**New insights into and limitations of the molecular phylogeny in the taxon-rich land snail genus *Montenegrina* (Mollusca: Gastropoda: Clausiliidae)**

***Short title:* Molecular phylogeny of the genus *Montenegrina***

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**Abstract**

Rock-dwelling gastropods are usually patchily distributed in limestone habitats, presumably have low active and passive dispersal ability and often represent narrow-ranged endemic taxa. Their current taxonomy is predominantly shell morphology based, and it remains unknown whether the morphologically differentiated and geographically separated populations represent true species.

In this study, we analysed the hyperdiverse door snail genus *Montenegrina*. Based on the current taxonomy defined by shell morphology, it contains 29 species and 106 subspecies distributed in the Balkan region. The constructed phylogenetic tree using three mitochondrial markers was used to test whether it agrees with the current taxonomy.

In this comprehensive tree, about half of the species and subspecies are monophyletic. Some of the paraphylies could be reasonably resolved by taxonomic changes, i.e. some subspecies should be reassigned or raised to species level. Other incongruences probably arose due to introgression even between distant clades. The histone genes turned out to be unsuitable for elucidating the phylogeny of *Montenegrina*. In the species delimitation tests, considerably more MOTUs were delimited than the number of presently described species. The present data indicate that (1) a shell morphology­-based taxonomy and taxon recognition can be problematic in such a large and morphologically highly variable genus; (2) the potential error due to incomplete sampling presents a problem in a genus as variable as *Montenegrina*; (3) multi-locus analyses should be conducted to arrive at a better basis for species delimitation; (4) integrative approaches including genetic as well as morphological/anatomical data from a comprehensive geographic sample are necessary.

**Introduction**

In certain areas of the Earth such as South East Asia, karstic regions show the highest biodiversity compared to other nearby geological regions (Clements, Sodhi, Schilthuizen, & Ng, 2006). In Europe as well, e.g., in the Calcareous Alps or the Balkans, karst areas represent biodiversity hotspots (Kryštufek & Reed, 2004; Hewitt, 2011). Although karst areas cover only a small part of the landmass, they provide specific habitats and are receiving increasing consideration in national conservation programs. Rock-dwelling gastropods significantly contribute to local species diversity in karst areas, for example the species rich genera *Albinaria* Vest, 1867 (Dimopoulou, Antoniou, Mylonas, Vardinoyiannis, & Poulakakis, 2017), *Alopia* Adams & Adams, 1855 (Fehér, Németh, Nicoara, & Szekeres, 2013), *Cristataria* Vest, 1867 (Páll-Gergely, Szekeres, Fehér, Asami, & Harl, 2019) and *Montenegrina* Boettger, 1877 (Fehér & Szekeres, 2016b). Obligate rock-dwelling gastropods exhibit limited active and passive dispersal ability, with long-distance dispersal mainly involving passive mechanisms (Uit de Weerd, Schneider, & Gittenberger, 2005), although detailed studies on the degree of passive and active dispersal and on particular strategies are scarce. These gastropods provide perfect examples of narrow-range taxa with presumably low dispersal ability. They are mostly restricted to limestone outcrops resulting in a very patchy distribution, and their habitat type can therefore be considered as island-like (Fehér & Szekeres, 2016b). Frequently, high morphological variability characterizes rock-dwelling snails. In most cases their taxonomy has been based on morphological traits, especially certain shell characters, yet taxonomic determination using such traits is sometimes ambiguous. A problematic factum in morphology-based taxonomy is that conspicuous morphological characters, e.g., shell coiling direction or ribbing, may appear several times independently, sometimes in distantly related taxa. This can make it difficult to assess whether similarity is due to a phylogenetic relationship or merely represents homoplasy (Uit de Weerd & Gittenberger, 2004; Uit de Weerd, Piel, & Gittenberger, 2004; Gittenberger & Uit de Weerd, 2006; Haase & Misof, 2009; Fehér et al., 2013; Kornilios, Stamataki, & Giokas, 2015; Páll-Gergely, Asami, & Sólymos, 2019). For some gastropod taxa the phylogeny based on DNA sequence data agrees with morphologically differentiated groups (Conde-Padín, Grahame, & Rolán-Alvarez, 2007; Harl, Páll-Gergely, et al., 2014). Nonetheless, in some taxonomic groups this is only partially true (Gittenberger & Uit de Weerd, 2006; *Agathylla*, Adams & adams, 1855:Fehér et al. 2013*;* *Pyramidula* fitzinger, 1833: Kirchner et al. 2016; Razkin et al. 2017) and in others no congruence between shell morphology and phylogenetic tree could be found (*Clausilia dubia:* Jaksch, 2012; *Trochulus hispidus*: Duda et al,. 2014). Conspicuous traits (like the presence/absence of ribs, which is longitudinal sculpturing on the shell) are frequently used by taxonomists, but are not necessarily phylogenetically informative traits. Moreover, other more specific (qualitative) characters such as plicae or lamellae, shell height or shell shape are sometimes too variable and not diagnostic.

Clausiliidae, or door snails, are the most speciose land snail family. They feature a unique structure – the clausilium – which is a calcareous plate for closing the aperture. Hence, many special conchological traits are present within this family. The high variability of these traitshas been the focus of several morphological studies in Clausiliidae (Szybiak & Lesniewska, 2008; Welter-Schultes, 2010). In a study on *Clausilia dubia* draparnaud, 1805, a morphologically extremely diverse species, Jaksch (2012) revealed rather high genetic diversity which, however, failed to reflect conchological differences or geographic groups. Especially in taxon-rich groups, where comprehensive sampling is a major challenge, the actual phylogenetic relationships and diversity behind high morphological variation have rarely been investigated (Hausdorf & Nägele, 2016; Scheel & Hausdorf, 2012). Therefore, the congruency of the morphology-based taxonomy with the phylogenetic framework often remains untested.

The genus *Montenegrina* is a taxon-rich but geographically narrow-ranged genus with specific habitat preferences (all species are obligate rock-dwelling and dependent on limestone) and a patchy, island-like distribution. It is a member of the door snail subfamily Alopiinae and comprises – according to the last revision – 29 species and 106 subspecies (Fehér & Szekeres, 2016b). The distribution range encompasses the western parts of the Balkan Peninsula (about 70.000 km2), extending from the southernmost parts of the Dinaric Mountains to the northern part of the Pindos Mountains. In obligate limestone-dwelling gastropods with presumably limited capacity of active and passive dispersal, karst outcrops function as ‘islands’ where isolated populations live (Gittenberger, 2007). According to Gittenberger´s assumption, highly diversified rock-dwelling gastropods represent examples of non-adaptive radiations (1991, 2004), i.e., it is unlikely that the high number of taxa is due only to (and consequently correlates with) numerous different ecological niches. Especially for *Montenegrina* the ecological conditions for the different populations appear to be very similar at first sight: they are restricted to limestone rocks and the general climate is supposedly rather similar over the distribution range. Some taxa, however, seem to have additional requirements, e.g., preferring somewhat higher elevations or are restricted to sites near the coastline. Recently, based on a huge data set on species occurrence, Fehér et al. (2018) confirmed the hypothesis of a non-adaptive mode of radiation in *Montenegrina*.

In an evolutionary sense, subspecies are usually considered as intermediate stages of an ongoing speciation process (Wu, 2001). In taxonomy the rank subspecies, albeit discussed for many years, usually regards to conspecific populations occupying distinct breeding ranges, which are diagnostically distinct from each other (Patten & Unitt, 2002).The majority of (land snail) subspecies, however, have been distinguished based on slight morphological differences only (geographic morphotypes) despite lacking information on contact zones or gene flow between subspecies. Páll-Gergely, Asami, et al. (2019) showed a correlation between shell complexity and the number of described subspecies. Unsurprising in *Montenegrina*, many of the presumably isolated populations that slightly differ morphologically have been described as subspecies. Whether and which of these morphologically differentiated and geographically separated populations should be classified as species instead of subspecies remains to be explored. The problem of species delimitation in gastropods has been controversially discussed in many taxa (Dépraz, Hausser, & Pfenninger, 2009; Falniowski, Szarowska, Glöer, & Pešić, 2012; Köhler & Johnson, 2012; Prévot, Jordaens, Sonet, & Backeljau, 2013). *Montenegrina*, as a morphologically highly diverse genus, is a prominent example for the challenging task to delimit species. To test the extent to which its phylogeny is congruent with its taxonomy, which has so far been established solely based on shell morphology, we conducted a comprehensive genetic analysis covering the whole distribution range and including almost all taxa of the genus. If available, material from the type locality was used. The phylogenetic tree reconstructions are based on three mitochondrial (*mt*) markers (*COI*, *16S* and *12S*) as well as on nuclear (*nc*) histone gene sequences (*histone H3 gene*, intergenic spacer *IGS*, *histone H4 gene*).

Within this study on the genus *Montenegrina* we addressed the following questions: (1) Is the current (shell based) taxonomy in accordance with clades in the phylogenetic trees? (2) Which proportion of the presently accepted species and subspecies are monophyletic? (3) What can be the main causes of non-monophyly? (4) Does the *mt* based phylogeny agree with that based on histone gene sequences? (5) How large are distances within and between groups (populations, subspecies and species)? (6) How reliable are species delimitation methods for assignments in the case of *Montenegrina*? The results of the present study (*mt* phylogeny, *nc* data, diversity) are an important basis for a broader integrative re-evaluation of the taxonomy of *Montenegrina* including shell morphology and genital anatomy, which could also have implications for other species-rich gastropod taxa. The synthesis of all these new taxonomical insights are summarised in the accompanying paper by (De Mattia, Fehér, Mason, & Haring, 2020).

**Material & Methods**

**Collecting Sites and Distribution of *Montenegrina***

The distribution range of the genus *Montenegrina* is restricted to the western Balkan Peninsula and includes the coastal regions of Montenegro (and reaching approx.150–160 km into the inland), Albania, western North Macedonia, and north-western Greece (Figure 1). Material was collected across the whole distribution range at 368 localities (Fehér et al., 2018), including all 29 species and 104 out of the 106 subspecies of the genus (Two populations of *M. perstriata* and one of *M. laxa* could not be assigned to subspecies yet and are indicated as *M. perstriata* ssp*.* and *M. laxa* ssp*.* in the trees, Figures 15, 16). In total, 823 specimens were used for DNA analysis, of which 408 were already used by Fehér et al., (2018). Most of them were specimens preserved in ethanol, while 40 specimens were mummified animals/bodies in dry shells, which were collected up to 112 years ago. All material is deposited in museum collections (Natural History Museum Vienna – NHMW, Hungarian Natural History Museum – HNHM, The Stuttgart State Museum of Natural History – SMNS, see Supporting Information Table S1). For one taxon (*M. hiltrudae dennisi*,Mdn) a published DNA sequence from the NCBI GenBank was included to complete the data set (GenBank Accession Nr. AY4255). As an outgroup, we initially used *Vallatia vallata* (Mousson, 1859) and subsequently added other genera (e.g., *Alopia* or *Clausilia*). Outgroup rooting resulted in very low bootstrap values of major clades, which might be due to substitutional saturation (specifically in the *COI* sequences). Finally, we decided to root the tree with clade G (which resulted in the same topology as obtained with midpoint rooting; data not shown). *Vallatia vallata* was also used as outgroup for the genetic distance calculations.

* Suggested position Figure 1

**Laboratory procedures**

For DNA extraction a small piece of tissue was used from ethanol-preserved specimens and whole specimens were used for the dry shell extraction. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer’s protocol. For extractions from dry shells the protocol was slightly adapted because multiple volumes of Lysis Buffers 1 and 2 were used to cover the whole shell. Moreover, the incubation time was extended to overnight. All following steps were performed using the original amount of ingredients as stated in the manufacturer’s protocol.

For phylogenetic analyses, partial sequences of three mitochondrial genes were amplified by PCR: (1) the *cytochrome c oxidase subunit I* gene (*COI*, 705 bp amplicon length, 791 specimens in total, 382 newly added herein), the *16S rRNA* gene (*16S*, 884–905 bp, 257 specimens in total, 16 newly added herein), and the *12S rRNA* gene (*12S*, 721–757 bp, 93 specimens in total, 15 newly added herein). The two rRNA genes were amplified only for representatives of those taxa expected to improve support for the branches, hence the lower numbers of these sequences. Furthermore, the DNA quality was low in some samples, which reduced the number of samples of successful *12S* amplifications. In addition, the nuclear gene region of the histone genes *H3* and *H4* as well as the intergenic spacer region (*IGS*) between them were amplified and sequenced (*H3-IGS-H4*). Due to double peaks and length variation in these marker sequences, several PCR products had to be cloned prior to sequencing and three clones were sequenced for each of these individuals (1101 bp, 198 sequences from 175 specimens). The primers used together with the annealing temperatures are given in Table 1. For PCR, TopTaq DNA Polymerase (Qiagen) was used, and for *COI* and *12S* MgCl2 (3mM) was added. The PCR profile started with an initial denaturation for 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C, annealing for 30 sec and elongation for 60 sec at 72°C, terminated by a final elongation phase of 10 min at 72°C. The *H3-IGS-H4* section was cloned for several specimens (see above), and for these, PCR was repeated using the proofreading Platinum® Taq DNA Polymerase High Fidelity (Invitrogen). This helped to avoid PCR errors in the cloned sequences leading to incorrect interpretations regarding variation of this marker sequence. These PCRs were run with a slightly different PCR profile (94°C for 2 min, 40 cycles of 94°C for 15 sec, 57°C for 30 sec, 68°C for 90 sec, no final elongation). Subsequently, PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Düsseldorf, Germany) and cloned (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA). Sequencing (both directions) was performed by LGC Genomics (Berlin, Germany) using the PCR primers, except for *H3-IGS-H4*,where internal primers were used (Mont\_insH4left, Mont\_insH3right). Cloned PCR products were sequenced using M13 universal primers. For all individually analysed sequences, DNA extraction type as well as GenBank Accession numbers (KU307511–KU308245, additional Accession numbers will be added in proof) are given in detail in Supporting Information Table S1.

* Suggested position Table 1

**Phylogenetic reconstruction of *Montenegrina***

All sequences were edited in Geneious 8.1.3 (Kearse et al., 2012). *COI* sequences could be unambiguously aligned (655 sites) as there were no insertions or deletions, while the separate alignments of *16S*, *12S* and *H3-IGS-H4* were performed in Geneious using the MUSCLE alignment algorithm and edited manually. The lengths of *16S* and *12S* alignments were 765 and 680 sites, respectively. Thereafter, the three alignments of the mitochondrial genes were concatenated in Geneious, resulting in a final alignment of 2102 bp (Supporting Information Alignment S1), including all available sequences (Table S1). As it was necessary to provide figures of partial trees, the *mt* data set was subdivided according to the major clades and the analyses were run separately (Clades AF, BC, D, E, G, HI, KLJ, Supporting Information Alignments S2 – S8). Each separated alignment was manually edited. For better display, some subsets had to be further separated and/or rearranged, but this was only done graphically. The length of the *H3-IGS-H4*alignment was 847 bp (*H3*: positions 1­310, IGS: 311­585, H4: 586­847). For network calculation, positions with gaps were excluded from the alignment using the online version of Gblocks (Castresana, 2000).

DNA sequences of the *COI* genes were translated to amino acid sequences using the invertebrate mitochondrial genetic code option of the MEGA6 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Alignment and homology analysis were done with the same program. Amino acid positions are numbered from the N-terminus of the complete protein.

PARTITIONFINDER 1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012) was used to select the appropriate partitioning scheme and models of sequence evolution for the *mt* phylogeny. The list of nucleotide evolution models was restricted to those available in the programs used for further analyses. The following 5-partition schemes and models were used: *COI* 1st codon position: GTR+G, *COI* 2nd codon position: HKY+I, *COI* 3rd codon position: GTR+G, *16S*: GTR+G, *12S*: GTR+G.

Bayesian trees were calculated using MRBAYES 3.2.1 (Ronquist et al., 2012) with a ten-chain analysis (one cold, nine heated) run for 5×106 generations each, and trees were sampled every 100th generation. After discarding the first 25% of trees as burnin, a 50% majority rule consensus tree was calculated from the remaining trees. With the *H3-IGS-H4* alignment, a simple NJ-tree was calculated in MEGA version 6 (Tamura et al., 2013). Various other trees were calculated with this data set as well, but the outlook of these trees was more complicated to interpret and lacked resolution due to very low distances between haplotypes and haplogroups (data not shown). Therefore, a Median Joining Network (Bandelt, Forster, & Röhl, 1999) calculated in POPART 1.7. (Leigh & Bryant, 2015) is shown, as this best illustrates the data. For the species delimitation tests, an ML tree was constructed with 742 *COI* sequences (655 bp) using the online version of PhyML 3.0 (Guindon et al., 2010), applying Smart Model Selection (SMS) with the Bayesian Information Criterion (Lefort, Longueville, & Gascuel, 2017) and approximate likelihood-ratio test (aLRT, Anisimova & Gascuel, 2006) to obtain branch support values.

**Calculations of inter- and intraspecific distances**

Average *p*-distances of the *COI* dataset within and between clades, species and subspecies were calculated in MEGA6 (Tamura et al., 2013). To allow comparisons with previous work (Fehér et al., 2018), we used the same letter code for the 13 clades (see results). The highly distant lineage of *M. sporadica sporadica*, which is the sister group to clades B and C and represented by a single sequence only, was excluded from these calculations.

**Species delimitation tests**

We used the *COI* dataset (742 sequences) to test two different molecular methods of *de novo* delimitation of molecular operational taxonomic units (MOTUs), a clustering (Automatic Barcode Gap Discovery, ABGD, (Puillandre, Lambert, Brouillet, & Achaz, 2012) and a tree-based method (Poisson Tree Processes, PTP; Zhang, Kapli, Pavlidis, & Stamatakis, 2013). ABGD relies on pairwise sequence distances between specimens to determine the number of MOTUs within a dataset. ABGD employs a two-phase system which initially divides sequences into OTUs based on a statistically inferred barcode gap (i.e., initial partitioning), and subsequently conducts a second round of splitting (i.e., recursive partitioning). The analysis was carried out on the ABGD Web Server (<http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html>) with the following settings: Pmin – Pmax (range of prior intraspecific divergence): 0.01– 0.1 with 20 steps in between, X (relative gap width): 0.05, Nbin: 10 and K2P distances with the default Ts/Tv ratio (2.0). Due to the ability to set a range for the prior intraspecific divergence, such ABGD typically produces a range of MOTU counts.

PTP is a tree-based method employing a non-ultrametric gene tree as input for the analysis. It models branching events in terms of number of substitutions and conducts a search for the transition points where the branching pattern changes from an among-species to a within-species branching pattern. Unlike the more commonly used Generalized Mixed Yule Coalescent (GMYC) method (Pons, Barraclough, Gomez-Zurita, & Cardoso, 2006), PTP directly uses the number of substitutions and therefore does not require ultrametricity. It was found that PTP outperforms GMYC (Zhang et al., 2013). The analysis was run on the bPTP web server (<http://species.h-its.org/ptp/>) with 500000 MCMC generations and with the default burn-in (0.1).

To evaluate how MOTU divisions match morphospecies assignments, a simple metric, was calculated as follows: Each individual is compared to all individuals (including itself), resulting in an n×n data matrix (*C*), where matrix cells (*Cij*) could take on four different values depending on whether the two individuals of the given pair are in the same species or not and whether they are in the same MOTU or not.

*Cij* = (1,1) if i­th and j­th individuals belong to the same morphospecies and are in the same MOTU;

*Cij* = (1,0) if i­th and j­th individuals belong to the same morphospecies but not the same MOTU;

*Cij* = (0,1) if i­th and j­th individuals belong to different morphospecies but are in the same MOTU;

*Cij* = (0,0) if i­th and j­th individuals belong to different morphospecies and are in different MOTUs.

By definition, the matrix values in the axis (*Cii*, i.e. when an individual is compared to itself) take the value (1,1). For each individual (i.e. in each row of the matrix from *Ci1* to *Cin*) we calculated the rate of (1,1) counts to the sum of (1,1), (1,0) and (0,1) counts using the COUNTIF formula of MS Excel.

Thereafter, morphospecies–MOTU match values were calculated for each morphospecies (MMMspec) as an average of the individual MMMi values. MMMspec takes the maximum value (1.0) when all individuals of a given morphospecies, and no individuals of any other morphospecies, are binned into the same MOTU.

**Results**

**Phylogenetic analyses – Overview Topology**

In the phylogenetic analyses, 823 specimens, representing 104 out of the 106 known taxa of the genus, were analysed. To provide an overview of the phylogenetic relationships within *Montenegrina*, a BI tree was calculated based on all three *mt* marker sequences (Figure 2). In this overview tree, species were collapsed into triangles because the data set was quite large, covering 28 species (104 subspecies). Detailed partial trees are presented below. To enable comparisons with previous work (Fehér et al., 2018), we used the same letter code for the 13 clades (letters A–L, D subdivided into D1 and D2; Note that D1 is not monophyletic in the overall tree in contrast to the tree presented in Fehér et al. (2018)). Most of the clades obtained maximum support values, except clades D, E and K. Most clades comprise three to four species; only one clade (A) represents just one species. In contrast, one species (*M. skipetarica*) with 15 subspecies ranges over two clades (B and C). The taxon-richest clade L includes 4 species and 15 subspecies. The designation of these clades is necessary to describe the results and the positions of the various taxa in this complex tree, but as a matter of fact the clades are not expected to correspond completely with taxonomy or reflect taxonomic hierarchy. When comparing current taxonomy based on morphology with the phylogenetic results gained by molecular genetic data, an important issue is monophyly. Overall, many of the taxa are more or less congruent with the gene tree and represent separate clades or subclades. Nonetheless, there are several cases of paraphyly, where species are distributed over more than one clade or intermingled with representatives of other species (Supporting Information Table S2). At the species level, 16 of the 28 species analysed appeared to be monophyletic (at the subspecific level 67 out of 104 were monophyletic). Some of the exceptions (i.e., the “problematic taxa”) are described below.

* Suggested position Figure 2

**Genetic distances of *COI* sequences**

To provide an overview on intrageneric distances in *Montenegrina* and differentiation of clades, we calculated average *p*-distances within and between clades (Table 2). The mean distances between clades in the data set of the *mt* gene *COI* ranged from 11.7% to 21.5% (17.3–21.9% to the outgroup, *V. vallata*). The clade with the lowest maximum within *p*-distances is J (11.7%), while clade E has the highest distance (22.7%).

Examinig the *p*-distances within species (comprehensive data and ranges of distances are given in Table 3), high values for maximum intraspecific distances in *COI* were found within *M. skipetarica* and *M. perstriata* (both 21.3%), followed by *M. sporadica* with 20.9%. The species with the lowest values are *M. apfelbecki*, *M. haringae and M. lillae* with 0%, followed by *M. zilchi* with 0.4%, yet for these samples only a small sample size was available. Mean and maximum distances within the species are also illustrated in Figure 3. The highest mean values were recorded in *M. perstriata* (14.7%) followed by *M. skipetarica* (12.9%) and *M. fuchsi* (12.7%). As the phylogenetic analyses in some cases showed discrepancies with respect to taxonomy due to outlying sequences (causing paraphylies as described below), we calculated the distances again after exclusion of those sequences (Figure 3). After this procedure, the highest mean distances within the species were still high, slightly below 12%. A similar picture was obtained by calculating mean distances between subspecies (within the same species) (Figure 4). Apart from the problematic taxa (due to paraphylies) the mean distances between subspecies were up to 9.5%.

* Suggested position Table 2 and Table 3

Figure 5 provides an overview on the mean and maximum *p*-distances within subspecies of the same species. That figure also shows the results after excluding paraphyletic subspecies. For ten subspecies no within *p*-distances could be calculated because only one sequence was available. A further 24 out of the 104 subspecies analysed showed no intra-subspecific variation at all (Supporting Information Table S3). Interestingly, for the other 70 subspecies, the maximum intra-subspecific distances ranged from 0.4% up to 18.7%, the latter value being nearly as high as values found for intraspecific distances.

* Suggested position Figures 3, 4 and 5

**Amino acid substitutions in the *COI* protein**

Whereas most of the *COI* mutations did not alter the encoded protein, some of them caused amino acid replacements that are conserved in entire clades. *Montenegrina* exhibits nine such positions (31, 41, 47, 69, 93, 106, 130, 131, 132). Replacements that are conserved in multiple clades are at position 41 (Cys in Clades A to F, Val in Clade G, Ser in Clades H to L), 93 (Met in Clades A to F, Leu in Clades G to L), and 132 (Leu in Clades A to G, Ile in Clades H, I, K, L, Met in Clade J) (Supporting Information Figure S1). Intriguingly, taxa of Clade G carry amino acid replacements, unique to this clade, at multiple positions (Val at 41, Asn at 47, Gly at 130, Asn at 131). The conserved replacements of possible evolutionary significance coincide with, and clearly delineate, the three main branches of the *COI* tree.

**Partial trees –– concordance and problems**

In the following we describe the various clades and subclades which are shown in several partial trees (Figures 6–15). Thus, the order of clades in the text is not alphabetical, but according to how they are combined in the partial trees. The distribution of clades is illustrated in Figure Supporting Information S2 A­E, the coordinates can be accessed online (Fehér & Szekeres, 2016a).

The most basal split in the tree separates clade G (Figure 6) from the rest. It contains four species, which are all monophyletic, with the exception of *M. sporadica*. While only *M. sporadica tropojana* is placed within clade G, *M. sporadica sporadica* has a very distant position in the tree as sister group to clades B and C (Figure 7, in the following termed clade BC). The mean *p*-distance between the two subspecies is very high (20.4%). Both taxa have a very small distribution range and their known localities are only about 10 km apart (north-eastern Albania and north-western Kosovo) (Supporting Information Figure S2 A). In general, they live in close vicinity to the three other taxa of Clade G, and the distribution area of this clade is separated from all other *Montenegrina* populations. The geographic distance to the nearest populations of Clade B and C is about 70 km.

The remaining part of the overall tree (Figure 2) is divided into two major groups, one comprising clades A to F and the other comprising clades H to L.

Clades B and C (Figure 7) contain only one species, *M. skipetarica*, which is one of the most taxon-rich species. In the tree most of the 15 subspecies are monophyletic. The two clades show a geographic pattern (Supporting Information Figure S2 A): With two exceptions the subspecies of clade B are distributed in northern Albania and those of clade C in southern Albania. The exceptions are *M. skipetarica csikii*, which is located in northern Albania but appears in clade C, and *M. skipetarica pindica* from southern Albania, which appears in clade B. The intraspecific distances within *M. skipetarica* are 12.9%. Considering the two phylogenetic clades B and C separately, the p values become only slightly lower (B 10.6%, C 11.9%). Only four subspecies turned out to be not monophyletic (*M. skipetarica pifkoi*, *M. skipetarica voidomatis*, *M. skipetarica gurelurensis*, *M. skipetarica puskasi*). *Montenegrina skipetarica gurelurensis* and *M. skipetarica puskasi* are found in two sister subclades (mean distance 3.3%) but are genetically mixed up with each other. They have a very restricted distribution areas and live in parapatry with a small contact zone. As mentioned above, the sister group to clades B and C isformed by the nominate subspecies of *M. sporadica*, while the other subspecies *M. sporadica tropojana* is located in clade G.

* Suggested position Figures 6 and 7

Clade F (Figure 8; Supporting Information Figure S2 B) shows a clear picture comprising three well-differentiated species. *Montenegrina subcristata* is the largest species in terms of distribution area and number of known populations within the whole genus. It is morphologically very variable, but the two formerly described subspecies have recently not been recognized any longer (Fehér & Szekeres, 2016b). Also the *mt* markers did not show any subspecific differentiation, although the intraspecific distances are rather high (7.6%). In comparison, *M. cattaroensis*,which is, besides *M. subcristata*, the second species occurring in Montenegro, has a mean intraspecific distance of 4.7% (three subspecies). *Montenegrina cattaroensis* inhabits several localities along the coastline of Montenegro and occurs close to *M. subcristata*. Only one subspecies, *M. cattaroensis cattaroensis*, is clearly differentiated in the tree, the other two are intermingled. The third species within this clade is *M. haringae*, which forms a highly distant lineage. It is known from only two localities on the coastline of northern Albania.

* Suggested position Figure 8

The closely related Clades D and E are all very taxon-rich (in contrast to the overall tree, subclade D1 is a monophylum in the partial tree). Three species and eleven subspecies are present in Clade D1 (Figure 9), three species and eight subspecies in Clade D2 (Figure 10), and four species with 13 subspecies in Clade E (Figure 11). In total, only four of those 10 species are monophyletic. Most of the taxa in Clades D2 and E are located in the area around the Lakes Ohrid and Prespa in south North Macedonia and Albania, a few taxa occur further south as far as northern Greece (Supporting Information Figure S2 C). Only the monophyletic *M. apfelbecki* is found in northern Albania. This species is known solely from the type material and could be extracted only from dry mummies. The taxa of Clade D1 occur more in the western parts of southern Albania and northern Greece.

Clade D1 (Figure 9) comprises the seven subspecies of *M. rugilabris*, which all form separated subclades. The distribution area stretches from southern Albania to northern Greece over about 100 km. Two other species are intermixed within *M. rugilabris*, rendering this species paraphyletic: *M. janinensis* from the area of Lake Pamvotida in Greece, which forms a monophyletic clade, and *M. fuchsi.* All three taxa show very similar distances between (sub)species, ranging from 7.1% to 15.6% (except *M. rugilabris lambdaformis* and *M. rugilabris welterschultesi* with only 1.3%). *Montenegrina fuchsi* also occurs in southern Albania, close to some populations of *M. rugilabris*. *Montenegrina fuchsi* itself is paraphyletic, as three subspecies emerge as separate lineages within *M. rugilabris* and one subspecies (*M. fuchsi muranyii*) appears in clade K next to *M. tomorosi*.

Clade D2 (Figure 10) comprises mainly seven subspecies of *M. dofleini*. Nonetheless, it is paraphyletic due to the presence of three other species in this clade: *M. hiltrudae robusta* is intermingled with specimens of *M. dofleini kastoriae*. Geographically the populations are only about 15 km apart. In addition, four specimens of *M. hiltrudae desaretica* appear next to the *M. dofleini prespaensis/sinosi* group, whereas the remaining *M. h. desaretica* specimens appear in clade E. Furthermore, *M. stankovici* clusters together with *M. dofleini pinteri* (of which only one population is known; 1.9% distance between). The latter two taxa co-occur at a locality at Lake Ohrid (NMN) in very close proximity, but they seem to prefer different microhabitats: as *M. stankovici* only lives very close to the water surface, *M. dofleini pinteri* in higher regions of the rocks. Another inconsistency with current taxonomy is *M. dofleini wagneri* which forms a separate branch within Clade E.

Clade E (Figure 11) consists of *M. apfelbecki*, *M. zilchi*, *M. dofleini wagneri* and *M. hiltrudae.* The former two taxa are known only from their type localities and form well-separated lineages. These two taxa are also distantly distributed from all other taxa of clades D1, D2 and E, one marking the northernmost occurrence (*M. apfelbecki*) and one the most southern (*M. zilchi*). *Montenegrina dofleini wagneri* is the sister group of *M. hiltrudae*, while the other subspecies of *M. dofleini* are intermingled in D2. Of the eleven subspecies of *M. hiltrudae*, nine are present only in Clade E, while *M. hiltrudae robusta* and *M. hiltrudae desaretica* are present in Clade D2 (see above). *Montenegrina hiltrudae* has a rather small distribution area, covering mainly the area around Lake Prespa. While *M. hiltrudae* in Clade E is distributed in several distinct subclades, there is no strict congruence between subspecies and subclades. Some subspecies are not well separated: *M. hiltrudae desaretica* is split up in two subclades (and one population of *M. h. desaretica* even shows up as sister group to *M. dofleini prespaensis/sinosi* in clade D2, resulting in a mean intraspecific p-distance of 10.4%; see above).

* Suggested position Figures 9, 10 and 11

Clade A (Figure 12) represents *M. attemsi* with its two subspecies, which inhabit small distribution ranges in North Macedonia. Their known localities are only about 30 km apart and very isolated from any other *Montenegrina* populations (Supporting Information Figure S2 B). Clade Ais well separated from its sister group (D1+D2+E+F).

In the second major group of clades, clade H (Figure 13) turned out to be taxonomically one of the most complicated clades. It contains four species with seven subspecies. The predominant species within this clade are *M. sturanyana* and *M. perstriata*. *Montenegrina sturanyana* with three subspecies shows a close relationship with *M. timeae* (itself forming a monophyletic group), but the nominate subspecies, *M. sturanyana sturanyana*, clusters next to the *M. perstriata* complex*.* Geographically, all those taxa occur quite close to each other in western North Macedonia and central to southern Albania, although *M. timeae* inhabits lower regions, whereas *M. sturanyana* ssp*.* occurs in higher altitudes (Supporting Information Figure S2 D). *Montenegrina perstriata* turned out to be polyphyletic as its eleven subspecies are scattered over four different clades (H, I, K, L). There is no clear geographic differentiation between the taxa of the different clades. The mean genetic distance within this species is 14.7%. Two of the three *M. perstriata* subspecies in clade H form a monophyletic group (*M. perstriata occidentalis*, *M. perstriata plenostoma*), but *M. perstriata drimica* forms separated groups. Calculating the *p*-distances only for the *M. perstriata* specimens in clade H yields a lower value (8.8%). Nonetheless, the phylogenetic relationships appear quite complex because several individuals of *M. perstriata drimica* appear in clade K next to *M. perstriata radikae*. Within the *M. perstriata* group in clade H, one individual of another species, *M. drimmeri*, clusters with *M. perstriata drimica.* All other individuals of *M. drimmeri* are present in clade L.

Clade I (Figure 13) comprises three species and eleven subspecies. *Montenegrina helvola* is monophyletic in the *mt* gene tree (intraspecific *p*-distance: 7.5%) and four of the five subspecies are monophyletic, whereas *M. helvola ornata* is paraphyletic. The differentiation within this species reflects the geographic distribution of subspecies. Whereas *M. helvola* lives more in the west from central to southern Albania, the populations of *M. nana* occur very close to each other in eastern Albania (Supporting Information Figure S2 D). *Montenegrina nana* forms a distinct clade but is paraphyletic due to three samples of *M. perstriata callistoma* within this clade*.* Finally, *M. perstriata* forms a separated group with two distinct lineages, but the three subspecies are not clearly differentiated.

Clade J (Figure 12; Supporting Information Figure S2 E) contains only two species and one subspecies. *Montenegrina minuscula* is monophyletic. The sister group of *M.* *minuscula* is one particular population from the Tomorr Mountains that is shell morphologically classified as *M. tomorosi tomorosi*. It is located over 100 km away from the known *M. minuscula* populations. The remaining *M. tomorosi tomorosi* samples are all in clade K, resulting in the highest maximum intra-subspecific *p*-distance of 18.7%.

* Suggested position Figures 12 and 13

Clade K (Figure 14) comprises four species and altogether nine subspecies. While six subspecies turned out to be monophyletic, only one species is monophyletic (*M. grammica*). All three subspecies of *M. grammica* show a very small distribution area and are located very distant to each other and to all other taxa in this clade (Supporting Information Figure S2 E). *Montenegrina grammica improvisa* and *M. grammica erosszoltani* live in northern Albania, about 20 km apart from each other, whereas *M. grammica grammica* occurs in northern Greece. Unfortunately, for each subspecies only one (*M. grammica improvisa*) or two specimens (*M. grammica grammica*, *M. grammica erosszoltani*) were available. The maximum intraspecific distance is 14.2%.This clade also contains *M. fuchsi* (which is otherwise located in clade D1), with its fourth subspecies *M. fuchsi muranyii*, which emerges within the *M. tomorosi*-group (*p-*distances between *M. f. muranyii* and *M. tomorosi* range from 6.7% to 8.7%). *Montenegrina fuchsi muranyii* occurs in the Tomorr Mountain area along with *M. tomorosi*, whose subspecies are all very closely grouped together and mixed up in the phylogenetic analyses. The exception is one *M. tomorosi* population which clustered with the distantly related *M. minuscula* (Clade J, see above). Finally, *M. perstriata* is represented in Clade K with two subspecies: *M. perstriata radikae* as well as the remaining samples of *M. perstriata drimica*.

Clade L (Figure 15) comprises the highest number of taxa, representing four species and 15 subspecies. Only one species (*M. soosi*) is clearly monophyletic. The four subspecies of *M. perstriata* also form a monophyletic group within Clade K but as described above, other taxa of this species occur in two other clades. Furthermore, there is a large subclade representing *M. laxa* with a mean intraspecific genetic distance of 7.5%. From the nine subspecies of *M. laxa*, four are not monophyletic and the monophyly of the whole species is broken only by *M. drimmeri*. *Montenegrina drimmeri* itself is also not monophyletic because other samples were found in Clade H. Geographically, all populations of this clade are located in Albania (Supporting Information Figure S2 E). *Montenegrina drimmeri* is known from one locality only (Lumi i Murrës) in close proximity to *M. laxa disjuncta*.

* Suggested position Figures 14 and 15

**Comparison with *nc* markers**

To test whether a nuclear marker sequence would reflect the mitochondrial phylogenetic tree, the histone gene section *H3-IGS-H4* was used. Histone gene sequences (*H3* or *H4*) have been used earlier in phylogenetic studies of snails, but the IGS has rarely been employed so far (e.g., Harl et al. 2014). As outlined in the following section, the analysis of this marker sequence turned out to be challenging and the results are difficult to interpret. Initially, there seemed to be hints for hybridisation and we therefore continued to collect more data. At a certain stage, however, we ceased to generate further sequences because we could not exclude that paralogous gene clusters were involved (see discussion). Nonetheless, as *histone* genes are widely used genetic marker sequences, it seemed important to show these results in detail and to discuss their information content and problems of interpretation.

Altogether, the *H3*-*IGS-H4* section was analysed in 174 individuals. In several individuals, where direct sequencing delivered unreadable sequences due to length variation of different copies, PCR products were cloned and several clones were sequenced. The distribution of taxa and their sequence variation within the various haplogroups are illustrated in a network in Figure 16. Eight haplogroups, some of which contain only a few sequences, are described in the following. Since haplogroups and *mt* clades are not completely congruent, we numbered the haplogroups (1­8) for the following description of the results. One haplogroup (1) appears highly diverged, separated from the nearest haplogroup 6 by a branch of 12 substitutions. It contains sequences representing the *mt* clades B, H, J, K, L. Besides haplogroup 4 (corresponding with Clade F), which is separated by a branch of 11 substitutions, the other haplogroups are more closely related. Four small haplogroups correspond (almost) exactly with *mt* clades: Haplogroups 2, 3, 4, 5 (Clades A, B, F and G). For group 3 there are some exceptions because some individuals with *mt* haplotypes of clade B are also found in *H3-IGS-H4* haplogroups 1 and 6. Haplogroup 7 consists of individuals with D and E *mt* haplotypes, and haplogroup 6 comprises individuals with *mt* haplotypes of *mt* clades C, D, E, plus one individual of clade B. Finally, haplogroup 8 contains individuals of *mt* clades H, I, K and L. This group is subdivided into four subgroups (which themselves are not congruent with respect to the four clades). In several cases, two different haplotypes were found in one individual, in a few cases even three (e.g., Mds-618-03). In the quite distinct haplogroups 7 and 6, many samples of *mt* clades D1 and D2 are separated, including differing haplotypes from single individuals. In haplogroups 1 and 8, *H3-IGS-H4* haplotypes are shared by individuals belonging to up to four *mt* clades. Altogether, only *mt* clades A, B, F and G clearly correspond to single haplogroups in the *nc* network.

* Suggested position Figure 16

**Species delimitation**

PTP delimited 218 MOTUs. ABGD delimited different numbers of MOTUs with differing prior maximal distances (P): 196 (P= 0.0100), 193 (P= 0.0113), 192 groups (P= 0.0127), 185 (P= 0.0144), 174 (P= 0.0162), 162 (P= 0.0183), 148 (P= 0.0207), 143 groups (P= 0.0234), 141 (P= 0.0264), 138 (P= 0.0298), 124 (P= 0.0336), 101 (P= 0.0379), 89 (P= 0.0428), 77 (P= 0.0483), 72 (P= 0.0546), 65 (P= 0.0616), 26 (P= 0.0695) and 1 (P= 0.0785).

Dividing schemes that split *Montenegrina* into many (~200) MOTUs kept only those species together of which only one (*M. apfelbecki*, *M. haringae*, *M. lillae*, *M. zilchi*) or just a few populations (*M. stankovici*, *M. okolensis*) are known. Larger species were mostly split into smaller groups representing narrow –ranged subspecies or even local populations. Those divisions that split *Montenegrina* into fewer MOTUs did it in an asymmetric manner (compare the results in Supporting Information Table S4, Supporting Information with the tree in Figure 2): For example the first ABGD step delimited 26 MOTUs, which is almost equal to the number of accepted morphospecies, but these 26 MOTUs were not congruent with the shell-based taxonomy of Fehér & Szekeres (2016b). One very large MOTU contained the whole clades D1, D2, E and F, representing approximately half of all sequences. Another MOTU consisted of the whole clades K and L (114 sequences), another one comprised clade I (62 sequences), and finally another large MOTU comprised clade H (45 sequences). All those MOTUs encompasses a considerable number of described taxa. At the same time, clades A, B, C and G were split into several small MOTUs containing subspecies or closely related populations: e.g., *M. attemsi* was split into subspecies (*M. attemsi attemsi* and *M. attemsi jakupicensis*) in this step.

We calculated morphospecies–MOTU match values (*MMM*spec) for all morphospecies by all MOTU divisions (resulting in 26 to 218 MOTUs) (Supporting Information Table S4). Out of the 16 non-problematic *Montenegrina* species (which seemed to be doubtless based on shell morphology and form a monophyletic clade in the tree), 13 have a maximum *MMMspec* value (1.0) at least in one of the divisions. Out of these species, *M. apfelbecki*, *M. okolensis*, *M. haringae* and *M. lillae* got maximum *MMMspec* values at all dividing schemes. *Montenegrina. subcristata* got maximum *MMMspec* values at the 65 and 72 groups’ divisions only, whereas *M. stankovici* got maximum *MMMspec* values at the most detailed divisions. It is noteworthy that *M. attemsi*, *M. grammica* and *M. helvola* did not get maximum *MMMspec* values at any of the divisions.

**Discussion**

The mitochondrial phylogeny of the genus *Montenegrina* was in many parts, but not entirely, congruent with the morphology-based taxonomy of the genus (Supporting Information Table S2). As there are several reported cases for such a phenomenon, including some related genera of the family Alopiinae (Giokas, 2000; Uit de Weerd & Gittenberger, 2004), this finding was not surprising. In general, the *COI* tree shows some well-recognizable zoogeographic patterns of different clades (see below).

In *Montenegrina*, character states of variable amino acid positions coincide with main groups of clades. The fact that, within *Montenegrina*,several amino acid changes in the *COI* protein were deduced from the DNA sequence is in contrast with a single variable site found in the genus *Alopia* (Fehér et al., 2013). Nonetheless, it remains speculative whether these replacements are conserved in a clade-specific manner due to episodes of positive selection, as has been shown for several animals and various *mt* genes (Garvin et al., 2017; Garvin, Bielawski, Sazanov, & Gharrett, 2015; Slimen, Allio, & Jacques, 2016).

The subspecific differentiation in many cases agrees with the *mt* clades of the tree presented here, whereas at the species level more than 1/3 of the species are problematic (see Table S2). The question arises whether some of the species described so far comprise more than one (some of them possibly cryptic) species. A high number of paraphylies might support this assumption although, importantly, a lack of monophyly might have various causes (Boettger, 1932; Funk & Omland, 2003). These include uninformative marker sequences (see Sauer & Hausdorf, 2012 concerning single locus vs. multi-locus markers), incomplete lineage sorting, introgression (Harl, Haring, & Páll-Gergely, 2019; Koch, Neiber, Walther, & Hausdorf, 2017; Lammers et al., 2013) or budding speciation (Kruckenhauser et al., 2014). The category of subspecies deserves some elucidation. In malacology, taxonomists often do not stick to strict definitions of subspecies but rather make arbitrary decisions concerning species or subspecies rank, based on the degree of morphological differences. We attempted to base the present study on as many taxa (species and their described subspecies) as possible. When we queried monophyly of subspecies, this was done because of the assumption that the genus *Montenegrina* might in fact harbour undetected (cryptic) species. Thus, the trees should reveal whether described species are monophyletic, but also if single subspecies represent distinct lineages. The fact that we found many paraphyletic subspecies within species, which are themselves monophyletic in the *mt* tree (e.g., *M. cattaroensis*, *M. grammica*), might easily be explained by gene flow as well as incomplete lineage sorting, and might even put into question some of the described subspecies.

Altogether, the task to resolve *Montenegrina* taxonomy has limitations that may be ascribed to several phenomena. For example, homoplasy (or even convergence) of shell traits may impede shell –based taxonomy to some extent. Concerning *mt* DNA-based phylogenetic reconstruction, introgression due to hybridization (recent hybridization after secondary contact or old hybridization events) may blur a clear topology of phylogenetic trees. The hope that inconsistencies between *mt* tree and taxonomy could be interpreted with the help of the *histone* gene sequences was mostly in vain. Even though *histone* gene sequences have been used (with varying success) in previous studies on gastropods (Armbruster, Boehme, Bernhard, & Schlegel, 2005; Harl, Páll-Gergely, et al., 2014; Harl, Duda, Kruckenhauser, Sattmann, & Haring, 2014), the present study exemplifies the remaining difficulties in the search for informative nuclear marker genes. Unfortunately, the *H3*-*IGS*-*H4* data proved neither suitable for a phylogenetic reconstruction in *Montenegrina*, nor provided conclusive results concerning hybridization. While some main clades corresponded with haplogroups in the *nc* network, there were several mixed haplogroups composed of representatives of various clades; this might be interpreted as an indication for paralogous histone gene clusters. Recombination events between paralogous gene clusters (see Harl et al., 2019) could blur the pattern even further. Unfortunately, we currently know little about the genomic organization of histone gene clusters in molluscs. While Li et al. (2005) reported only one cluster in *Chlamys farreri* jones & preston, 1904, García-Souto, Pérez-García, Morán, & Pasantes (2015) showed that the number and location of the gene clusters varied in venerid clams. Gastropods have not been analysed in detail with respect to the organisation of their histone gene clusters. Although the median-joining network cannot easily be divided into two or more paralogous loci, the fact that the divergent haplogroup 1 contains representatives of five *mt* clades is difficult to explain based solely on introgression. For single individuals the results could also be explained by hybridization, but it seems impossible to disentangle the various potential reasons for the observed patterns in the network: e.g., introgression due to hybridization, incomplete lineage sorting, paralogous gene clusters, or even recombination between divergent copies (see Harl et al., 2019). Possibly all those factors contributed to the observed pattern. Genomic data would be helpful to unveil the number and locations of histone genes/gene clusters in *Montenegrina.*

An important conclusion of this study is that the possible error due to incomplete sampling is a problem especially in a genus as huge and hypervariable as *Montenegrina*. The present study is based on a very comprehensive sampling. In view of the complex phylogenetic trees, it becomes clear that sampling effort may considerably affect the inferred systematic framework (tree topology, clade compositions, distribution ranges). We found highly distinct lineages occurring in very restricted, isolated areas, sometimes within the range of other lineages. Such local lineages might be easily missed during collecting trips. This small-structured phylogeographic pattern, together with the high morphological variability of *Montenegrina*, makes its phylogenetic reconstruction specifically prone to sampling effects.

In the following, we discuss some examples of incongruences, resulting taxonomic problems and their interpretation. Importantly, a solely *mt*-based phylogeny as presented here cannot fully resolve these questions. As shown in a survey of the diverse marine deep-sea gastropod genus *Bolma* risso, 1826 (Castelin et al., 2017), interpreting traditional as well as molecular genetic investigations separately can easily lead to biased results concerning species delimitation. We refrained from drawing any taxonomic conclusions within this study. A thorough anatomical investigation was conducted in the accompanying paper by De Mattia et al., (2020), and further genetic investigations are ongoing. A synthesis of genetic data with genital anatomy and shell-based taxonomy is presented in a separate study (De Mattia et al., 2020).

**High intraspecific divergence without paraphyly**

For some monophyletic *Montenegrina* species, extremely high distances between subspecies raise the question whether some of them should be classified as separate species. An extreme example is *M. skipetarica* with its 15 assigned subspecies. This species clearly showed up as a monophyletic group, but the maximum intraspecific distance of 21.3% suggests that it might include more than one species. It spans over two clades that show, except for two subspecies, a clear geographic pattern, and each of the clades is further divided into subclades, which in general correspond to subspecies. In this case the genetic results agree with the morphological determination, and they also support a taxonomic split between the two clades. Notably, even if the species was split into two species (corresponding to clades C and B), the intraspecific distances would remain very high (mean >10%; maximum >18%). Further species with high intraspecific distances show a general congruence between current taxonomy and genetics: *M. subcristata* (maximum intraspecific distance 13%), which has the largest distribution area and the largest number of known populations within the whole genus (Figure 8). It is morphologically very variable and several subspecies have been described up to now, but Fehér & Szekeres, 2016b revoked this subspecific division because the morphological differences allowed no clear delineation of two (or more) morphotypes. The *mt* DNA tree supports this concept, because we found no clear differentiation of particular shell traits between intraspecific clades. Another example for a very high intraspecific *mt* distance is *M. helvola* (maximum intraspecific distance 14%) with five subspecies,where in general (with one exception) there is good agreement between the DNA –based tree and taxonomy. High intraspecific distances have been reported from other land snail taxa (Thomaz, Guiller, & Clarke, 1996; Watanabe & Chiba, 2001; Sauer & Hausdorf, 2012; Scheel & Hausdorf, 2012; Harl, Páll-Gergely, et al., 2014; Kruckenhauser et al., 2014) and thus do not necessarily prove the existence of cryptic species.

**Incongruences between shell-based taxonomy and genetic tree**

The observed incongruences between shell morphology and genetics were of two different types: either the shell morphology was clearly distinguishable, but the phylogenetic results showed very close relationships and no clear differentiation, or the shell morphology did not allow any different taxonomic assignment, but in the genetic analysis the specimens showed up in different positions, sometimes very far from each other.

Good examples for the first case are some of the co-occurring taxon pairs. Within *Montenegrina* there are some cases where two different species co-occur syntopically or at least occur in very close vicinity (parapatrically). One case already described above is the co-occurrence of *M. dofleini pinteri* and *M. stankovici*, which form monophyletic sister groups. Their morphological differentiation might reflect their differentiation into different ecological niches: one very close to the water surface and the other in higher parts of the rock. Their genetic similarity could be due either to recent divergence or to introgression by hybridization. Another example is the two subspecies *M. skipetarica gurelurensis* and *M. skipetarica puskasi*. Morphologically they can be easily distinguished: *M. skipetarica gurelurensis* is densely ribbed and *M. skipetarica puskasi* is smooth. While *M. skipetarica puskasi* has a large rangewith several occurrences in a 50–100 km2 range, *M. skipetarica gurelurensis* occurs at one site only, parapatrically with one of the *M. s. puskasi* populations. The *mt* DNA data does not reflect the clear morphological differentiation between these two subspecies. They are distributed in three lineages, the average between *p*-distance being 3.3%, which might be a hint that the morphological separation occurred only recently and very fast, as is known from other snails (Fehér et al., 2013; Páll-Gergely, Asami, et al., 2019). The presence of a small contact zone, which harbours morphologically transitional specimens, implies ongoing gene flow between two formerly separated subspecies leading to introgression of *mt* haplotypes.

An example for the second type is *M. sporadica* with its two subspecies *M. sporadica sporadica* and *M. sporadica tropojana*. The latter was recently described by Fehér & Szekeres (2016) and assigned to *M. sporadica* due to its morphological similarity and the close geographic occurrence in northern Albania and Kosovo, which is far away from all other *Montenegrina* species. In the phylogenetic analyses, however, the two subspecies are widely separated in two different clades (between *p*-distance 20.4%). Thus, it seems unlikely that these two taxa belong to the same species, and their conchological similarity could be ascribed to convergence or homoplasy. Nonetheless, introgression cannot be ruled out. The latter scenario would require hybridization between highly distinct lineages and large geographic distances (see figure S2A) and should be tested with a larger sample size and nuclear markers. Unfortunately, in the present study only a few individuals of *M. s. sporadica* were available, from which we could not amplify the *H3-IGS-H4* sequence (which might have provided additional hints).

In some cases, the results clearly indicate a taxonomic problem or incorrect assignment. Due to some apparent morphological similarities (small size, neck structure and sculpture, lunella shape and position), *M. fuchsi muranyii* (clade K) was assigned to *M. fuchsi*, of which all other subspecies are located in clade D1. The genetic analyses showed two individualsof *M. fuchsi muranyii* as sister group to *M. tomorosi*, and the rest nested within *M. tomorosi*. As all the *M. tomorosi* populations as well as the *M. fuchsi muranyii* population inhabit the region of the Tomorr Mountain in Albania, which is quite distant to the known populations of *M. fuchsi*, the close genetic relationship makes sense from a phylogeographic perspective. As the morphology of *M. fuchsi muranyii* is also similar to *M. tomorosi*, we suggest that it could be reassigned to *M. tomorosi muranyii*.

In summary, the results clearly indicate that taxonomic changes are necessary in several cases. Some of the observed paraphylies detected by the molecular genetic analysis could be reasonably resolved by changing the taxonomy. Nonetheless, taking these actions without considering other traits would be too hasty. In the present study, we refrained from drawing taxonomic consequences. This was done in a separate study evaluating the genetic results together with detailed genital anatomical investigations and shell morphology and considering geographic distribution (De Mattia et al., 2020). Future multilocus analyses might test the *mt* phylogeny presented here and possibly close open sampling gaps and further improve the basis for taxonomic decisions.

**Automated species delimitation**

The present study on *Montenegrina* exemplified that species delimitation based solely on distances and the presence of a barcoding gap is a risky task. This was already emphasized earlier for various other groups of animals such as land snails (Köhler & Johnson, 2012), moths (Kekkonen, Mutanen, Kaila, Nieminen, & Hebert, 2015) or lizards (Hofmann et al., 2019). As ABGD offered a range of possible MOTU counts between 26 and 196, one would need a different source of information to choose the most appropriate grouping scheme. It is common practice for the result of one molecular species delimitation method to be evaluated by a different delimitation method as well (e.g. ABGD and PTP; Razkin et al., 2017). If we use the PTP result for this purpose, we should accept the most detailed division (196 MOTUs) as being most appropriate. This number even exceeds the number of described subspecies in *Montenegrina*. Raising all those MOTUs to species level without further support from other sources of information such as shell or genital morphology would appear to be a rather arbitrary strategy. In contrast, the lowest number of MOTUs (26) delimited by ABGD is almost equal to the number of accepted morphospecies. Examining the results of these analyses in detail, however, revealed that there was little correspondence with the current taxonomy: some of those MOTUs contained several described species and split some well-defined species into several MOTUs.

A taxonomically complex group such as *Montenegrina* naturally comprises problematic and ambiguous taxa. If species delimitation tools are used to supplement the morphology-based approach, we might expect that the former are in accordance with preconceptions about the non-problematic groups. In such cases, we more readily trusted the same source of information concerning problematic species. However, the DNA –based species delimitation methods applied in the current study provided rather contradictory results for *Montenegrina*. One potential reason for this finding might lie in the island-like and fragmented distribution: on one hand this leads to high levels of genetic differentiation and small-ranged species, but, on the other hand, in some cases, to extremely high intraspecific genetic distances.

Similarly inconsistent results of species delimitation methods were reported recently (Luo, Ling, Ho, & Zhu, 2018; Sukumaran & Knowles, 2017). Luo et al. (2018) showed that in some circumstances PTP and GMYC yielded high rates of false positives. Further research is required for a better understanding of the behaviour of molecular species-delimitation methods (Luo et al., 2018). As Sukumaran & Knowles (2017) pointed out “the results of molecular species delimitation should be interpreted alongside other lines of evidence, such as comparative morphology, population genetics, and ecology”. If at all, such methods may contribute to an integrative taxonomy approach for species delimitation, but not replace it, particularly not in a speciose and highly diverse genus such as *Montenegrina*.

**Dispersal and possible gene flow in *Montenegrina***

The fragmented distribution due to habitat requirements, together with low dispersal potential, certainly triggers speciation in *Montenegrina*. Although such a low dispersal potential is often proposed for land snails, it has rarely been tested. In *Albinaria*,Giokas & Mylonas (2004) reported high site fidelity. In a study on *M. subcristata*,population densities and dispersal activity were monitored over one season (Bulatović et al., 2019). Their data indicates that this species of *Montenegrina* has high site fidelity (on average 1.6 m over one season), with only a few individuals covering longer distances over the season (max. 8 m). Local temperature and humidity seem also to influence population density, and apparently suitable rock habitats might not be appropriate due to microclimatic conditions. Thus, the patchy distribution of *Montenegrina* probably reflects a combination of habitat requirements, limestone rock being only one of them.

The fact that, mostly, only one species is present at each site favours a non-adaptive radiation in *Montenegrina*,as has been proposed for *Albinaria* by Gittenberger (1991) and recently confirmed by Fehér et al. (2018). Furthermore, competitive exclusion of species /subspecies seems to be the predominant pattern. At the same time, the results point to more frequent gene flow than implied by current taxonomy. This raises the question whether the number of true species is actually lower than currently accepted. Concerning closely related populations, hybridization of subspecies, specifically in contact zones, may result in introgression as well as intermediate morphotypes (e.g., *M. skipetarica* *puskasi* / *M. skipetarica* *gurelurensis*), and several of those more or less differentiated subspecies/populations may be viewed as potential species *in* *statu nascendi*. Regarding specific groups possibly consisting of hybridizing populations (e.g., *M. dofleini prespaensis* and *M. hiltrudae desaretica* in the Lake Prespa area; see De Mattia et al., 2020), thorough investigations on large samples using microsatellites are underway to assess gene flow. Some results even lead to asking whether hybridization across very distant lineages may sometimes occur (e.g., *M. sporadica*). Under the Biological Species Concept (Mayr, 1942) – which we know is in many cases not applicable – this would lead to splitting species. Therefore, in the case of *Montenegrina* we prefer to adhere to the Fitness Species Concept (Hausdorf, 2011). The amount of gene flow between species could be investigated by comprehensive analyses (using genomic data of large samples) of such taxon pairs. Overall, however, the general lack of co-occurrence of *mt* haplogroups points rather at the existence of many small-ranged species. Regardless which species concept is followed, the essential question would be whether hybridisation and introgression are only sporadic events.

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**Figure Legends**

**Figure 1.** Distribution range of *Montenegrina*, comprising the western part of the Balkan Peninsula.Black dots: sample localities. BIH – Bosnia Herzegovina, HRV – Croatia, MNE – Montenegro, SRB – Serbia, XKX – Kosovo, NMN – North Macedonia, ALB – Albania, ITA – Italia, GRC – Greece

**Figure 2.** BI tree based on the concatenated *mt* data set (*COI*, *12S*, *16S*, rooted with clade G). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. More details are shown in the partial trees. Asterisks: species occurring in more than one clade. Numbers (before dash): subspecies included, as well as total numbers of subspecies (after dash).

**Figure 3.** Mean *p*-distances within species (*COI* data set). Numbers in brackets: individuals included per species. The fully coloured bars show mean distances, the shadows show maximum distances; in case of obvious phylogenetic discrepancies (causing paraphylies as described in the text), distances were recalculated after excluding those sequences (red bars before, green bars after exclusion; blue bars no recalculation needed; wp – species recalculated).

**Figure 4.** Mean *p*-distances between subspecies of the same species (*COI* data set). Numbers in brackets (before dash): subspecies included, as well as total numbers of individuals used (after dash). In case of obvious phylogenetic discrepancies (causing paraphylies as described in the text) distances were recalculated after excluding those sequences (red bars before, green bars after exclusion; blue bars no recalculation needed; wp – species recalculated).

**Figure 5.** Mean *p*-distances within subspecies (*COI* data set). Numbers in brackets: included individuals per subspecies. The fully coloured bars show mean distances, the shadows show maximum distances; in case of obvious phylogenetic discrepancies (causing paraphylies as described in the text), distances were recalculated after excluding those sequences (red bars before, green bars after exclusion; blue bars no recalculation needed; wp – species recalculated).

**Figure 6.** BI tree of Clade G based on the concatenated *mt* data set (*COI*, *12S*, *16S*, midpoint rooted). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. Asterisks: species occurring in more than one clade.

**Figure 7.** BI tree of Clades B+C as well as *M. s. sporadica* based on the concatenated *mt* data set (*COI*, *12S*, *16S*). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. Asterisks: species occurring in more than one clade. For this partial tree, instead of midpoint rooting, *M. s. sporadica* was used to root the tree. Midpoint rooting had placed this lineage between clades B and C (in contrast to the topology of the comprehensive tree), which might have been due to the comparatively short branch leading to *M. s. sporadica.*

**Figure 8.** BI tree of Clade F based on the concatenated *mt* data set (*COI*, *12S*, *16S*, rooted with Clade A). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated.

**Figure 9**. BI tree of Clade D1 based on the concatenated *mt* data set (*COI*, *12S*, *16S*, rooted with Clade D2). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. Asterisks: species occurring in more than one clade.

**Figure 10.** BI tree of Clade D2 based on the concatenated *mt* data set (*COI*, *12S*, *16S*, rooted with Clade D1). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. Asterisks: species occurring in more than one clade.

**Figure 11.** BI tree of Clade E based on the concatenated *mt* data set (*COI*, *12S*, *16S*, midpoint rooted). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. Asterisks: species occurring in more than one clade.

**Figure 12.** BI tree of Clades A+J based on the concatenated *mt* data set (*COI*, *12S*, *16S*, Clade A rooted with Clade F, Clade J rooted with Clades H+I). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. Asterisks: species occurring in more than one clade. Overview tree on the left shows the position of the two clades.

**Figure 13.** BI tree of Clades H+I based on the concatenated *mt* data set (*COI*, *12S*, *16S*, midpoint rooted). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. Asterisks: species occurring in more than one clade, #: single sequences within other (sub)species.

**Figure 14.** BI tree of Clade K based on the concatenated *mt* data set (*COI*, *12S*, *16S*, rooted with Clade J). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. Asterisks: species occurring in more than one clade.

**Figure 15**. BI tree of Clade L based on the concatenated *mt* data set (*COI*, *12S*, *16S*, rooted with Clade J). Colours correspond to those in the distribution maps (Supporting Information Figure S1). PP values (> 0.80) of major nodes are indicated. Asterisks: species occurring in more than one clade.

**Figure 16.** Median-Joining Network based on the histone *H3-IGS-H4* sequences. Colours correspond to those in the trees and the distribution maps (Supporting Information Figure S1). Haplogroups are numbered 1-8, letters in brackets indicate clade represented in the haplogroups. Single sequences are indicated by their short lab code, for shared haplotypes the number of included sequences of each taxon is given in brackets. Cloned sequences are marked with an asterisk and, if there is more than one sequence, the three different clones are indicated at the end of the lab code (i-iii).

**Supporting Information:**

**Figure S1**. Amino acid substitutions of the *COI* protein coinciding with major clades in the *COI* DNA-based cladogram. Specific amino acids at positions 41, 93 and 132 are identified with their three –letter codes.

**Figure S2 A-E.** Distribution maps of *Montenegrina* by clades. **A:** Clades B – yellow, C – orange, BC – white, G – grey. **B:** Clades A – brown, E – red, F – green. **C:** Clades D1 – light red, D2 – dark red, E – red. **D:** Clades H – dark blue, I – light blue. E: Clades K – pink, Clade L – violet, Clade J – turquoise. BIH – Bosnia Herzegovina, HRV – Croatia, MNE – Montenegro, SRB – Serbia, XKX – Kosovo, NMN – North Macedonia, ALB – Albania, ITA – Italia, GRC – Greece

**Table S1.** Specimens used in the phylogenetic analyses and their sampling localities. For all mitochondrial (*COI, 16S, 12S*) and nuclear (*H3-IGS-H4*) sequences, the GenBank accession numbers are given, \* – already published sequences (Fehér et al. 2018). Type status indicates if the material is type material. Collection numbers and the clade (A-L in the phylogenetic tree) are indicated. ME – Montenegro, KS – Kosovo, NM – North Macedonia, AL – Albania, GR – Greece. $ – *COI* and *16S* sequences of individuals of the same locality were merged. # – extraction from mummified bodies in dry shells.

**Table S2.** Overview of all included species and their clade assignment. X: the (sub)species is monophyletic, accompanied by a comment on their mono- or paraphyly.

**Table S3.** Genetic diversity and average *p-*distances (maximum and mean) within subspecies of *Montenegrina* in the *COI* data set. Seq. nr. – number of sequences included *p*-distances.

**Table S4.** Calculated morphospecies–MOTU match values (*MMM*spec) for all morphospecies for all MOTU divisions (ABGD and PTP). Bold letters: taxa with no paraphyly problem in the phylogenetic analysis.

**Alignment S1** Concatenated *mt* data (*COI – 16S – 12S*)

**Alignment S2** Concatenated *mt* data of clades A and F

**Alignment S3** Concatenated *mt* data of clades B, C and BC

**Alignment S4** Concatenated *mt* data of clades E

**Alignment S5** Concatenated *mt* data of clades D

**Alignment S6** Concatenated *mt* data of clades G

**Alignment S7** Concatenated *mt* data of clades HI

**Alignment S8** Concatenated *mt* data of clades KLJ

**Alignment S9** Histone gene sequence *H3-IGS-H4*

**Table 1.** Primer sequences for amplification and sequencing of *COI*, *12S*, *16S* and *H3-IGS-H4* as well as the used annealing temperatures (Ta).

|  |  |  |  |
| --- | --- | --- | --- |
| **Primer name** | **Primer Sequence (5'-3')** | **Ta** | **Reference** |
| ***COI*** |  |  |  |
| COIfolmerfwd | GGTCAACAATCATAAAGATATTGG | 46 | Duda et al., 2011 |
| COIschneckrev | TATACTTCTGGATGACCAAAAAATCA |  | Duda et al., 2011 |
| ***16S*** |  |  |  |
| 16SLOrc\_fw1 | TTACCTTTTGCATAATGGTTAAACTA | 52 | Harl, Páll-Gergely, et al., 2014 |
| 16SLOrc\_rev | CGGTCTGAACTCAGATCATG |  | Harl, Páll-Gergely, et al., 2014 |
| ***12S*** |  |  |  |
| 12SGastfwd2 | CCTCTACTTTGTTACGACTTATCT | 54 | Cadahía et al., 2014 |
| 12SGastrev1 | TAAGCTGTAGGGCTCATAAC |  | present study |
| *H3-IGS-H4* |  |  |  |
| OrcH3Right1 | TGGGCATGATGGTGACACGCT | 54 | Cadahía et al., 2014 |
| OrcH4Left1 | GTGCGTCCCTGGCGCTTCA |  | Harl, Páll-Gergely, et al., 2014 |
| *H3-IGS-H4* ***intern*** |  |  |  |
| Mont\_insH4left | CACGCAATACCTTGCGATGAC | / | present study |
| Mont\_insH3right | GGATTTTCTAGCTGCCTTGGT |  | present study |

**Table 2.** Genetic diversity and average *p*-distances within and between clades in the *COI* data set. The mean distances within clades are given in the diagonal and printed bold. Seq. nr. – number of sequences included; Out – Outgroup

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| clade | seq. nr. | max within | A | B | BC | C | D1 | D2 | E | F | G | H | I | J | K | L | Out |
| **A** | 10 | 12.0 | **6.7** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **B** | 58 | 19.6 | 18.0 | **10.6** |  |  |  |  |  |  |  |  |  |  |  |  |  |
| **BC** | 2 | 0.0 | 16.6 | 13.1 | **0** |  |  |  |  |  |  |  |  |  |  |  |  |
| **C** | 40 | 18.2 | 16.7 | 15.0 | 14.0 | **11.9** |  |  |  |  |  |  |  |  |  |  |  |
| **D1** | 51 | 16.4 | 17.7 | 17.9 | 15.4 | 16.4 | **10.6** |  |  |  |  |  |  |  |  |  |  |
| **D2** | 122 | 12.4 | 16.1 | 16.8 | 14.6 | 15.9 | 11.7 | **5.9** |  |  |  |  |  |  |  |  |  |
| **E** | 88 | 22.7 | 18.3 | 17.2 | 14.6 | 16.5 | 13.8 | 13.1 | **12.4** |  |  |  |  |  |  |  |  |
| **F** | 132 | 16.4 | 15.6 | 17.1 | 16.00 | 17.4 | 15.0 | 12.8 | 15.4 | **9.5** |  |  |  |  |  |  |  |
| **G** | 31 | 20.9 | 19.9 | 20.9 | 19.9 | 21.5 | 19.3 | 20.0 | 20.3 | 19.4 | **12.2** |  |  |  |  |  |  |
| **H** | 47 | 18.7 | 19.5 | 19.5 | 18.4 | 18.7 | 18.9 | 18.4 | 17.9 | 19.5 | 19.9 | **11.2** |  |  |  |  |  |
| **I** | 63 | 18.7 | 18.6 | 19.9 | 16.9 | 18.3 | 18.9 | 18.2 | 18.3 | 19.3 | 20.7 | 17.3 | **11.9** |  |  |  |  |
| **J** | 7 | 11.6 | 21.1 | 19.6 | 17.6 | 21.0 | 18.9 | 18.5 | 17.6 | 19.1 | 17.9 | 16.7 | 17.9 | **5.8** |  |  |  |
| **K** | 65 | 20.9 | 18.7 | 19.7 | 17.00 | 18.3 | 18.6 | 18.0 | 18.7 | 19.4 | 18.7 | 16.0 | 17.3 | 15.6 | **9.7** |  |  |
| **L** | 74 | 16.4 | 19.4 | 20.2 | 18.7 | 18.7 | 20.1 | 19.0 | 19.0 | 19.9 | 20.6 | 17.0 | 17.2 | 19.5 | 15.9 | **10.2** |  |
| **Outgroup** | 1 | n/c | 21.6 | 20.9 | 17.3 | 21.4 | 18.7 | 19.0 | 18.7 | 21.9 | 19.6 | 19.9 | 20.6 | 18.0 | 17.5 | 19.2 | **n/c** |

**Table 3.** Genetic diversity and average *p-*distances and ranges of distances within species in the *COI* data set. The mean distances within clades are given in the diagonal and printed bold. Seq. nr. – number of sequences included

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| species | seq. nr. | max within | apf | att | cat | dof | dri | fuc | gra | har | hel | hil | jan | lax | lil | min | nan | oko | per | pro | rug | ski | soo | spo | sta | stu | sub | tim | tom | zil | Out |
| apfelbecki | 11 | 0.0 | **0.0** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| attemsi | 10 | 12.0 | 20.7 | **6.7** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| cattaroensis | 26 | 7.1 | 17.6 | 17.3 | **4.7** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| dofleini | 91 | 13.8 | 14.9 | 16.1 | 14.5 | **5.4** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| drimmeri | 7 | 16.0 | 21.2 | 18.6 | 18.4 | 17.9 | **4.7** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| fuchsi | 15 | 19.6 | 19.0 | 18.3 | 17.8 | 14.6 | 18.0 | **12.7** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| grammica | 4 | 14.2 | 18.0 | 18.8 | 19.9 | 17.4 | 15.4 | 18.0 | **9.9** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| haringae | 5 | 0.0 | 16.4 | 18.5 | 15.4 | 14.3 | 20.6 | 16.6 | 20.0 | **0.0** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| helvola | 24 | 14.2 | 19.0 | 20.0 | 20.5 | 18.2 | 15.6 | 19.2 | 17.7 | 20.5 | **7.5** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| hiltrudae | 74 | 16.0 | 17.0 | 17.6 | 15.9 | 11.9 | 17.7 | 15.6 | 18.9 | 16.3 | 17.8 | **1.4** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| janinensis | 8 | 3.1 | 18.0 | 17.2 | 13.3 | 9.8 | 16.8 | 12.7 | 19.2 | 16.0 | 18.1 | 10.5 | **1.2** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| laxa | 46 | 14.7 | 21.4 | 19.3 | 18.8 | 19.4 | 10.6 | 19.9 | 16.5 | 21.2 | 16.8 | 19.0 | 20.6 | **7.5** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| lillae | 5 | 0.0 | 22.7 | 21.2 | 21.6 | 21.0 | 21.2 | 20.4 | 20.1 | 21.3 | 21.1 | 22.1 | 21.2 | 23.1 | **0.0** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| minuscula | 5 | 3.6 | 18.9 | 21.5 | 19.1 | 18.8 | 17.5 | 19.3 | 16.3 | 18.8 | 16.7 | 16.9 | 18.3 | 20.2 | 18.0 | **2.1** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| nana | 13 | 6.7 | 20.0 | 18.8 | 20.5 | 17.2 | 15.8 | 18.7 | 18.2 | 20.5 | 12.6 | 18.9 | 19.0 | 18.2 | 22.4 | 17.9 | **3.7** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| okolensis | 5 | 0.9 | 19.1 | 18.5 | 18.2 | 17.6 | 18.8 | 17.6 | 17.4 | 16.0 | 18.2 | 17.4 | 16.7 | 19.6 | 15.1 | 14.3 | 19.1 | **0.4** |  |  |  |  |  |  |  |  |  |  |  |  |  |
| perstriata | 83 | 21.3 | 19.9 | 19.0 | 19.3 | 18.2 | 15.3 | 18.9 | 16.1 | 19.2 | 16.2 | 18.1 | 18.6 | 15.9 | 22.7 | 17.8 | 16.9 | 18.1 | **14.7** |  |  |  |  |  |  |  |  |  |  |  |  |
| prokletiana | 17 | 12.0 | 22.9 | 19.8 | 20.2 | 20.4 | 19.5 | 19.5 | 18.5 | 19.8 | 20.3 | 20.3 | 18.4 | 20.2 | 18.8 | 18.1 | 22.8 | 13.3 | 20.4 | **7.3** |  |  |  |  |  |  |  |  |  |  |  |
| rugilabris | 32 | 14.7 | 15.8 | 17.6 | 15.9 | 11.5 | 18.1 | 14.0 | 18.9 | 15.0 | 19.0 | 13.1 | 10.8 | 19.8 | 20.7 | 18.8 | 19.7 | 17.2 | 18.8 | 19.7 | **9.7** |  |  |  |  |  |  |  |  |  |  |
| skipetarica | 98 | 21.3 | 18.5 | 17.5 | 18.6 | 16.5 | 19.3 | 18.2 | 18.4 | 19.2 | 19.0 | 16.4 | 16.4 | 19.6 | 22.5 | 19.6 | 20.3 | 18.7 | 19.3 | 21.6 | 17.3 | **12.9** |  |  |  |  |  |  |  |  |  |
| soosi | 8 | 6.2 | 21.0 | 19.4 | 19.0 | 19.2 | 11.8 | 18.4 | 16.4 | 20.3 | 16.7 | 18.8 | 18.7 | 13.3 | 21.6 | 17.9 | 17.1 | 18.6 | 15.2 | 20.0 | 19.8 | 19.1 | **3.9** |  |  |  |  |  |  |  |  |
| sporadica | 6 | 20.9 | 18.1 | 19.1 | 19.7 | 17.7 | 19.5 | 18.1 | 17.7 | 19.0 | 19.2 | 17.2 | 16.8 | 20.2 | 17.6 | 16.5 | 20.3 | 11.3 | 19.3 | 17.3 | 17.6 | 18.1 | 19.2 | **11.4** |  |  |  |  |  |  |  |
| stankovici | 27 | 1.8 | 14.7 | 16.7 | 13.6 | 8.1 | 17.6 | 14.3 | 17.6 | 14.3 | 19.0 | 12.7 | 10.7 | 19.0 | 21.8 | 17.3 | 19.4 | 18.4 | 18.9 | 20.6 | 11.4 | 16.4 | 19.4 | 17.8 | **0.3** |  |  |  |  |  |  |
| sturanyana | 8 | 15.1 | 21.1 | 19.5 | 21.4 | 19.3 | 15.8 | 19.3 | 15.0 | 22.6 | 16.4 | 18.9 | 18.9 | 18.0 | 20.9 | 16.2 | 17.3 | 19.2 | 16.7 | 20.1 | 20.5 | 19.2 | 18.4 | 21.1 | 18.9 | **9.6** |  |  |  |  |  |
| subcristata | 101 | 12.9 | 16.8 | 15.1 | 12.5 | 12.5 | 19.2 | 16.3 | 18.0 | 14.6 | 19.0 | 14.4 | 13.5 | 20.0 | 19.4 | 18.9 | 19.4 | 17.2 | 19.5 | 19.5 | 14.9 | 16.8 | 20.4 | 18.6 | 12.1 | 19.5 | **7.6** |  |  |  |  |
| timeae | 6 | 6.2 | 19.6 | 19.1 | 21.4 | 18.0 | 15.6 | 18.4 | 14.4 | 21.3 | 16.1 | 17.8 | 18.3 | 17.6 | 19.6 | 15.4 | 16.5 | 18.0 | 16.0 | 19.5 | 19.4 | 18.2 | 17.5 | 19.6 | 18.1 | 8.8 | 18.8 | **2.7** |  |  |  |
| tomorosi | 49 | 18.7 | 17.6 | 18.4 | 19.9 | 18.0 | 14.5 | 16.3 | 14.7 | 19.9 | 17.5 | 18.8 | 18.4 | 16.4 | 20.8 | 14.9 | 16.5 | 17.3 | 16.5 | 18.1 | 18.0 | 19.2 | 14.5 | 18.2 | 17.8 | 16.4 | 19.4 | 15.4 | **7.4** |  |  |
| zilchi | 4 | 0.4 | 13.9 | 19.4 | 16.7 | 11.5 | 19.1 | 16.6 | 18.4 | 17.7 | 20.4 | 14.0 | 12.2 | 21.5 | 23.4 | 18.7 | 19.9 | 20.3 | 20.3 | 21.6 | 13.6 | 18.4 | 21.3 | 20.2 | 11.3 | 20.8 | 13.7 | 20.0 | 19.4 | **0.2** |  |
| Outgroup | 1 | 0.0 | 19.6 | 21.6 | 22.7 | 19.4 | 18.1 | 18.7 | 17.6 | 18.7 | 19.9 | 18.6 | 19.1 | 20.0 | 23.6 | 17.7 | 22.5 | 16.2 | 19.5 | 19.7 | 18.4 | 21.1 | 16.2 | 18.1 | 17.9 | 21.0 | 21.9 | 19.9 | 17.3 | 19.9 | **n/c** |