variability and its population sizes and levels of gene flow have been large enough to maintain it. Our genetic distances (calibrated according to Stepien et al. 1999) suggest that the haplotypes of C. kusceri diverged from each other between approximately 100 000–200 000 years ago, corresponding to this period.

In contrast, populations of dreissenoids inhabiting the cold temperate surface waters were subjected to extreme climatic variations. During the Pliocene and Pleistocene Ice Ages, surface-water species contracted into small isolated populations in restricted southerly refugia (Cooper et al. 1995; Hewitt 1996; Taberlet et al. 1998). Such populations underwent bottlenecks and much of their genetic variability was lost (Nei et al. 1975), apparently due to stochastic lineage extinctions (Avise 1994, 2000). Subsequent rapid range expansion during the interglacials may have further decreased genetic variability, according to the ‘leading edge hypothesis’ (Hewitt 1996, 2000). This hypothesis suggests that the first colonizing individuals to follow the retreating glaciers would have founded the new populations, leading to the rapid exponential growth of their genotypes (Hewitt 1996; Stepien et al. 1998). Subsequently arriving individuals would have contributed less to the gene pools of the new populations, which had already reached their carrying capacities (Hewitt 1996). This population genetic pattern apparently characterizes many r-selected colonizing species, which tend to be excellent invaders — such as Dreissena, Mytilopsis, and Corbicula (Morton 1996).

In contrast, the subterranean populations of C. kusceri have existed for 5 million years, apparently uninterrupted by environmental disturbances (Morton et al. 1998). These populations maintained higher genetic diversity, derived from the steady accumulation of random mutations in selectively neutral areas, as identified in our study. Since mtDNA is very sensitive to population bottlenecks (Avise 1994, 2000), the constant subterranean environment and lack of fluctuations in population sizes would have preserved selectively neutral genetic changes. High genetic variability despite the smaller population sizes of C. kusceri compared to its surface-dwelling relatives (including species of Dreissena, Mytilopsis, and Corbicula), suggests that it may be the long-term maintenance of population size, rather than the current one, that is crucial to retaining genetic diversity. This hypothesis merits considerable further testing.

Summary and conclusions

In our present-day human quest to rescue endangered species, we must keep in mind that once they have undergone marked reductions in effective population sizes, much of their genetic diversity is likely lost. This also is true of present-day large populations, which appear to have rebounded from past bottlenecks, such as Dreissena polymorpha. Such decreased genetic diversity may not negatively affect the survival of the species, as evidenced by the colonizing success of D. polymorpha.

Retention of variability in slowly evolving genes may be indicative of constant historic environments and sustained population sizes, as found in the cold underground caves and rivers of the Dinaric karst of the former Yugoslavia. In contrast, climatic changes and resultant range alterations in surface-level habitats during the Pleistocene Ice Ages likely led to genetic drift and founder effects that significantly reduced variability in other dreissenoid molluscs.

Changes in the genetic diversity and composition of populations, coupled with vicariant isolations — such as those produced by glaciation patterns — may lead to speciation (Avise 1994, 2000). In contrast, long-term maintenance of population size, a relatively constant reproductive rate, a presumably moderate gene flow, and K-selected species character traits coupled with isolation in an extreme environment can apparently lead to long-term retention of genetic diversity. Therefore, it is not predictive that a relatively small, specialized population will necessarily have low genetic diversity. Investigations of the genetic diversity of Congeria kusceri throughout its range will be valuable for further addressing these questions, along with analysis of nuclear DNA variation.

The cave faunas of the Dinaric karst of Slovenia, Croatia, and Bosnia-Herzegovina are highly endangered by ground-water pollution (Morton et al. 1998; Sket 1999). For example, the pit location from which our samples were collected has been used as a rubbish dump (Jalzic 1998). The K-selected population of C. kusceri houses significant genetic diversity that merits considerable further study, along with comparisons that are needed with other inhabitants of this unique environment (Sket 1999). Their 5 million years of troglobytic survival away from the extreme climatic fluctuations characteristic of surface-level aquatic habitats promises to reveal many secrets about the long-term effects of environmental constancy. Hopefully, we will not lose C. kusceri to anthropogenic disturbances before this can be accomplished.

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