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# On the origin and diversification of the stygobiotic freshwater snail genus *Hauffenia* (Caenogastropoda: Hydrobiidae) with special focus on the northern species and the description of two new species

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**Abstract.** During systematic surveys of groundwater snails in Slovakia, Hungary and Bosnia and Hercegovina two new species of the genus *Hauffenia*, *H. lozekiana* sp. nov. from a single locality in Slovakia, and *H. steffeki* sp. nov. with a small range in Bosnia and Hercegovina were discovered and are here described based on shell morphology, anatomy and DNA sequence data (COI, 16S rRNA, ITS2). The discovery of *H. steffeki* sp. nov. extends the range of the genus considerably towards the south. *Hauffenia lozekiana* sp. nov. appears to be a relict surviving within the range of the today widely distributed *H. kissdalmae*. Based on a time tree, we developed a scenario for the origin and diversification of the genus. The ancestor probably evolved in the Miocene on the Balkans and with the gradual desiccation of the Paratethys and its remnant water bodies diversified towards the north. Karstic and in particular alluvial connectivities together with changing courses of paleo-rivers probably played an important role for dispersal. Ecological observations suggest that the phreatic rhizosphere, the delicate net of tree rootlets and their exudates, are important for the existence of these groundwater snails.

**Keywords.** DNA taxonomy, groundwater, Miocene, Pannonian Sea, rhizosphere.

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## Introduction

Hydrobioid gastropods (sensu Davis 1979) are notorious for their extreme diversity on the one hand and the difficulties they pose for identification and inference of relationships due to their small size, simple shell morphology, and high degree of convergence on the other hand (e.g., Hershler & Ponder 1998; Wilke *et al.* 2000; Clark *et al.* 2003; Delicado *et al.* 2019). Molecular methods have revealed an increasing number of morphologically cryptic species and there are even genera which cannot be defined unambiguously based on shell morphological and anatomical data (e.g., Haase 2008; Zielske & Haase 2015; Delicado *et al.* 2021). Many valvatiform species of the family Hydrobiidae Stimpson, 1865 characterized by a more or less depressed shell and open umbilicus are illustrative examples for species having been taxonomically misplaced due to their superficial similarities. One of these genera that has kept changing its specific composition, hence range, is *Hauffenia* Pollonera, 1898 (see Bodon *et al.* 2001 and Rysiewska *et al.* 2017 for partial overviews of taxonomic changes). Based on anatomical and molecular data, this genus is confirmed in Austria, northern Croatia, Hungary, northeastern Italy, Slovakia, and Slovenia (Bodon *et al.* 2001; Šteffek *et al.* 2011; Falniowski & Szarowska 2015; Rysiewska *et al.* 2017).

Slovakia and Hungary were the latest countries to be added to the range of the genus. On the territory of Slovakia, empty shells of the genus were first discovered by Vojen Ložek in several springs in the Slovak Karst in 1985 (mentioned in an interview in Vesmír by Mitterová 1986), followed by several records from this region (Šteffek & Grego 2008; Grego & Šteffek 2010; Šteffek *et al.* 2011). The first record from the Bystrická Highland was also provided by Ložek (Ložek & Galvánek 1987) from Holocene travertine deposits in Dolná Mičiná. Later, empty shells were found among flotsam of the Driekyňa brook (Šteffek & Grego unpubl. data) and three localities of the lower Hron River (Čiliak & Šteffek 2013). In Hungary, Majoros and Krolopp have found valvatiform hydrobiid shells in the Aggtelek Karst region in the 1990s (Gábor Majoros pers. com.), but the first published record is only from 2008, when *Hauffenia kissdalmae* Erőss & Petró, 2008 was described (Erőss & Petró 2008).

Among the species listed in MolluscaBase (2021), *H. lucidula* (Angelov, 1967) from Bulgaria and *H. edlaueri* (Schütt, 1961) from southern Dalmatia (Croatia) have only been allocated to *Hauffenia* based on their general shell morphology (Schütt 1961; Angelov 1967; see also Georgiev 2013). As their ranges are clearly disjunct from the range outlined above, their generic affiliation is doubtful and subject to anatomical and molecular investigation. Other species originally described as *Hauffenia* and later already transferred to other genera are listed in MolluscaBase (2021).

During the last 19 years, spring and groundwater snails have been systematically collected across Slovakia, Hungary as well as Bosnia and Hercegovina. Many samples contained individuals which most likely belong to *Hauffenia*. Screening these samples molecularly revealed two unknown species – one from Slovakia, the other one from Bosnia and Hercegovina – which are described here as new to science. These investigations including newly collected material from Austria as well as published DNA sequences retrieved from GenBank also gave rise to the development of a scenario of the origin and dispersion of the genus with special focus on the species occurring in Hungary and Slovakia.

## Material and methods

### Sampling

Sampling was systematically carried out by screening sandy sediment fractions of 0.2–3 mm from spring heads or caves according to Grego *et al.* (2017). Sand samples were either sorted still wet under a stereo microscope and living specimens preserved in 99% ethanol or fixed in ethanol on the spot. The samples were obtained from the localities listed in Table 1 (Figs 1–5).

### Phylogenetic analyses

Identifying species of *Hauffenia* based on the shell is hardly possible and the inference of relationships definitely impossible. Therefore, we aimed at a phylogenetic reconstruction based on standard DNA sequence data. However, as in quite a large proportion of our samples from Hungary and Slovakia cytochrome oxidase I (COI) could not be amplified, we sequenced only 16S rRNA (16S) and the internal transcribed spacer 2 (ITS2) across the comprehensive set of samples including two species of *Kerkia* Radoman, 1978 as outgroup (Table 1). Prior to sequencing, DNA was extracted using the E.Z.N.A. Mollusc DNA kit (Omega Bio-Tek). For polymerase chain reactions (PCR) we used the primer pairs 16Sar and 16Sbr (Palumbi *et al.* 1991) as well as ITS2-f [5′-CTAGCTGCGAGAATTAATGTGA-3′ (Wade & Mordan 2000)] and ITS2-r [5′-GGTTTCACGTACTCTTGAAC-3′ (Nekola *et al.* 2015)], respectively. The reaction volumes of 11 µL contained 1 µL of DNA solution (~20 ng), 5 µL of HS MyTaqTM RedMix (Bioline), 0.40 µL of 1% BSA, 0.25 µL of each primer (from a 10 pmol stock solution), and 4.10 µL water. PCR temperature profiles were: for 16S, denaturation at 95°C for 1 min, 10 touch-down cycles comprising 20 s denaturation at 95°C, annealing starting at 60°C and dropping by 1 degree per cycle for 20 s, as well as 1 min extension at 72°C, followed by another 25 cycles with annealing at 51°C and a final extension at 72°C for 5 min; ITS2 was amplified in 30 cycles after initial denaturation at 95°C for 1 min with each cycle comprising denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1.5 min, again followed by a final extension at 72°C for 5 min. For COI and a limited number of specimens, we achieved the best results using the primer pair L1490-Alb [5′-ACTCAACGAA TCATAAAGATATTGG-3′ (Gittenberger *et al.* 2004) and BCO [5′- GTATCGGCTGTAAAATAAGC-3′ (Haase *et al.* 2003)] originally developed for landsnails (Haase *et al.* 2021). The PCR mix conformed to those of the other genes. The PCR profile comprised the following steps: denaturation at 95°C for 3 min, 30 cycles denaturation at 95°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 1.5 min, and a final extension at 72°C for 7 min. Exonuclease I and shrimp alkaline phosphatase were used to purify the PCR products. For cycle sequencing we used the ABI Big Dye Terminator Reaction Mix 3.1 (Thermo Fisher) and the PCR primers. Sequences were compiled on an ABI 3130xl Genetic Analyzer (Thermo Fisher), proof-read with Geneious ver. R10.2 (www.geneious.com) and BioEdit ver. 7.0.5.3 (Hall 1999) and submitted to GenBank (Table 2). For alignments, we used MAFFT ver. 7 (Katoh *et al.* 2019) with default settings. Identical sequences were identified in DAMBE 72.152 (Xia 2018) and omitted. The final alignments comprised 522 (16S), 782 (ITS2), and 535 (COI) bp, respectively.

Phylogenetic trees were reconstructed for 16S and ITS2 separately as well as jointly across the comprehensive set of specimens and for all three concatenated genes based on the intersect of available individuals in maximum likelihood (ML) and Bayesian frameworks using W-IQ-TREE (Trifinopoulos *et al.* 2016) and MrBayes ver. 3.2.6 (Ronquist *et al.* 2012), respectively. Substitution models were fitted to both genes by a fast version of PartitionFinder (Lanfear *et al.* 2012) implemented in W-IQ-TREE and by jModeltest ver. 2.1.4 (Darriba *et al.* 2012) for MrBayes ver. 3.2.6. Both approaches identified HKY + I, K2P + Γ + I, and HKY + Γ as best-fitting models for 16S, ITS2, and COI, respectively. The W-IQ-TREE analysis ran with linked edges and 1000 replicates of ultrafast bootstrapping (Minh *et al.* 2013). MrBayes was run for 10 mio generations logging every 1000th tree, a burn-in of 25%, and otherwise default settings. Convergence of parameter estimates was monitored by the diagnostics implemented in MrBayes.

**Table 1** (continued on next three pages). Locality data. Type localities highlighted in grey. Abbreviations: Alt = altitude; AT = Austria; BiH = Bosnia and Herzegovina; Cond = conductivity; HU = Hungary; ID = locality number; SeqNo = sequence number; SK = Slovakia. *no sequence* refers to live collected material where sequencing failed.

| **ID** | **SeqNo** *material* | **Species** | **Country** | **Orographic unit** | **Locality** | **Latitude** | **Longitude** | **Alt** m | **T** °C | **pH** | **Cond** µS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | **1593** | *H. kissdalmae* | **SK** | Rimava Basin | Bretka, Melegvíz Spring at mouth of Muráňka Gorge | 48°29.3639′ | 20°20.2940′ | 203 | 18.9 | 7.390 | 505 |
| **2** | **1604** | *H. kissdalmae* | **SK** | Rimava Basin | Meliata, spring Teplica in Bajzova Valley | 48°31.4487′ | 20°19.1422′ | 235 | 14.2 | 6.957 | 649 |
| **3** | **1626** | *H. kissdalmae* | **SK** | Slovak Karst | Gemerská Hôrka, spring in centre of village | 48°32.1290′ | 20°22.6900′ | 217 | 14.0 | 7.039 | 624 |
| **4** | **1603, 1627, 1636, 1639–1641, 1670, 1671** | *H. kissdalmae* | **SK** | Slovak Karst | Gemersá Hôrka, Päťročenka Spring | 48°32.2610′ | 20°22.7200′ | 224 | 10.2 | 7.774 | 639 |
| **5** | **1582** | *H. kissdalmae* | **SK** | Slovak Karst | Plešivec, Sudená studňa Spring | 48°34.3023′ | 20°24.0597′ | 259 | 10.9 | 7.408 | 557 |
| **6** | **1654–1656** | *H. kissdalmae* | **SK** | Slovak Karst | Kunova Teplica, Závodná jaskyňa Cave | 48°36.4350′ | 20°23.4520′ | 250 | 10.1 | 7.154 | 510 |
| **7** | **1633, 1635** | *H. kissdalmae* | **SK** | Slovak Karst | Kunova Teplica, Hučiaca vyvieračka, karst spring | 48°37.5245 | 20°23.3992′ | 267 | 9.7 | 7.502 | 453 |
| **8** | **1617, 1619** | *H. kissdalmae* | **SK** | Slovak Karst | Plešivec, Čepkov I, karst spring | 48°33.2930′ | 20°25.1540′ | 232 | 11.0 | 7.344 | 596 |
| **9** | **1631, 1632** | *H. kissdalmae* | **SK** | Slovak Karst | Plešivec, Malá studňa, intermittent karst spring | 48°33.1060′ | 20°25.8300′ | 246 | 12.2 | 7.119 | 672 |
| **10** | **1611–1613, 1648–1650** | *H. kissdalmae* | **SK** | Slovak Karst | Gombasek, Čierna vyvieračka, karst spring | 48°33.7675′ | 20°27.9192′ | 268 | 10.2 | 7.392 | 610 |
| **11** | **1597–1599, 1653** | *H. kissdalmae* | **SK** | Slovak Karst | Slavec, Malá studňa under Plešivec Plateau | 48°33.1177′ | 20°25.8168′ | 241 | 10.6 | 7.674 | 227 |
| **12** | **1621–1623** | *H. kissdalmae* | **SK** | Slovak Karst | Brzotín, Stará vyvieračka, intermittent karst spring | 48°36.5230′ | 20°28.2490′ | 275 | 10.0 | 7.266 | 966 |
| **13** | **1651–1653** | *H. kissdalmae* | **SK** | Slovak Karst | Slavec, Vodovodná vyvieračka, karst spring | 48°35.1360′ | 20°28.7740′ | 240 | 10.4 | 7.476 | 510 |
| **14** | **1638** | *H. kissdalmae* | **SK** | Slovak Karst | Brzotín, Hradná vyvieračka, karst spring | 48°36.4450′ | 20°29.2020′ | 261 | 9.8 | 7.222 | 530 |
| **15** | **1628–1630** | *H. kissdalmae* | **SK** | Slovak Karst | Kečovo, Kečovská vyvieračka, karst spring | 48°30.0120′ | 20°29.1370′ | 356 | 9.3 | 7.132 | 638 |
| **16** | **1585** | *H. kissdalmae* | **SK** | Slovak Karst | Silická Jablonica, Strašná studňa Spring 1 km W of village | 48°33.1090′ | 20°36.3080′ | 258 | 9.5 | 7.362 | – |
| **17** | **1602** | *H. kissdalmae* | **SK** | Slovak Karst | Drnava, Bleskový prameň, karst spring | 48°37.8970′ | 20°40.3460′ | 525 | 9.5 | 7.400 | 796 |
| **18** | **1605–1607** | *H. kissdalmae* | **SK** | Slovak Karst | Kováčová, Kováčovská vyvieračka, karst spring | 48°38.3645′ | 20°41.8270′ | 458 | 9.5 | 7.362 | 384 |
| **19** | **1620** | *H. kissdalmae* | **SK** | Turňa Basin | Turňa nad Bodvou, Zsigárt Spring | 48°36.5290′ | 20°50.9050′ | 196 | 10.1 | 7.979 | 453 |
| **20** | **1691–1693** | *H. kissdalmae* | **SK** | Turňa Basin | Zádiel, well in the village | 48°36.9133′ | 20°49.9481′ | 251 | 7..3 | 7.730 | 540 |
| **21** | **1600, 1601** | *H. kissdalmae* | **HU** | Aggtelek Karst | Tornanádaska, Rongyos-kút Spring | 48°34.0208′ | 20°48.3360′ | 170 | 15.2 | 7.675 | 478 |
| **22** | **1677, 1678** | *H. kissdalmae* | **HU** | Aggtelek Karst | Szögliget, Puska Pál Spring | 48°31.4888′ | 20°36.5745′ | 310 | – | – | – |

**Table 1** (continued). Locality data.

| ID | SeqNo *material* | Species | Country | Orographic unit | Locality | Latitude | Longitude | Alt m | T °C | pH | Cond µS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **23** | **1594** | *H. kissdalmae* | **HU** | Aggtelek Karst | Tornanádaska, Kastélykerti Spring | 48°33.7948′ | 20°47.0570′ | 170 | 11.2 | 7.351 | 551 |
| **24** | *shells* | *H. kissdalmae* | **SK** | Slovak Karst | Gemerská Teplica, vyvieračka Hlavište, karst spring | 48°36.2740′ | 20°17.7300′ | 252 | 11.7 | 7.419 | 571 |
| **25** | *shells* | *H. kissdalmae* | **SK** | Slovak Karst | Gemerský Sad, spring Rybník | 48°34.4934′ | 20°19.3921′ | 251 | 10.4 | 7.203 | 692 |
| **26** | *shells* | *H. kissdalmae* | **SK** | Slovak Karst | Vidová, Vidovská vyvieračka, karst spring | 48°33.8600′ | 20°26.4050′ | 238 | 10.3 | 7.235 | 588 |
| **27** | *no sequence* | *H. kissdalmae* | **SK** | Slovak Karst | Gombasek, Biela vyvieračka, karst spring | 48°34.0400′ | 20°28.0860′ | 255 | 10.4 | 7.200 | 608 |
| **28** | *no sequence* | *H. kissdalmae* | **SK** | Slovak Karst | Brzotín, Nová vyvieračka, karst spring | 48°36.5910′ | 20°28.2700′ | 267 | 9.7 | 7.262 | 530 |
| **29** | *shells* | *H. kissdalmae* | **SK** | Slovak Karst | Krásnohorská Dlhá Lúka, spring Buzgó | 48°37.1070′ | 20°35.1720′ | 326 | 9.4 | 7.448 | 1720 |
| **30** | *shells* | *H. kissdalmae* | **SK** | Slovak Karst | Krásnohorská Dlhá Lúka, spring Pod Lomom | 48°37.0790′ | 20°35.3930′ | 328 | 9.0 | 7.251 | 1863 |
| **31** | *shells* | *H. kissdalmae* | **SK** | Slovak Karst | Krásnohorská Dlhá Lúka, spring Občasný | 48°37.0720′ | 20°35.4460′ | 329 | – | – | – |
| **32** | *no sequence* | *H. kissdalmae* | **SK** | Slovak Karst | Drnava, Drienový prameň, spring in the village | 48°38.3560′ | 20°39.4210′ | 382 | 9.5 | 7.362 | 442 |
| **33** | *shells* | *H. kissdalmae* | **SK** | Slovak Karst | Silická Jablonica, Čierna Studňa, karst spring | 48°33.7390′ | 20°38.2820′ | 233 | 10.1 | 7.147 | – |
| **34** | *shells* | *H. kissdalmae* | **SK** | Slovak Karst | Hrhov, Žemeň kút, spring | 48°35.1020′ | 20°43.8150′ | 204 | 12.9 | 7.154 | – |
| **35** | *shells* | *H. kissdalmae* | **SK** | Slovak Karst | Hrhov, Hrhovská Vyvieračka, karst spring | 48°36.5800′ | 20°44.9430′ | 233 | 11.1 | 7.417 | 508 |
| **36** | *no sequence* | *H. kissdalmae* | **SK** | Turňa Basin | Včeláre, Teplica Spring under road E-571 | 48°35.9960′ | 20°48.6410′ | 205 | 10.1 | 7.979 | 588 |
| **37** | *no sequence* | *H. kissdalmae* | **HU** | Aggtelek Karst | Bódvaszilas, Kis-Vecsem Spring | 48°33.0829′ | 20°43.8749′ | 175 | 11.7 | 7.576 | 561 |
| **38** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Bódvaszilas, Kökény-berki Spring | 48°32.9315′ | 20°43.4426′ | 240 | – | – | – |
| **39** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Bódvaszilas, Miklós-hegy alatti-forrás, karst spring | 48°32.0587′ | 20°43.1625′ | 205 | – | – | – |
| **40** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Bódvaszilas, Vecsem Spring | 48°33.0508′ | 20°43.8967′ | 170 | 11.9 | 7.600 | 558 |
| **41** | **1596** | *H. kissdalmae* | **HU** | Aggtelek Karst | Perkupa, Dobódél, Sárkány Spring | 48°29.8629′ | 20°43.1455′ | 160 | – | – | – |
| **42** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Teresztenye, Kinizsi Spring with cave | 48°26.8315′ | 20°36.0729′ | 245 | – | – | – |
| **43** | **1674, 1675** | *H. kissdalmae* | **HU** | Aggtelek Karst | Szalonna, Meleg Spring | 48°27.0044′ | 20°43.2085′ | 150 | 16.6 | 7.617 | 465 |
| **44** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Égerszög, village spring near picnic place | 48°26.6136′ | 20°35.0032′ | 240 | – | – | – |
| **45** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Szögliget, Hideg-kút Spring | 48°31.9659′ | 20°38.6158′ | 228 | – | – | – |
| **46** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Szögliget, Lakatos Spring | 48°32.7016′ | 20°40.2546′ | 293 |  | 8.100 | 607 |
| **47** | *no sequence* | *H. kissdalmae* | **HU** | Aggtelek Karst | Szögliget, Csörgő Spring (Parokia) | 48°31.4156′ | 20°40.6977′ | 175 | 11.2 | 7.379 | 595 |
| **48** | *no sequence* | *H. kissdalmae* | **HU** | Aggtelek Karst | Szögliget, Gizi néni-kútja, well | 48°31.2681′ | 20°40.7914′ | 180 | – | – | – |
| **49** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Szögliget, Szeszfőzdei Spring | 48°31.2049′ | 20°40.9128′ | 180 | – | 7.400 | 654 |
| **50** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Hidvégardó, Melegvíz Spring | 48°34.0568′ | 20°48.5600′ | 168 | – | – | – |

**Table 1** (continued). Locality data.

| ID | SeqNo *material* | Species | Country | Orographic unit | Locality | Latitude | Longitude | Alt m | T °C | pH | Cond µS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **51** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Perkupa, Hideg-kút Spring | 48°28.1917′ | 20°41.8178′ | 156 | – | – | – |
| **52** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Tornakápolna, Vízfakadás-forrás, spring | 48°27.7506′ | 20°36.5457′ | 300 | – | – | – |
| **53** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Jósvafó, Barladla Jósva forrás, spring | 48°29.0585′ | 20°32.4325′ | 271 | 12.6 | 7.355 | 527 |
| **54** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Jósvafó, Béke Barlang Cave Spring, | 48°27.6500′ | 20°32.5667′ | 342 | 10.3 | 7.324 | 548 |
| **55** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Jósvafő, Jósva Spring (Hosszú-alsó-barlang cave entrance) | 48°29.0151′ | 20°32.5338 | 223 | 10.1 | 7.657 | 521 |
| **56** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Jósvafő, inside Kossuth Cave (behind Nagy-Tohonya spring) | 48°29.2822′ | 20°33.0853 | 210 | – | – | – |
| **57** | *shells* | *H. kissdalmae* | **HU** | Aggtelek Karst | Jósvafő, Nagy-Tohonya Spring | 48°29.2571′ | 20°33.0196 | 210 | 13.5 | 7.319 | 573 |
| **58** | *shells* | *H. kissdalmae* | **HU** | Bükk Mountains | Mályinka, Ámor Spring | 48°08.2547′ | 20°30.7612 | 415 | – | – | – |
| **59** | *shells* | *H. kissdalmae* | **HU** | Bükk Mountains | Mályinka, Bakó-sara alatti II. Spring | 48°07.8015′ | 20°30.8791 | 540 | – | – | – |
| **60** | *shells* | *H. kissdalmae* | **HU** | Bükk Mountains | Mályinka, Három-kút Spring | 48°08.1339′ | 20°31.5238 | 490 | – | – | – |
| **61** | **1614–1616, 1642–1644** | *H. kissdalmae* | **SK** | Bystrica Highland | Slovenská Ľupča; Driekyňa, Vyvieranica, Karst spring | 48°45.1560′ | 19°17.2950′ | 420 | 9.4 | 7.132 | 529 |
| **62** | **1663–1665** | *H. kissdalmae* | **SK** | Bystrica Highland | Banská Bystrica, Iliáš, spring and well at N side of the village | 48°42.1747′ | 19°08.5832′ | 336 | 9.4 | 7.096 | 777 |
| **63** | **1685–1687** | *H. kissdalmae* | **SK** | Bystrica Highland | Banská Bystrica,spring at N foot of Mt. Urpín above railway | 48°43.7501′ | 19°08.3315′ | 362 | 9.4 | 7.131 | 563 |
| **64** | *shells* | *H. kissdalmae* | **SK** | Bystrica Highland | Čačín, Zolná Valley, spring behind waterwork | 48°40.1475′ | 19°15.4553′ | 428 | 10.7 | 7.250 | 504 |
| **65** | *shells* | *H. cf. lozekiana* * | **SK** | Bystrica Highland | Dolná Mičiná, Mičinské travertíny, Holocene tarvertine deposit | 48°40.0254′ | 19°13.7779′ | 386 | – | – | – |
| **66** | *shells* | *H. kissdalmae* | **SK** | Horehronské Podolie | Nemecká, spring under the Motorest | 48°48.5834′ | 19°24.3266′ | 421 | 8.6 | 7.318 | 443 |
| **67** | *shells* | *H. kissdalmae* ** | **SK** | Hron Valley | Revište, Hron River, flotsam sediments | 48°31.2645′ | 18°43.6325′ | 223 | – | – | – |
| **68** | *shells* | *H. kissdalmae* ** | **SK** | Hron Valley | Vozokany, Hron River, flotsam sediments | 48°00.5083′ | 18°39.9617′ | 126 | – | – | – |
| **69** | *shells* | *H. kissdalmae* ** | **SK** | Hron Valley | Čata, Hron River, flotsam sediments | 47°57.5085′ | 18°39.6855′ | 117 | – | – | – |
| **70** | **1679** | ***H. kissdalmae*** | **HU** | Börzsöny Mts | Kissmaros, Csömöle Valley, **Őz** forrás; well and spring | 47°50.3500′ | 19°00.4700′ | 145 | – | – | – |
| **71** | **1610** | ***H. lozekiana* sp. nov.** | **SK** | Bystrica Highland | Poniky/Oravce, Kalinovský prameň (Kalinovec), spring | 48°42.0270′ | 19°15.9520′ | 482 | 9.2 | 7.121 | 55 |
| **72** | **1592** | *H. steffeki* sp. nov.. | **BiH** | Grmeč | Bosnian Federation, Dabar, Dabarska Pećina cave in Donji Dabar near Bankovići, sand on the cave bottom | 44°42.6000′ | 16°38.3100′ | 173 | – | – | – |
| **73** | **1589–1591, 1672** | ***H. steffeki* sp. nov.** | **BiH** | Grmeč | Bosnian Feration, Donji Kamengrad, 4 km W of Sanski Most; spring and well | 44°46.7682′ | 16°33.9859′ | 228 | – | – | – |

**Table 1** (continued). Locality data.

| ID | SeqNo *material* | Species | Country | Orographic unit | Locality | Latitude | Longitude | Alt m | T °C | pH | Cond µS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **74** | **1680, 1681** | *H. steffeki* sp. nov. | **BiH** | Grmeč | Bosnian Federation, Spring Krivina, Lušči Palanka- Miljevci 400 m on road R405 towads Sanski Most, on right side of road | 44°45.7345′ | 16°27.0528′ | 413 | – | – | – |
| **75** | *shells* | *H. steffeki* sp. nov. | **BiH** | Grmeč | Bosnian Federation, Bihać district, Vrelo Zdena 3 km W of Sanski Most | 44°45.7186′ | 16°37.8371′ | 176 | – | – | – |
| **76** | *shells* | *steffeki* sp. nov. | **BiH** | Grmeč | Bosnian Federation, Izvor Banovac between Gorice and Naprelie, left side of the road | 44°46.8065′ | 16°30.0365′ | 375 | – | – | – |
| **77** | **1666, 1667** | *H. wienerwaldensis* | **AT** | Oberösterreichische Voralpen | upper spring in the valley above the Konrad Lorenz Forschungsinstitut, south of Grünau im Almtal | 47°48.8500′ | 13°56.85′ | 569 | – | – | – |
| **78** | **1682** | *H. wienerwaldensis* | | Wienerwald | Döbling, Vienna, broken pipe from old walled spring at N slope of Latisberg, Nesselbach drainage system | 48°16.2900′ | 16°18.97′ | 410 | – | – | – |
| **79** | **2148** | *H. kerschneri* | | Oberösterreichische Voralpen | Nationalpark Oberösterreichische Kalkalpen, Quelle (=spring) JÖA, 110425/ABOL/558 | 47°46.9440′ | 14°26.055′ | 810 | – | – | – |
| **80** | *shells* | *H. wienerwaldensis* | | Oberösterreichische Voralpen | W of In der Krems, drainage system of river Krems, Quelle (= spring) KREMS, 110425/ABOL/517 | 47°51.4530′ | 14°6.2850′ | 637 | – | – | – |
| **81** | *shells* | *H. wienerwaldensis* | | Wienerwald | Kaumberg, near Forellenhof | 48°00.1740′ | 15°55.9800′ | 530 | – | – | – |
| **82** | **1673** | *H. wienerwaldensis* | | Wienerwald | Alland, Glashütte, Reithquelle | 48°03.5940′ | 16°3.8040′ | 260 | – | – | – |
| **83** | **1688-1690** | *H. wienerwaldensis* | | Wienerwald | Zobeltgraben, Neusiedl, Bezirk Baden | 47°54.8330′ | 16°4.9833′ | 390 | – | – | – |
| **84** | *shells* | *H. wienerwaldensis* | | Wienerwald | Spring Wiener Graben | 48°07.6000′ | 16°12.6400′ | 366 | – | – | – |

* data from Ložek & Galvánek 1987

** data from Čiliak & Šeffek 2013

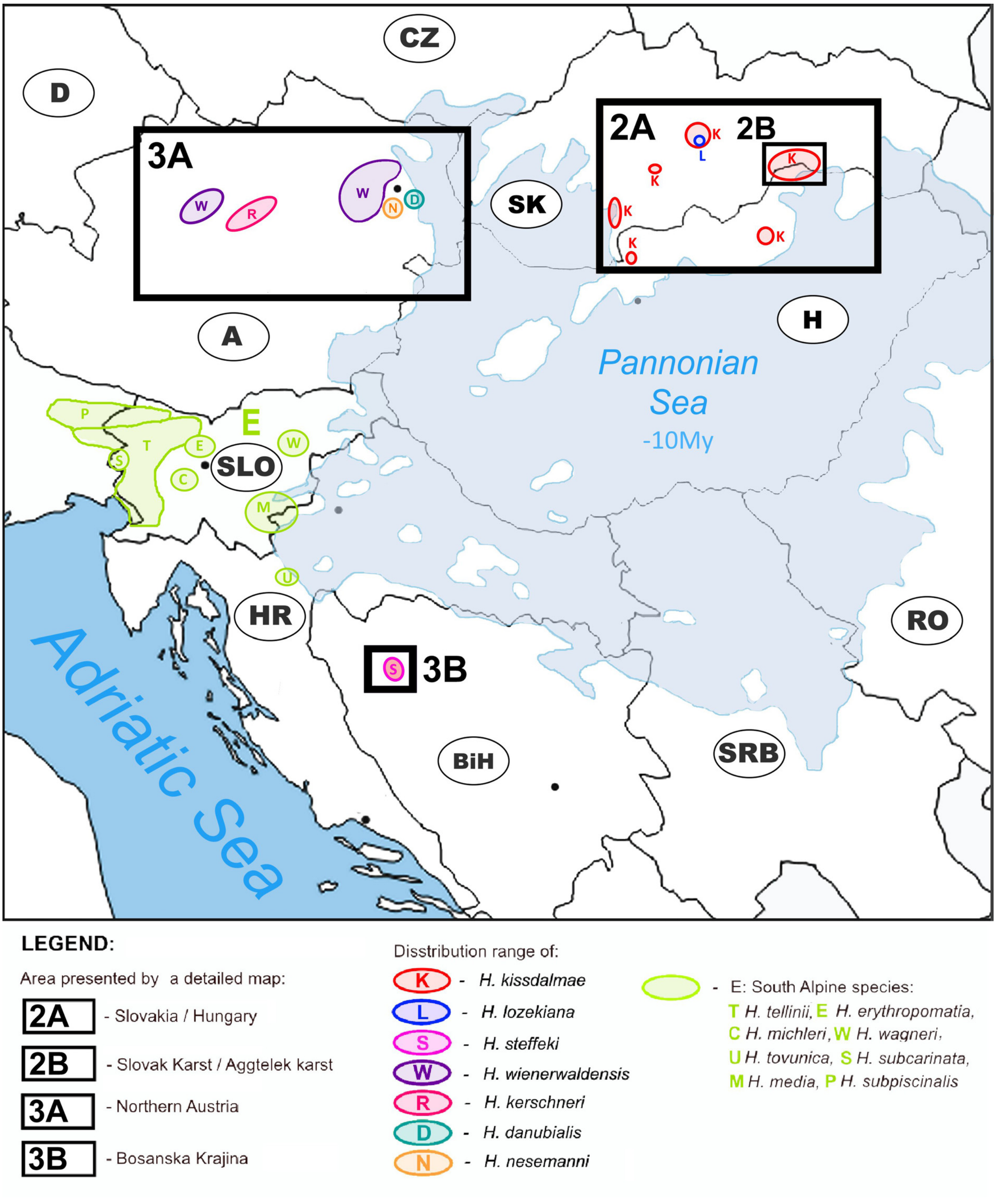

**Fig. 1.** Current distribution of *Hauffenia* Pollonera, 1898 in relation to the extension of the Pannonian Sea 10 Ma.

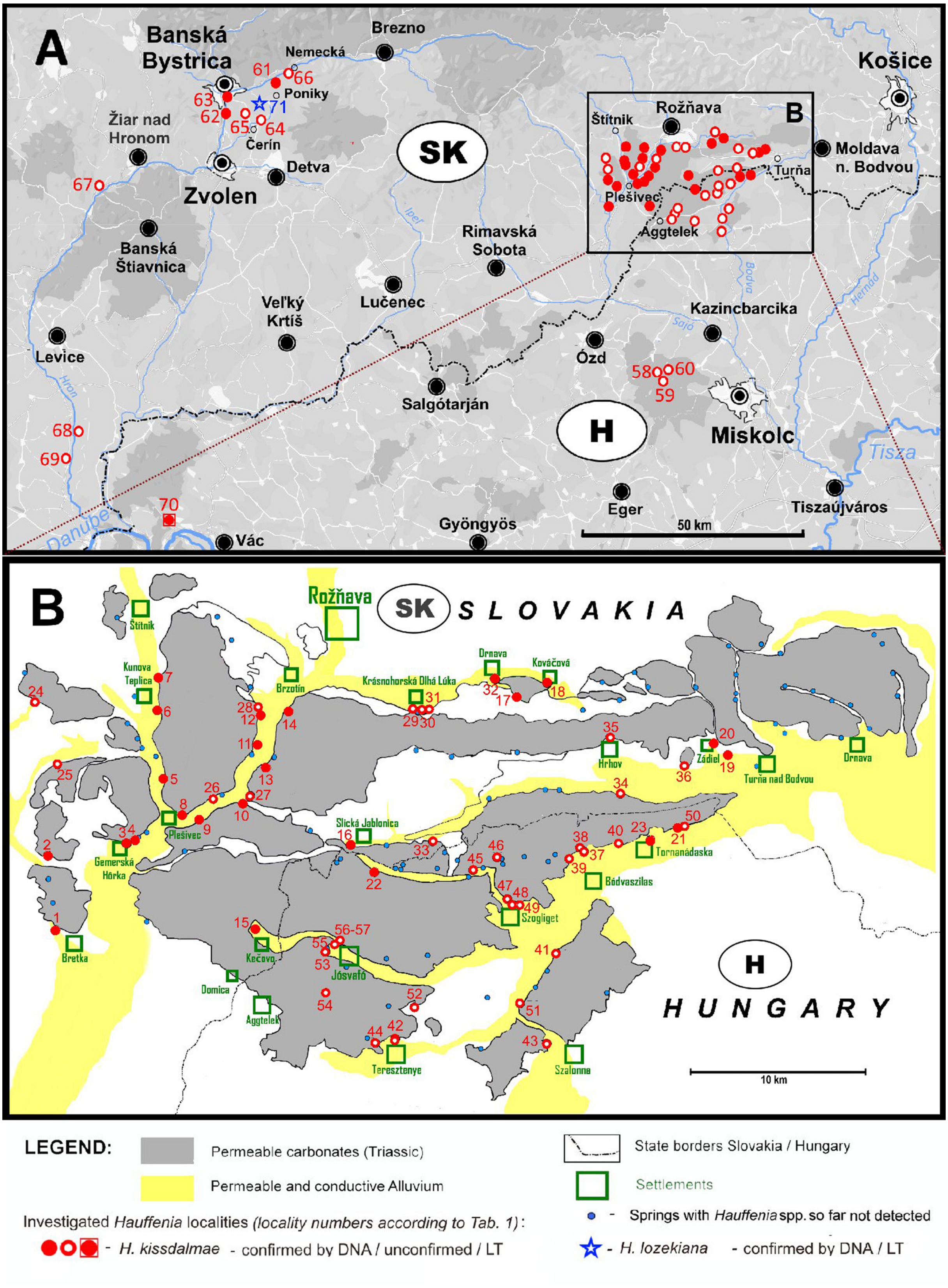

**Fig. 2.** Localities in Slovakia (A) and Hungary (B). Abbreviation: LT = type locality.

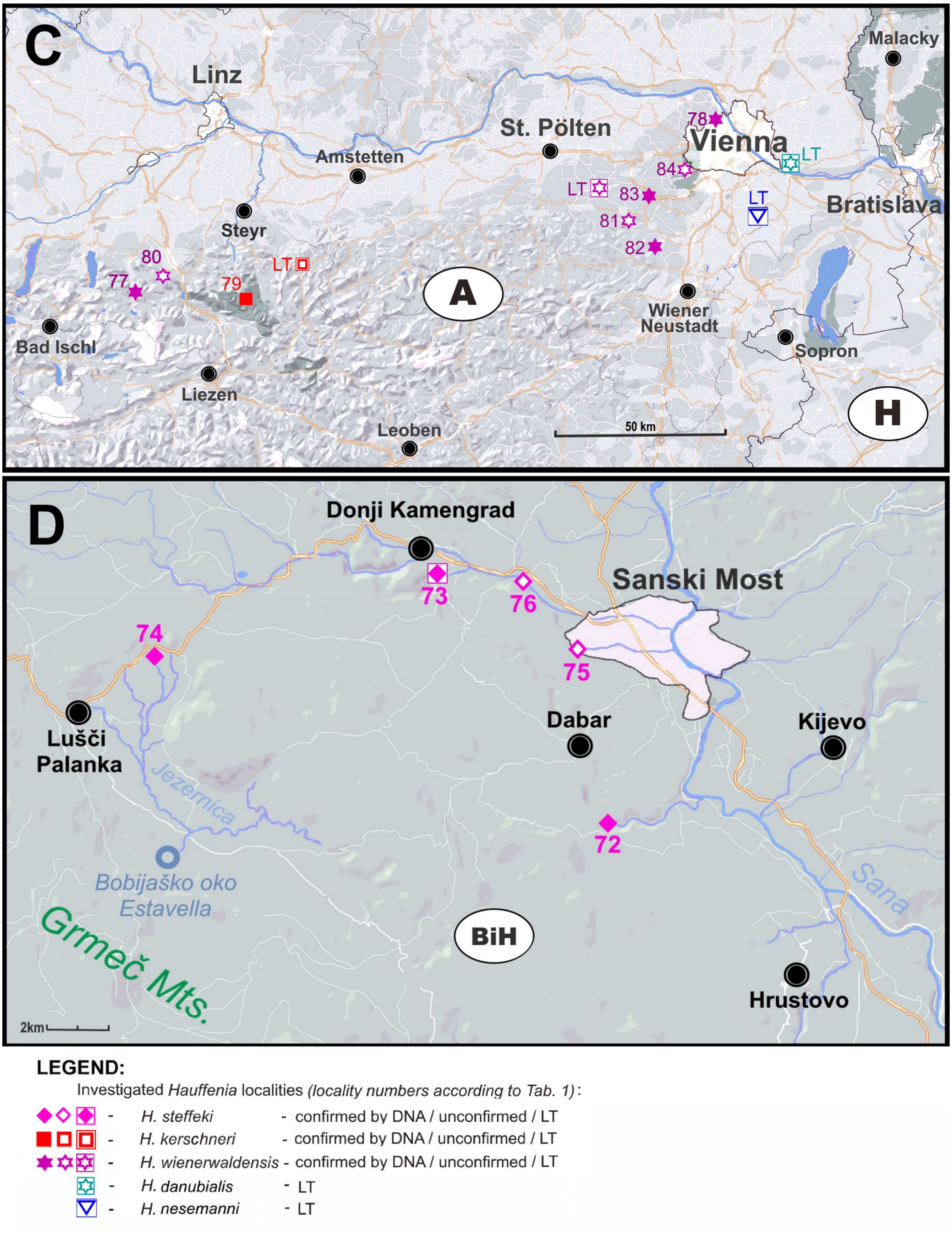

**Fig. 3.** Localities in Austria (C) and Bosnia and Herzegovina (D). Abbreviation: LT = type locality.

**Fig. 4.** Selected localities of *H. kissdalmae* Erőss & Petró, 2008. Image numbers correspond to locality numbers in Table 1.

### Time tree

In order to infer the temporal scenario of the diversification of *Hauffenia*, we reconstructed a time tree in BEAST ver. 2.6.3 (Bouckaert *et al.* 2019) based on COI, because this gene was available in GenBank for the largest number of species (Table 2). As already mentioned, the set of new COI sequences of Austrian, Slovak, Hungarian and Bosnian species was smaller than for 16S and ITS2 because a number of specimens failed to amplify. The new sequences were aligned together with the sequences of other species of *Hauffenia* as well as sequences of *Peringia ulvae* (Pennant, 1777) and *Salenthydrobia ferrerii* (Wilke, 2003) retrieved from GenBank (Table 2) using Clustal W (Thompson *et al.* 1994) implemented in BioEdit. The latter two species were used for time calibration as their split is the only plausibly dated case in hydrobiids (Wilke 2003; see below). There are Miocene fossils attributed to *Hauffenia* (Papp 1954; Reischütz 2000; Anistratenko & Anistratenko 2009; Bandel 2010; Harzhauser *et al.* 2014); however, these allocations are doubtful because hydrobiid genera can rarely be identified based on shell morphology. The final alignment was trimmed to 535 positions. Substitutions were not saturated according to the test of Xia *et al.* (2003) executed in DAMBE. The best fitting substitution model of those implemented in BEAST suggested by jModeltest based on the Bayesian Information Criterion was again HKY + Γ. The analysis in BEAST was set up using BEAUTi. We selected the birth-death model as tree prior deciding against the more commonly used Yule-model because narrow range habitat specialists such as most hydrobiids probably have a fairly high species turnover. The model comparison in Path sampler, an app provided with BEAST, remained ambiguous. For either model, we did not manage to let the marginal likelihood estimates fluctuating within the same range converge. Since the strict clock was rejected by MEGA X (Kumar *et al.* 2018), the log normal relaxed clock model was implemented. The split of *P. ulvae* and *S. ferrerii* associated with the Messinian salinity crisis 5.96–5.33 Ma (Krijgsman

**Fig. 5.** Type locality of *H. lozekiana* sp. nov. (71) and Dabarska Pećina, a locality of *H. steffeki* sp. nov. (72) (see Table 1).

**Table 2** (continued on next page). GenBank accession numbers of newly sequenced specimens. Abbreviations: Loc = locality; Seq = Sequence Number (see Table 1). Individuals with sequences identical to one of those listed here are given in Fig. 6 and Supp. file 1 (Figs S1, S3).

| **COI** | | | | **16S** | | | |
|---|---|---|---|---|---|---|---|
| **Species** | **Loc** | **Seq** | **GenBank** | **Species** | **Loc** | **Seq** | **GenBank** |
| *H. kerschneri* | 79 | 2148 | MZ408290 | *H. kerschneri* | 79 | 2148 | MZ424110 |
| *H. kissdalmae* | 01 | 1593 | MZ408291 | *H. kissdalmae* | 01 | 1593 | MZ424111 |
| *H. kissdalmae* | 02 | 1604 | MZ408292 | *H. kissdalmae* | 02 | 1604 | MZ424112 |
| *H. kissdalmae* | 04 | 1603 | MZ408293 | *H. kissdalmae* | 04 | 1639 | MZ424113 |
| *H. kissdalmae* | 05 | 1582 | MZ408294 | *H. kissdalmae* | 15 | 1628 | MZ424114 |
| *H. kissdalmae* | 10 | 1611 | MZ408295 | *H. kissdalmae* | 19 | 1620 | MZ424115 |
| *H. kissdalmae* | 20 | 1691 | MZ408296 | *H. kissdalmae* | 20 | 1691 | MZ424116 |
| *H. kissdalmae* | 22 | 1677 | MZ408297 | *H. kissdalmae* | 22 | 1677 | MZ424117 |
| *H. kissdalmae* | 22 | 1678 | MZ408298 | *H. kissdalmae* | 61 | 1614 | MZ424118 |
| *H. kissdalmae* | 62 | 1663 | MZ408299 | *H. lozekiana* | 71 | 1610 | MZ424119 |
| *H. kissdalmae* | 62 | 1664 | MZ408300 | *H. steffeki* | 73 | 1589 | MZ424120 |
| *H. kissdalmae* | 63 | 1686 | MZ408301 | *H. steffeki* | 73 | 1672 | MZ424121 |
| *H. lozekiana* | 71 | 1610 | MZ408302 | *H. steffeki* | 74 | 1592 | MZ424122 |
| *H. lozekiana* | 71 | 1734 | MZ408303 | *H. steffeki* | 74 | 1681 | MZ424123 |
| *H. steffeki* | 72 | 1592 | MZ408304 | *H. wienerwaldensis* | 77 | 1666 | MZ424124 |
| *H. steffeki* | 73 | 1589 | MZ408305 | *H. wienerwaldensis* | 78 | 1682 | MZ424125 |
| *H. steffeki* | 73 | 1672 | MZ408306 | *Kerkia* sp. | – | 1578 | MZ424126 |
| *H. steffeki* | 74 | 1680 | MZ408307 | *Kerkia* sp. | – | 1581 | MZ424127 |
| *H. wienerwaldensis* | 78 | 1682 | MZ408308 | *Kerkia* sp. | – | 1587 | MZ424128 |
| *H. wienerwaldensis* | 82 | 1673 | MZ408309 | *Kerkia* sp. | – | 1588 | MZ424129 |
| *H. wienerwaldensis* | 83 | 1688 | MZ408310 | | | | |

| **ITS2** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Species** | **Loc** | **Seq** | **GenBank** | **Species** | **Loc** | **Seq** | **GenBank** |
| *H. kerschneri* | 79 | 2148 | MZ424134 | *H. kissdalmae* | 23 | 1594 | MZ424163 |
| *H. kerschneri* | 79 | 2149 | MZ424135 | *H. kissdalmae* | 23 | 1595 | MZ424164 |
| *H. kissdalmae* | 01 | 1593 | MZ424136 | *H. kissdalmae* | 41 | 1596 | MZ424165 |
| *H. kissdalmae* | 02 | 1604 | MZ424137 | *H. kissdalmae* | 61 | 1614 | MZ424166 |
| *H. kissdalmae* | 03 | 1626 | MZ424138 | *H. kissdalmae* | 61 | 1616 | MZ424167 |
| *H. kissdalmae* | 04 | 1603 | MZ424139 | *H. kissdalmae* | 61 | 1643 | MZ424168 |
| *H. kissdalmae* | 04 | 1636 | MZ424140 | *H. kissdalmae* | 62 | 1663 | MZ424169 |
| *H. kissdalmae* | 04 | 1639 | MZ424141 | *H. kissdalmae* | 62 | 1664 | MZ424170 |
| *H. kissdalmae* | 04 | 1640 | MZ424142 | *H. kissdalmae* | 62 | 1665 | MZ424171 |
| *H. kissdalmae* | 04 | 1641 | MZ424143 | *H. kissdalmae* | 63 | 1686 | MZ424172 |
| *H. kissdalmae* | 05 | 1582 | MZ424144 | *H. kissdalmae* | 63 | 1687 | MZ424173 |
| *H. kissdalmae* | 06 | 1654 | MZ424145 | *H. lozekiana* | 71 | 1610 | MZ424174 |
| *H. kissdalmae* | 06 | 1656 | MZ424146 | *H. steffeki* | 72 | 1592 | MZ424175 |
| *H. kissdalmae* | 08 | 1619 | MZ424147 | *H. steffeki* | 73 | 1589 | MZ424176 |
| *H. kissdalmae* | 11 | 1598 | MZ424148 | *H. steffeki* | 73 | 1590 | MZ424177 |

**Table 2 (**continued). GenBank accession numbers of newly sequenced specimens.

**ITS2**

| **Species** | **Loc** | **Seq** | **GenBank** | **Species** | **Loc** | **Seq** | **GenBank** |
|---|---|---|---|---|---|---|---|
| *H. kissdalmae* | 11 | 1599 | MZ424149 | *H. steffeki* | 73 | 1591 | MZ424178 |
| *H. kissdalmae* | 13 | 1651 | MZ424150 | *H. steffeki* | 74 | 1680 | MZ424179 |
| *H. kissdalmae* | 13 | 1652 | MZ424151 | *H. steffeki* | 74 | 1681 | MZ424180 |
| *H. kissdalmae* | 13 | 1653 | MZ424152 | *H. wienerwaldensis* | 77 | 1667 | MZ424181 |
| *H. kissdalmae* | 15 | 1628 | MZ424153 | *H. wienerwaldensis* | 78 | 1682 | MZ424182 |
| *H. kissdalmae* | 15 | 1629 | MZ424154 | *H. wienerwaldensis* | 82 | 1673 | MZ424183 |
| *H. kissdalmae* | 17 | 1602 | MZ424155 | *H. wienerwaldensis* | 83 | 1688 | MZ424184 |
| *H. kissdalmae* | 18 | 1605 | MZ424156 | *H. wienerwaldensis* | 83 | 1689 | MZ424185 |
| *H. kissdalmae* | 18 | 1606 | MZ424157 | *H. wienerwaldensis* | 83 | 1690 | MZ424186 |
| *H. kissdalmae* | 20 | 1691 | MZ424158 | *Kerkia* sp. | – | 1578 | MZ424130 |
| *H. kissdalmae* | 20 | 1692 | MZ424159 | *Kerkia* sp. | – | 1581 | MZ424131 |
| *H. kissdalmae* | 20 | 1693 | MZ424160 | *Kerkia* sp. | – | 1587 | MZ424132 |
| *H. kissdalmae* | 21 | 1600 | MZ424161 | *Kerkia* sp. | – | 1588 | MZ424133 |
| *H. kissdalmae* | 21 | 1601 | MZ424162 | | | | |

*et al.* 1999) separating the Mediterranean from the Atlantic Ocean was used for calibration (Wilke 2003). According to Wilke (2003), this divergence happened at the end of the crisis. However, it appears much more plausible, that the generic lineages started to separate already once the Mediterranean was cut off (Haase *et al.* 2007). As a consequence, we applied a uniform prior within the bounds of 5.33 to 5.96 Ma. BEAST was run twice with 20 mio generations and logging of every 1000th tree. Both runs were combined in LogCombiner with a burnin of 25% after convergence of parameter estimates had been controlled in Tracer ver. 1.7.1 (Rambaut *et al.* 2018). A maximum clade credibility tree based on the remaining 30 002 trees with mean node heights was constructed in TreeAnnotator.

In order to compare the topology of the ultrametric tree to unconstrained reconstructions, we conducted also a ML analysis using W-IQ-TREE with 1000 ultrafast bootstrap replicates and a Bayesian analysis in MrBayes based on the same substitution model. MrBayes ran with the same specifications as above.

### Network analysis

The relationships among 16S haplotypes of *H. kissdalmae* were also analyzed by a statistical parsimony network (Clement *et al.* 2002) reconstructed using PopART ver. 1.7 (Leigh & Bryant 2015) because a network probably represents close, intraspecific relationships better than a tree.

## Molecular diagnoses

We also incorporated sites of COI into the diagnoses of the two new species using the R-package QUIDDICH (R-core team; Kühn & Haase 2020). Because of the low number of fixed unique (type 1 characters sensu Kühn & Haase 2020) and the entire lack of type 2 characters (polymorphic sites whose states are not shared with any other taxon compared), we identified type 3 (polymorphic sites with at least one state not shared with any other taxon compared) and type 4 characters (fixed unique states in pairwise comparisons) as well. In addition, we calculated genetic p-distances in MEGA X with pairwise deletion of missing sites.

### Morphology

Up to 20 shells of the new species with at least 2.125 whorls deemed subadult to adult – in contrast to other truncatelloidean genera, *Hauffenia* does not develop a thickened apertural lip once fully-grown and sexually mature (e.g., Verhaegen *et al.* 2018) – were measured from photographs taken with a Zeiss SteREO Discovery ver. 20 dissecting microscope equipped with a Plan Apo S 0.63 × objective and a Zeiss Axio Cam MR3 using the program AxioVision LE (Zeiss). Shell height, shell width, aperture height and aperture width were measured parallel or perpendicular to the coiling axis. The number of whorls was counted to the nearest eighth (Kerney & Cameron 1979).

For scanning electron microscopy (SEM), shells, opercula and radulae were cleaned in ca 2.5% sodium hypochlorite. Penes were dissected free, dehydrated in ethanol, transferred to absolute acetone, and finally dried in hexamethyldisilazane (Nation 1983). Specimens were mounted with carbon tabs and coated with palladium/platinum in a Fisons Polaron SC7640 sputter coater prior to imaging in a Zeiss EVO LS10 SEM.

### Abbreviations of collections

HNHM = Hungarian natural History Museum, Budapest
JG = collection Jozef Grego, Banská Bystrica, Slovakia
NHMW = Natural History Museum Vienna, Austria
NMBE = Natural History Museum Bern, Switzerland
SMF = Senckenberg Research Institute and Nature Museum, Frankfurt, Germany
SMOPAJ = Slovak Museum of nature Protection and Caving, Liptovský Mikuláš, Slovakia
ZMH = Zoological Museum of Hamburg, Germany
ZPE = collection Zoltán Péter Erőss, Budapest, Hungary

## Results

### *Phylogenetic analyses*

The separate analyses of 16S and ITS2 in MrBayes gave largely equivalent results (Supp. file 1). Each species was well supported, but their relationships remained largely unresolved. Only the sister relationship of *H. steffeki* sp. nov. to *H. wienerwaldensis* Haase, 1992 received significant support based on 16S. In the joint analysis (Fig. 6), the picture remained similar. *Hauffenia steffeki* sp. nov. was now sister species of all other Austrian, Hungarian and Slovak species. The Austrian species formed a clade and *H. kissdalmae* and *H. lozekiana* sp. nov. another one. However, the posterior probabilities for inter-species relationships were all below 0.95. The single gene ML analyses produced a similarly ambiguous picture regarding species relationships as did the Bayesian analyses. In the joint analysis, *H. kissdalmae* was sister species to all other species, which formed a caterpillar in the order *H. lozekiana* sp. nov., *H. kerschneri* (Zimmermann, 1930), *H. wienerwaldensis*, and *H. steffeki* sp. nov. (Fig. 7). Again, species were well supported, but bootstrap values for their relationships were all < 70.

The analyses across all three genes including the intersect of available taxa only confirmed *H. steffeki* sp. nov. as sister species to all other species with significant support (Fig. 8; Supp. file 1). Whether the Austrian species had an exclusive common ancestor and whether *H. lozekiana* sp. nov. was sister species of them or *H. kissdalmae* remained inconclusive.

The time tree across all newly sequenced species, five species for which sequences were available from GenBank, as well as *Peringia ulvae* and *Salenthydrobia ferrerii* used for calibration, also displayed quite ambiguous relationships (Fig. 9). *Hauffenia steffeki* sp. nov. was identified as sister species to all other taxa, however, with only weak support. The remaining species formed two maximally supported clades, though, one consisting of all other species from the Balkans, the other one of the northern species. Within these clades, relationships had again only low posterior probabilities. Because of these ambiguities and because of the considerably large highest posterior density intervals, inferences of temporal events should be made cautiously. It is probably safe to state that all intraspecific differentiation is young and most splits fall into the late Pleistocene. Most speciation events, however, appear to have occurred during the Pliocene, some possibly already in the late Miocene. The origin of *Hauffenia* also seems to date back into the Miocene.

In contrast to the ultrametric tree, Bayesian and ML analyses yielded only two well supported major clades, one consisting of all Balkan species with *H. steffeki* sp. nov. branching off first, and the other one of the Austrian, Hungarian and Slovak taxa. Relationships within these clades remained largely inconclusive (Supp. file 1).

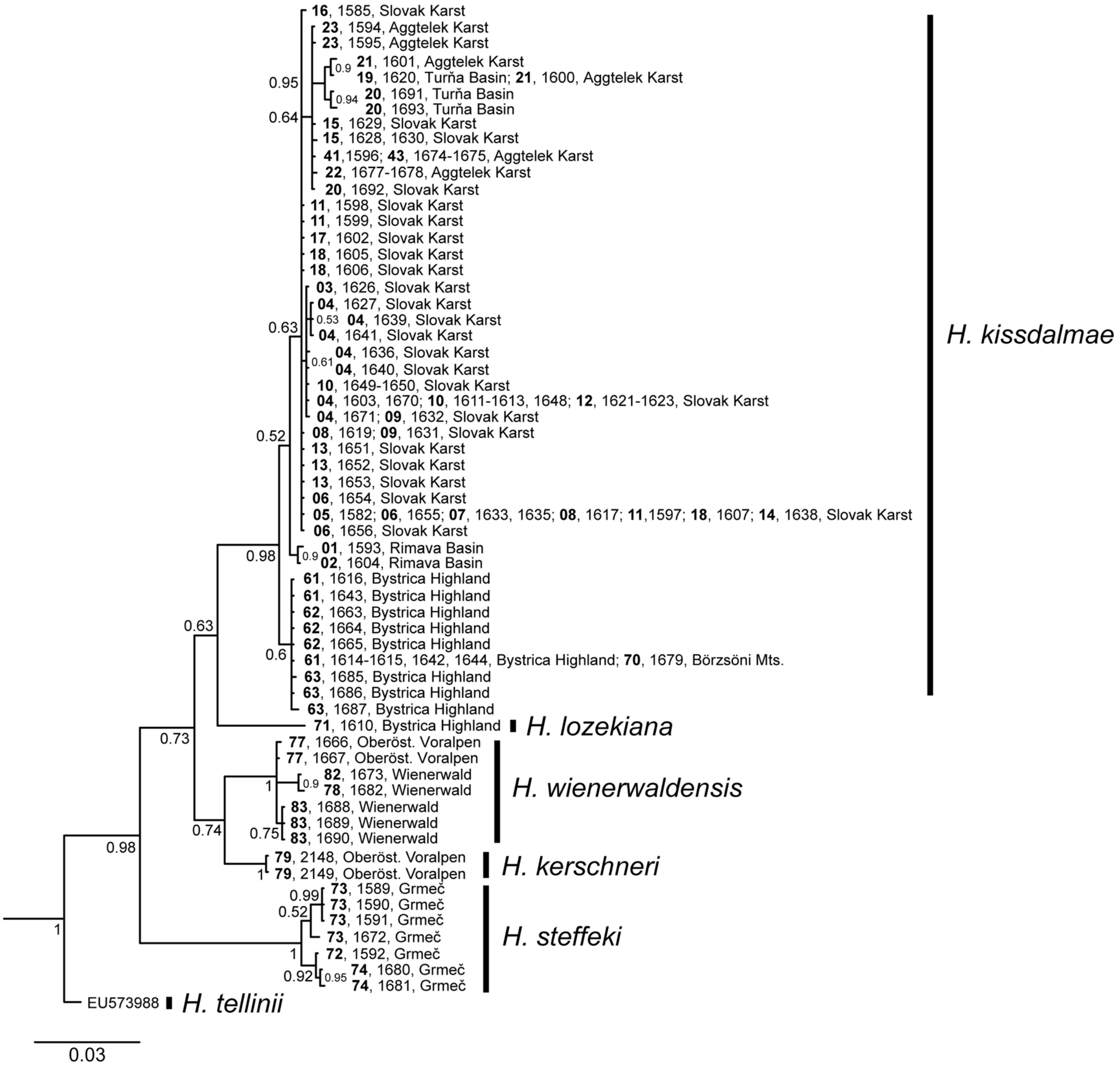

**Fig. 6.** Phylogenetic tree from Bayesian analysis in MrBayes based on 16S rRNA and ITS2 (outgroup pruned off). Specimens identified by two-digit locality code in bold, four digit individual DNA code, and orographic unit (see Table 1) or GenBank accession number (from Ponder *et al.* 2008); posterior probabilities at nodes; scale bar in substitutions per site.

In all of these analyses including data from GenBank, the sequences attributed to *H. erythropomatia* (Hauffen, 1856) were never monophyletic.

The statistical parsimony network of *H. kissdalmae* is presented in the context of the species' distribution in the following section.

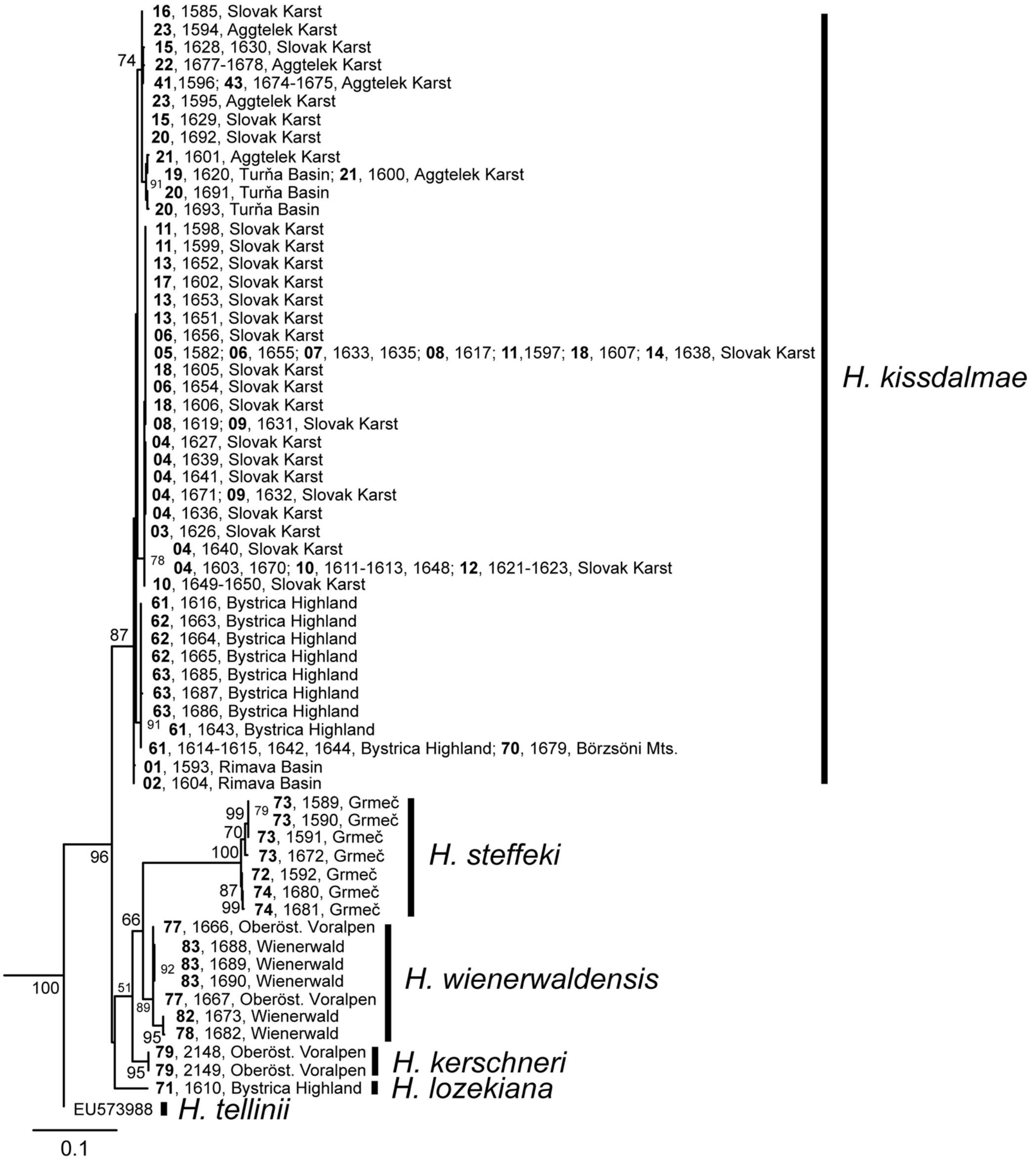

**Fig. 7.** Phylogenetic tree from maximum likelihood analysis in W-IQ-TREE based on 16S rRNA and ITS2 (outgroup pruned off). Specimens identified by two-digit locality code in bold, four digit individual DNA code, and orographic unit (see Table 1) or GenBank accession number (from Ponder *et al.* 2008); bootstrap support values at nodes > 50; not given for extremely short nodes within *H. kissdalmae* Erőss & Petró, 2008 and *H. wienerwaldensis* Haase, 1992; scale bar in substitutions per site.

### *Distribution and ecology of H. kissdalmae*

Based on the molecular data presented in this study, the range of this species extends considerably northeastward from the type locality to southeast Slovakia and northeast Hungary. The type locality Kismaros at the eastern slope of the Börzsöny Mountains (Erőss & Petró 2008) is rather an extreme point at the southwestern edge of the distribution. The main distribution of this species includes the large Mesozoic karstic formation of Gemersko-Turniansky kras (Gömor-Tornai karszt) known as Slovenský kras in Slovakia and Aggteleki karszt in Hungary, as well as in the adjacent parts of Turňa Basin and Rimava Basin. The 800 km$^2$ karst region is well known for its well karstified plateau-type of karst with

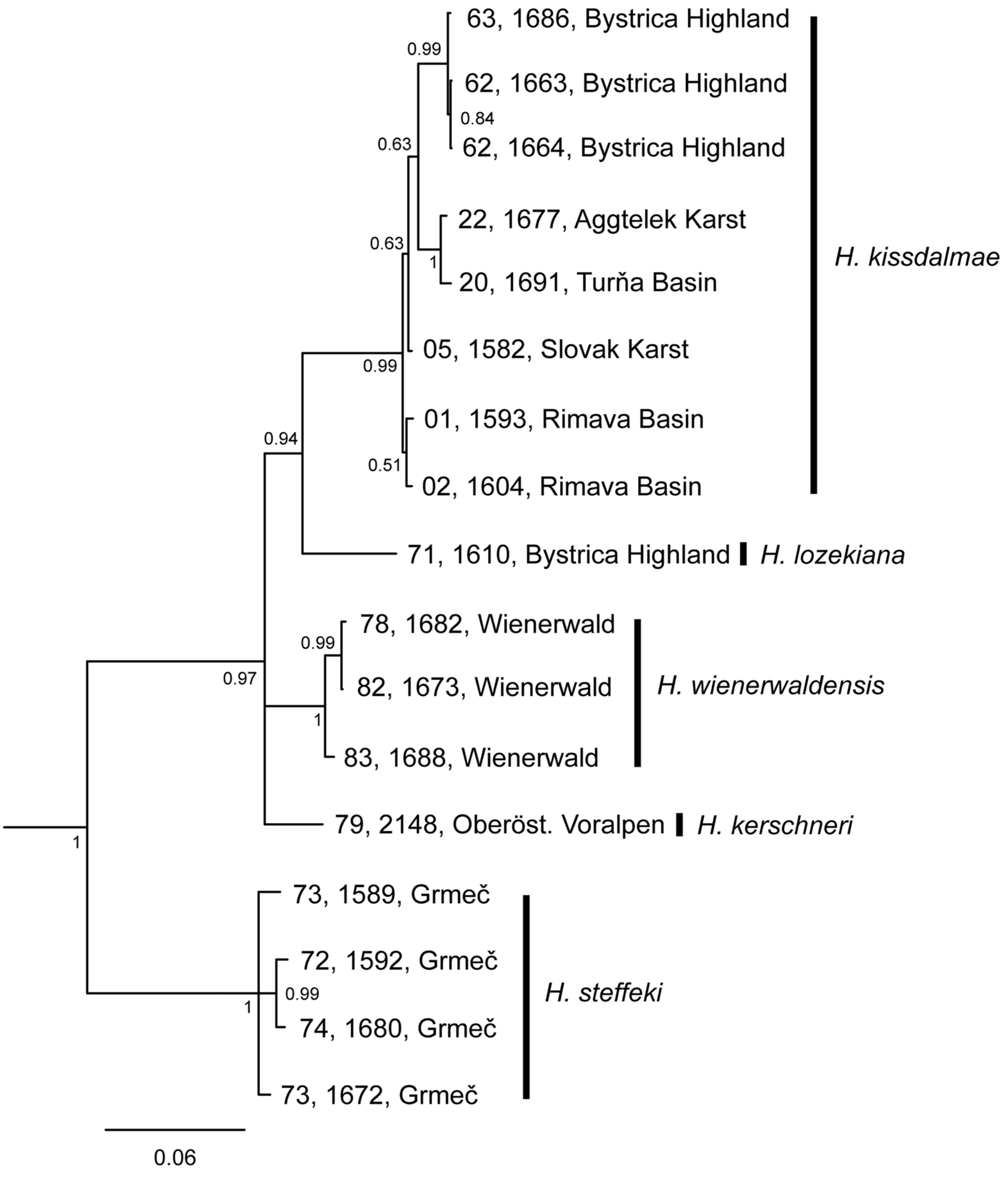

**Fig. 8.** Phylogenetic tree from Bayesian analysis in MrBayes based on COI, 16S rRNA and ITS2 (outgroup pruned off). Specimens identified by two-digit locality code in bold, four digit individual DNA code, and orographic unit (see Table 1); posterior probabilities at nodes; scale bar in substitutions per site.

numerous springs. The species was confirmed by molecular data in 33 spring localities in Slovakia (30) and Hungary (3) (Fig. 2). The sequencing of live collected material from further nine localities was not successful and at 24 other sites only empty shells were found. Due to geographical and hydrogeological relations among all sites we consider these records lacking molecular evidence as *H.* cf. *kissdalmae*. In the statistical parsimony network (Fig. 10), the populations from the Slaná River alluvium with its tributaries (rivers Muráň, Štítnik, Čremošná) and the western part of the Turňa Basin harbor the central, most common and therefore probably ancestral haplotype A. A clade containing haplotypes D and E from the Jósva and Ménes rivulets, respectively, is typical for the Bódva River alluvium in the eastern part of the range. Both rivulets as right tributaries of the Bódva River are characterized by discontinuous alluvial deposits and vanishing surface streams leaving long parts of the valleys without surface water. Therefore, the populations at the origins of the rivulets may be somewhat isolated. Haplotype C occurs close to the Bódva River, however, at a water temperature (ca 14°C; Table 1) which is remarkably higher than the average karst spring in the region (9.8°C). Also haplotypes F and G were collected in warm springs, Gemerská Hôrka and (14°C) Bretka (19°C), respectively. Whether the higher temperatures indicate local adaptation and/or are causal for the differentiation is unclear, though, as in in the Gemerská Hôrka spring haplotypes A and F were found sympatrically. More data from springs associated to Bódva alluvium would be needed to clearly understand the clade distribution.

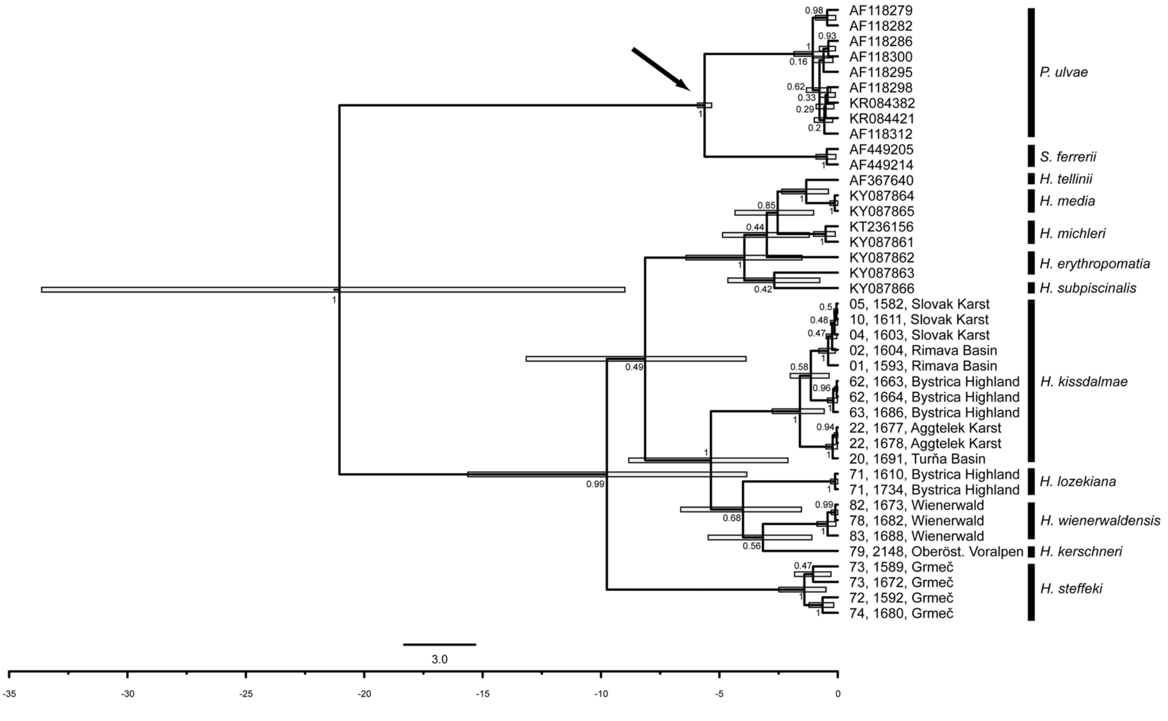

**Fig. 9.** Phylogenetic time-tree from Bayesian analysis in BEAST based on COI. Specimens identified by two-digit locality code in bold, four digit individual DNA code, and orographic unit (see Table 1) or GenBank accession number (from Wilke & Davis 2000; Wilke 2003; Ponder *et al.* 2008; Falniowski & Szarowska 2015; Barco *et al.* 2016; Rysiewska *et al.* 2017); posterior probabilities and highest posterior density intervals at nodes; arrow points at calibrated node; scale bar in substitutions per site; timeline on bottom in Ma before present.

The second center of distribution was found in the Valley of the Hron River in Slovakia (haplotype H), from Nemecká through Slovenská Ľupča, Banská Bystrica, Revište, and Vozokany towards its mouth into the Danube River. This geographically and hydrologically isolated group includes also the type locality of the species in Kismaros. A third isolated occurrence was just recently discovered at the northern foot of the Bükk Mountains (Hungary), but due to missing molecular data we only tentatively assigned these populations to *H. kissdalmae*.

## *Ecology*

After the first findings of empty shells in the Slovak Karst by Vojen Ložek, it was assumed, that the species is similar and related to some of the stygobiotic Slovenian and Croatian species (Mitterová 1986; Šteffek & Grego 2002, 2008) and inhabits the numerous caves and karst conduits of the region. The following intensive sampling of cave rivers in the region by Jozef Grego confirmed, that despite findings of numerous live specimens at the cave spring heads, the species was repeatedly not present in the cave streams except scarce dead shells near the entrance (sampled caves: Gombasek, Buzgó, Brzotínska, Misuka, Zúgó, Hrušovská, Vápená and Gemerskoteplická). For example, in the river cave Buzgó with one main direct cave outflow and three associated springs monitored in irregular intervals over 15 years, *Hauffenia* were consistently absent in the cave and in the direct outflow, but in the three springs associated to stony debris at least a higher number of empty shells and occasionally living specimens were found. This fact indicates, that despite the species being a stygobite adapted to underground habitats (missing eyes and pigmentation), it does not inhabit the caves itself, but is restricted to the zones of dispersed springs with stony debris (Grego & Šteffek 2010). It was first supposed that the main food source of the species was the chemolithotrophic bacterial film covering the deep spring debris, but later indications

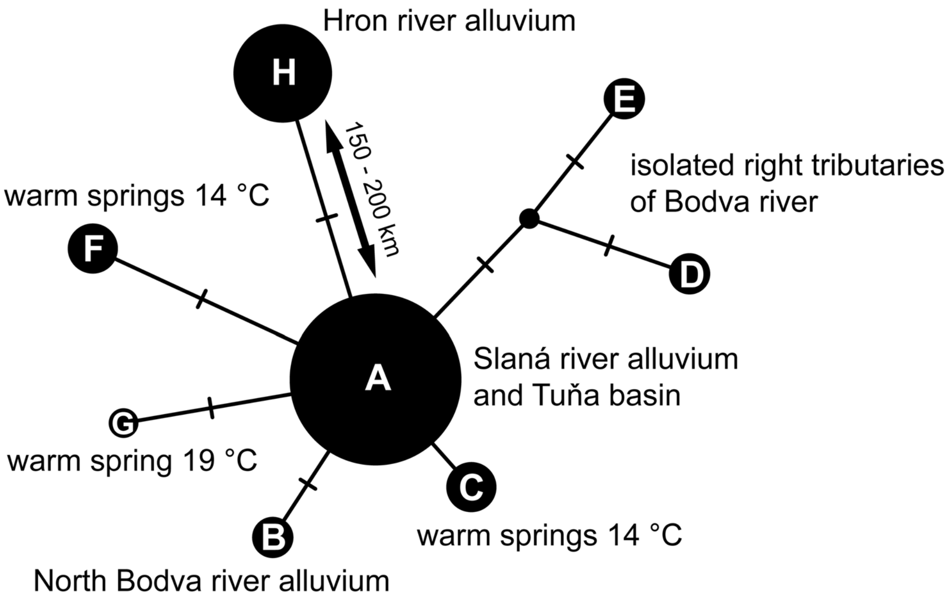

**Fig. 10.** Statistical parsimony network of *H. kissdalmae* Erőss & Petró, 2008 based on 16S rRNA. Haplotype circles A–H are proportional in size to the number of individuals with identical sequences. For comprehensive specimen information see Supp. file 1, Fig. S1. Crosslines and small circle represent non-sampled haplotypes.

suggested that the animals were associated with the delicate net of roots and rootlets within the spring zone. We called this specific hypogean habitat phreatic rhizosphere (Jasinska *et al.* 1996; Grego in press) where the mollusks and crustaceans likely feed on the root exudates (Badri & Vivanco 2009) or on the microbes supported by exudates or directly on live or decaying root tissue. The root exudates are probably a frequently underestimated hypogean food source considering that the trees are returning about 20–40% of the carbon fixed by photosynthesis to the soil microflora and mycorrhiza through exudates (Badri & Vivanco 2009). The association of the species to the phreatic rhizosphere was on the one hand directly confirmed by higher numbers of living individuals collected in habitats with roots (e.g., wells or spring heads), and on the other hand by long term monitoring (2012–2021) of the spring Ľadová Studňa in the Driekyňa valley, Bystrica Highlands, Slovakia. The sampling was done according to Grego *et al.* (2017). We took 250–280 g of wet sediment at the spring head at each sampling. This yielded a relatively high and stable count of live individuals (20–35) as well as a stable ratio to empty shells (0.8–0.86) until the first selective tree cut in the forest in about 50 m around the spring zone in autumn 2013. Under these altered conditions the population of *H. kissdalmae* declined (live individuals 12–20) and the population displayed a higher instability (ratio of live to empty shells 0.33–1.28). After the entire deforestation of the spring zone in autumn 2014, the population rapidly decreased to 3–5 live individuals per sample with a marked increase of empty shells (43) and four years later, the population apparently disappeared and now even empty shells became scarce (Fig. 11). These observations strongly support the association of *H. kissdalmae* to the rhizosphere and the importance of the dendroflora surrounding the spring zone.

Distribution range, molecular data and the considerations about habitat suggest that the species disperses along the alluvial river and rivulet beds represented by the hyporheic zone and the phreatic rhizosphere. Most likely the species is widespread under the tree belts framing the rivers along the alluvium, but live individuals can only be collected in springs or wells associated to the alluvium. The narrow specialization

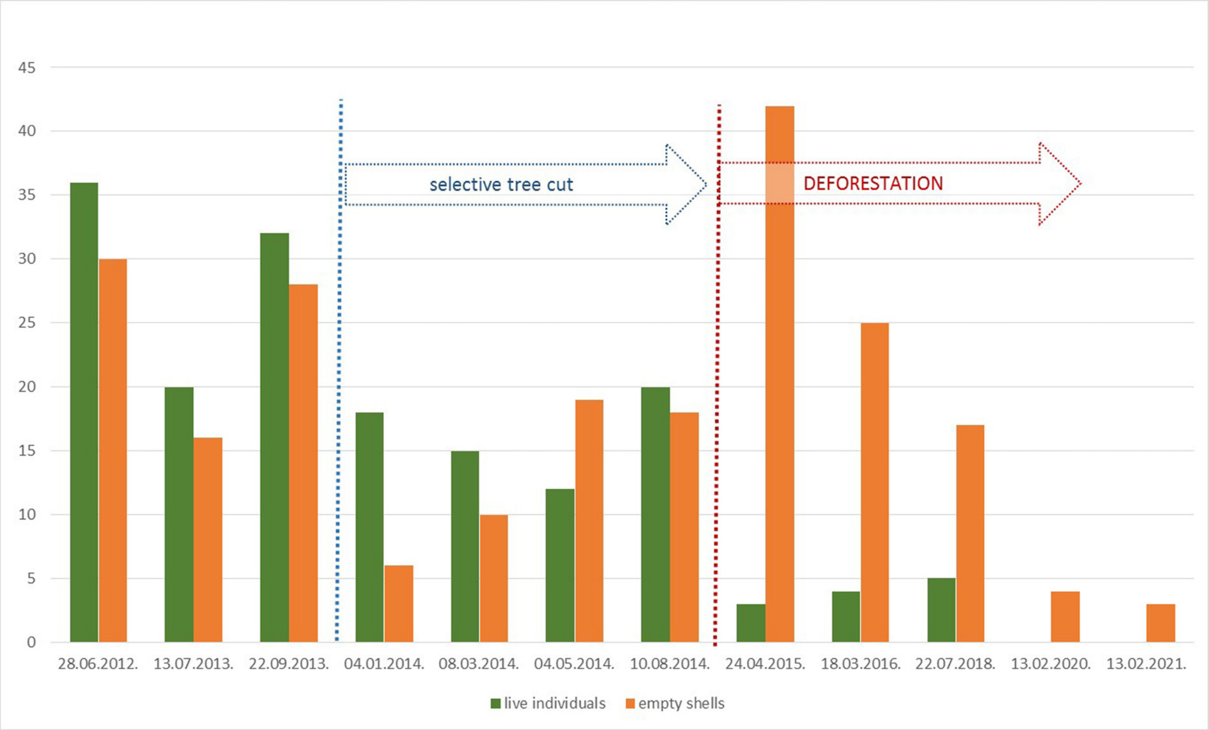

**Fig. 11.** Monitoring of *H. kissdalmae* Erőss & Petró, 2008 in spring Ľadová Studňa, Driekyňa Valley, Slovenská Ľupča, Slovakia (locality 61 from Table 1) from 2012–2021.

of *H. kissdalmae* to specific subterranean habitats makes it very sensitive to alterations of the habitat. The main threat is the deforestation of spring zones and river banks, which is frequently followed by artificial strengthening of the river banks or transformation of the river beds into artificial channels as well as building of dams and small power plants. This obviously leads to dramatic changes of the character of the hyporheic zone, including groundwater pollution.

## *Systematic descriptions*

The quality of fixation of the material was fairly heterogeneous. Therefore, anatomical descriptions had to remain incomplete. Curiously, all live collected adult specimens were male. The single female was immature.

Phylum Mollusca Linnaeus, 1758
Class Gastropoda Cuvier, 1795
Subclass Caenogastropoda Cox, 1960
Family Hydrobiidae Stimpson, 1865
Genus *Hauffenia* Pollonera, 1898

### ***Hauffenia lozekiana*** sp. nov.

urn:lsid:zoobank.org:act:63FE4F3F-32A5-4F83-869F-C72B6E2D56AE
Figs 2, 5 71a–b, 12A–C, 13A–B, 14A, 15A–C, 17A–B, 18; Tables 3–4

#### Diagnosis

The combination of valvatiform shell with eventually detached peristome and an only initially sculptured embryonic shell, a thin, yellow operculum without peg-like structure in the nucleus and the massive, trapezoidal penis, whose left side is flattened, distinguishes *H. lozekiana* sp. nov. from all other congeneric species. Only three diagnostic positions of the COI alignment were of type 1, a single of type 3, and 107 of type 4 (Table 3). Uncorrected genetic distances to congeners ranged from 0.077 (*H. kerschneri*) to 0.133 (*H. steffeki* sp. nov.; see Supp. file 3).

#### Etymology

*Hauffenia lozekiana* sp. nov. is dedicated to Vojen Ložek (26 Jul. 1925–15 Aug. 2020), renowned and influential Czech malacologist and paleontologist, who contributed significantly to Quaternary malacostratigraphy, Czechoslovakian zoogeography of mollusks and was the first to discover the genus *Hauffenia* in Slovakia.

#### Material examined

**Holotype** (Fig. 12A–C)

SLOVAKIA • Bystrica Highlands, vicinity of Poniky/Oravce, Kalinovský prameň (Kalinovec); 48°42.027′ N, 19°15.952′ E; alt. 482 m; 4 Jan. 2014; J. Grego leg.; karst spring; NHMW 113638. (Fig. 5 71a–b)

**Paratypes** (Fig. 13A–B)

SLOVAKIA • 5 specs; same collection data as for holotype; JG F1684 • 4 specs; same collection data as for holotype; NHMW 113639 • 2 specs; same collection data as for holotype; ZPE • 2 specs; same collection data as for holotype; 25 Jan. 2014; HNHM 105307 • 2 specs; same collection data as for holotype; JG F1649 • 2 specs; same collection data as for holotype; 18 May 2017; JG F1650 • 4 specs; same collection data as for holotype; 2 Jul. 2020; JG F1886 • 2 specs; same collection data as for holotype; NMBE 569388 • 2 specs; same collection data as for holotype; SMF 363255 • 2 specs; same collection data as for holotype; SMOPAJ 1/2021 • 2 specs; same collection data as for holotype; ZMH 140884 • 3 specs; same collection data as for holotype; 21 Dec. 2020; JG F1887.

### Description

Shell (Figs 12A–C, 13A–B, 14A; Table 4). Valvatiform, maximum height measured 1.03 mm, maximum width 1.58 mm, on average twice as wide as high, with up to 2.75 whorls, final part of last whorl may be detached, fairly variable in shape with a coefficient of variation of 13.31% for the height/width ratio; embryonic shell with 1.1–1.2 whorls, first 0.75 whorls wrinkled, sculpture then fading to become smooth; aperture almost round only slightly higher than wide, prosocline, lip continuous, not thickened; umbilicus wide.

Operculum. Thin, chitinous, yellow, almost circular with central nucleus, the latter without peg or peg-like modification.

External features. Epidermis without pigment, no eye spots visible, tentacles without particular ciliation.

Mantle cavity. 9 gill filaments (N = 1), osphradium short, oval, behind middle of gill.

Digestive system (Figs 15A–C, 16). Radula with formula R 4-5 1 4-5/1 1, L 4-6 1 5-6. M1 23-29, M2 15-20 (N = 3); intestine forming a long loop in roof of mantle cavity, the loop is additionally coiled, its appearance seems to vary depending on the presence and number of fecal pellets.

Female genitalia. Not observed; the only live collected female specimen was subadult, hence, the distal genitalia were not yet developed.

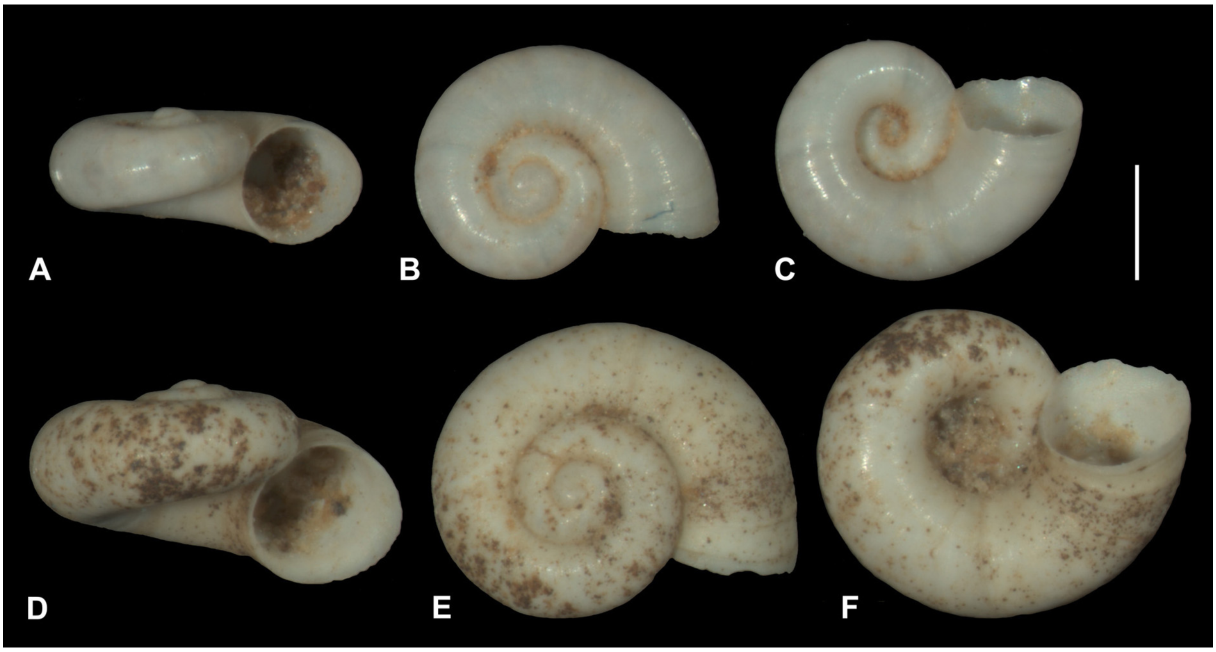

**Fig. 12.** Holotypes. **A–C**. *H. lozekiana* sp. nov. (NHMW 113638). D–E. *H. steffeki* sp. nov. (NHMW 113640). Scale bar = 500 µm.

Male genitalia (Figs 17A–B, 18). Not observed except for kidney-shaped prostate and penis; penis massive, trapezoidal, left side flattened, small, brown, probably chitinous stylet anterior right near penial opening.

### Habitat

The type locality is a small permanent karst spring emerging from the small karst massif of Drienok formed by Middle Triassic (Aegean–Ladinian) Wetterstein, Reifling and Gutenstein Limestones of the Silicikum Unit (Silica Nappe) at the contact zone to impermeable Early Triassic shales and sandstones of Szin and Bódvaszilas Members with a small discontinuous stripe of alluvial gravel at the bottom of the spring valley. The character of the type locality indicates the same habitat preference in the phreatic rhizosphere as discussed for *H. kissdalmae*.

### Distribution

Known only from the type locality so far.

### Conservation

There is only one known location and the area of occupancy is probably considerably smaller than 20 km$^2$. The combination of the restricted range and the threat imposed by the apparent trend of logging in the area around the type locality qualify for a threat assessment of Vulnerable (VU, D2) according to the IUCN Standards and Petitions Committee (2019).

### Remarks

The genetic distances of *H. lozekiana* sp. nov. from the more widespread *H. kissdalmae* and the Austrian species suggest that it is a relict probably from the earlier colonization of the Hron River Valley ca 4.5 Ma ago (see Discussion).

## ***Hauffenia steffeki*** sp. nov.

urn:lsid:zoobank.org:act:0637E21D-A9F8-429D-B025-2D3ED9E1E28E
Figs 2, 5 72a–b, 12D–E, 13C–D, 14B, 15D–F, 16, 17C–E, 19; Tables 3–4

### Diagnosis

*Hauffenia steffeki* sp. nov. is characterized by a valvatiform shell with eventually detached peristome and an only initially sculptured embryonic shell, a thin, orange operculum without peg-like structure in the nucleus and the massive, trapezoidal penis with a rounded lobe on the left side. There were nine diagnostic COI alignment positions of type 1, 13 of type 3, and 85 of type 4 (Table 3). Uncorrected genetic distances to congeners ranged from 0.108 [*H. michleri* (Kuščer, 1932)] to 0.141 (*H. kissdalmae*; see Supp. file 3).

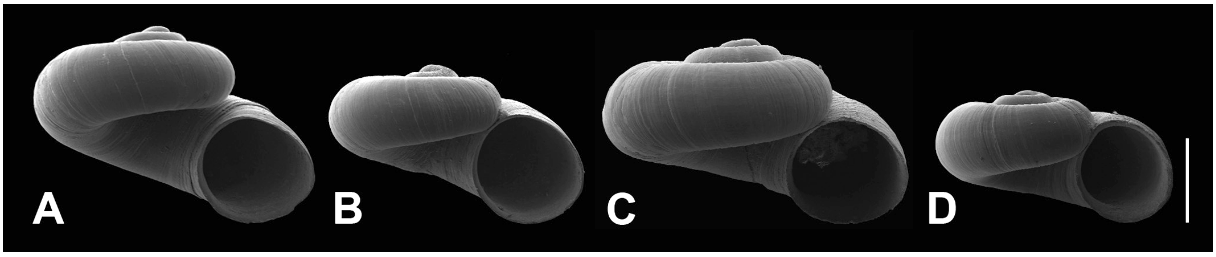

**Fig. 13.** Paratypes from type localities (SEM micrographs). **A–B**. *H. lozekiana* sp. nov. (NHMW 113639). **C–D**. *H. steffeki* sp. nov. (NHMW 113641). Scale bar = 500 µm.

**Table 3** (continued on next page). Diagnostic sites of types 1, 3, and 4 of COI for *H. lozekiana* sp. nov. and *H. steffeki* sp. nov. For reference alignment see Supp. file 2.

| Species | Type | Position/State | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *H. lozekiana* | 1 | 1 | 2 | 2 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 8 | 3 | 5 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 8 | 0 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | G | C | G | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 3 | 3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 4 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | C | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | 0 | 0 | 0 | 1 | 1 | 1 | 2 | 2 | 3 | 3 | 4 | 5 | 6 | 6 | 8 | 8 | 8 | 9 | 9 | 9 | 0 | 0 | 1 | 1 | 1 | 2 | 2 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 5 | 6 |
| | | 2 | 5 | 8 | 1 | 4 | 7 | 6 | 9 | 5 | 6 | 1 | 6 | 5 | 8 | 3 | 4 | 6 | 2 | 5 | 8 | 1 | 7 | 0 | 6 | 9 | 2 | 8 | 1 | 2 | 4 | 7 | 0 | 3 | 9 | 2 | 1 |
| | | A | C | A | C | T | A | T | T | G | C | T | A | T | C | T | C | A | A | T | A | A | T | G | A | T | A | A | T | T | A | A | A | G | A | T | C |
| | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 |
| | | 6 | 6 | 7 | 7 | 8 | 9 | 9 | 9 | 0 | 0 | 1 | 1 | 2 | 2 | 2 | 3 | 3 | 4 | 4 | 5 | 6 | 6 | 6 | 6 | 7 | 7 | 7 | 8 | 8 | 9 | 9 | 9 | 9 | 0 | 0 | 1 |
| | | 4 | 7 | 0 | 6 | 2 | 1 | 4 | 7 | 3 | 6 | 5 | 8 | 1 | 4 | 7 | 6 | 9 | 2 | 5 | 7 | 0 | 3 | 6 | 9 | 3 | 8 | 9 | 4 | 7 | 0 | 3 | 6 | 9 | 2 | 5 | 4 |
| | | A | T | A | T | T | T | G | C | T | A | G | A | T | T | A | G | A | G | C | G | A | A | C | C | T | T | A | C | A | T | C | T | T | A | T | A |
| | | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | | 3 | 3 | 3 | 4 | 4 | 4 | 5 | 5 | 7 | 7 | 8 | 8 | 9 | 9 | 0 | 0 | 0 | 1 | 1 | 1 | 2 | 4 | 5 | 6 | 6 | 6 | 6 | 6 | 7 | 7 | 7 | 7 | 8 | 8 | 8 | 9 |
| | | 2 | 3 | 5 | 3 | 4 | 7 | 0 | 9 | 1 | 4 | 0 | 6 | 2 | 5 | 1 | 4 | 7 | 0 | 6 | 9 | 2 | 0 | 8 | 1 | 2 | 4 | 5 | 7 | 0 | 3 | 4 | 6 | 0 | 5 | 8 | 1 |
| | | T | T | A | C | A | T | A | G | T | T | A | T | A | C | A | A | G | A | G | T | G | A | T | A | C | T | C | A | A | A | T | A | G | A | T | T |
| | | 4 | 5 | 5 | 5 | 5 | 5 | 5 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 9 | 0 | 0 | 1 | 3 | 3 | 3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 4 | 3 | 9 | 2 | 0 | 1 | 3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | T | G | A | G | C | G | A | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

**Table 3** (continued). Diagnostic sites of types 1, 3, and 4 of COI for *H. lozekiana* sp. nov. and *H. steffeki* sp. nov. For reference alignment see Supp. file 2.

| Species | Type | Position/State | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *H. steffeki* | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 4 | 4 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 0 | 2 | 2 | 4 | 0 | 3 | 7 | 6 | 6 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | 7 | 2 | 8 | 9 | 3 | 0 | 9 | 2 | 4 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | | G | G | G | C | A | G | G | T | G | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | 3 | 0 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 4 | 4 | 4 | 4 | | | | | | | | | | | | | | | | | | | | | | | |
| | | 2 | 7 | 8 | 1 | 4 | 4 | 7 | 7 | 6 | 0 | 3 | 8 | 9 | | | | | | | | | | | | | | | | | | | | | | | |
| | | 3 | 9 | 2 | 6 | 2 | 8 | 2 | 5 | 5 | 1 | 4 | 8 | 4 | | | | | | | | | | | | | | | | | | | | | | | |
| | | G | G | G | C | C | C | G | G | T | C | G | G | C | | | | | | | | | | | | | | | | | | | | | | | |
| | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | 0 | 0 | 1 | 1 | 1 | 2 | 3 | 3 | 4 | 5 | 6 | 6 | 8 | 8 | 8 | 9 | 9 | 9 | 1 | 1 | 1 | 3 | 3 | 3 | 3 | 4 | 4 | 5 | 6 | 6 | 6 | 7 | 7 | 8 | 9 | 9 |
| | | 2 | 8 | 1 | 4 | 7 | 6 | 5 | 6 | 1 | 6 | 5 | 8 | 3 | 4 | 6 | 2 | 5 | 8 | 0 | 6 | 9 | 1 | 2 | 4 | 7 | 0 | 3 | 2 | 1 | 4 | 7 | 0 | 6 | 8 | 1 | 4 |
| | | A | G | A | G | A | A | A | T | T | G | T | T | T | C | T | G | A | G | A | A | T | T | T | G | A | G | G | T | T | G | T | G | T | A | T | A |
| | | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 |
| | | 9 | 0 | 1 | 1 | 2 | 2 | 2 | 3 | 3 | 4 | 5 | 6 | 6 | 6 | 7 | 8 | 8 | 9 | 0 | 3 | 3 | 4 | 4 | 4 | 5 | 5 | 7 | 7 | 8 | 8 | 0 | 0 | 1 | 1 | 1 | 2 |
| | | 7 | 6 | 5 | 8 | 1 | 4 | 7 | 6 | 9 | 5 | 1 | 0 | 3 | 6 | 3 | 4 | 7 | 0 | 2 | 3 | 5 | 3 | 4 | 7 | 0 | 9 | 1 | 4 | 0 | 6 | 4 | 7 | 0 | 6 | 9 | 2 |
| | | T | A | G | A | T | A | A | A | G | C | T | T | T | T | T | T | G | T | G | T | A | T | G | T | A | G | T | T | A | T | A | G | A | G | G | G |
| | | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | | | | | | | | | | | | | | | | | | | |
| | | 4 | 5 | 6 | 6 | 6 | 7 | 7 | 7 | 8 | 8 | 9 | 0 | 0 | 1 | 3 | 3 | 3 | | | | | | | | | | | | | | | | | | | |
| | | 0 | 8 | 1 | 5 | 7 | 0 | 3 | 4 | 0 | 5 | 1 | 3 | 9 | 2 | 0 | 1 | 3 | | | | | | | | | | | | | | | | | | | |
| | | A | T | A | T | G | G | A | T | G | G | T | A | G | A | T | G | A | | | | | | | | | | | | | | | | | | | |

### Etymology

*Hauffenia steffeki* sp. nov. is named after Jozef Šteffek (1 Jan. 1952–19 Apr. 2013), ever-helpful friend and unforgettable Slovak malacologist, initiator of the intensive field work dedicated to the genus *Hauffenia* in Slovakia.

### Material examined

**Holotype** (Fig. 12D–E)

BOSNIA AND HERCEGOVINA • Bosnian Federation, Bihać district, Donji Kamengrad, 4 km W of Sanski Most; 44°46.7682′ N, 16°33.9859′ E; alt. 228 m; 24 Mar. 2016; J. Grego, G. Jakab and B. Šmída leg.; spring and well in dry canyon; NHMW 113640. (Fig. 5 72a, b)

**Paratypes** (Fig. 13C–D)

BOSNIA AND HERCEGOVINA – **Bosnian Federation** • 13 specs; same collection data as for holotype; JG F0679 • 10 specs; same collection data as for holotype; NHMW 113641 • 8 specs; same collection data as for holotype; 4 Apr. 2015; J. Grego, M. Grego, R. Lohaj and B. Šmída leg.; JG F0473 • 10 specs; same collection data as for holotype; 24–25 Mar. 2016; J. Grego, M. Olšavský, G. Jakab and B. Šmída leg.; HNHM 105308 • 33 specs; same collection data as for holotype; JG F0561 • 10 specs; same collection data as for holotype; NMBE 569389 • 10 specs; same collection data as for holotype; SMF 363256 • 10 specs; same collection data as for holotype; SMOPAJ 2/2021 • 10 specs; same collection data as for holotype; ZMH 140885 • 35 specs; Bosanska Krajina, Sanski Most district, Dabarska Pećina, cave in Donji Dabar near Bankovići; 44°42.6′ N, 16°38.31′ E; alt. 173 m; 25 Mar. 2016; J. Grego, M. Olšavský¸ G. Jakab and B. Šmída leg; sand on cave river bottom; JG0565 • 68 specs; same collection data as for preceding; 15 Mar. 2017; J. Grego, G. Jakab and B. Šmída leg.; JG F0692 • 12 specs; Bihać district, Spring Krivina, Lušči Palanka-Miljevci, 400 m from village toward Sanski Most, at right side of road R405; 44°45.7345′ N, 16°27.0528′ E; alt. 413 m; 15 Mar. 2017; J. Grego, G. Jakab and B. Šmída leg; JG F0675 • 3 specs; Bihać district, Vrelo Zdena 3 km W of Sanski Most; 44°45.7186′ N, 16°37.8371′ E; alt. 176 m; 15 Mar. 2017; J. Grego, G. Jakab and B. Šmída leg.; JG F1888 • 7 specs; Bihać district, Izvor Banovac between Gorice and Naprelie, left side of the road; 44°46.8065′ N, 16°30.0365′ E; alt. 375 m; 15 Mar. 2017; J. Grego, G. Jakab and B. Šmída leg.; JG F0668.

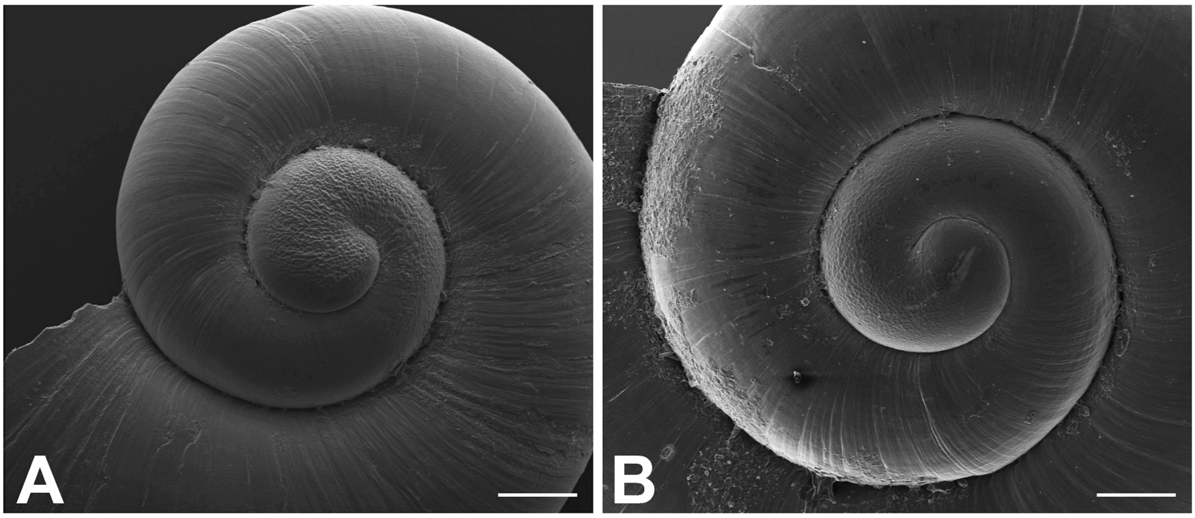

**Fig. 14.** Protoconchs (SEM micrographs). **A**. *H. lozekiana* sp. nov., paratype (NHMW 113639). **B**. *H. steffeki* sp. nov., paratype (NHMW 113641). Scale bars = 100 µm.

**Other material tentatively allocated to *H. steffeki***

BOSNIA AND HERCEGOVINA – **Bosnian Federation** • 1 spec.; Bihać district, Bastaško Vrelo, 5 km W of Drvar; 44°23.584′ N, 16°19.572′ E; alt. 527 m; 15 Mar. 2017; J. Grego, G. Jakab and B. Šmída leg.; JG F 0743. – **Republika Srpska** • 7 specs; Donja Pećka, Izvor Sana; 44°19.0302′ N, 16°50.1773′ E; alt. 446 m; 15 Mar. 2017; J. Grego, M. Olšavský, G. Jakab and B. Šmída leg.; sand on bank of river; JG F 0571/.

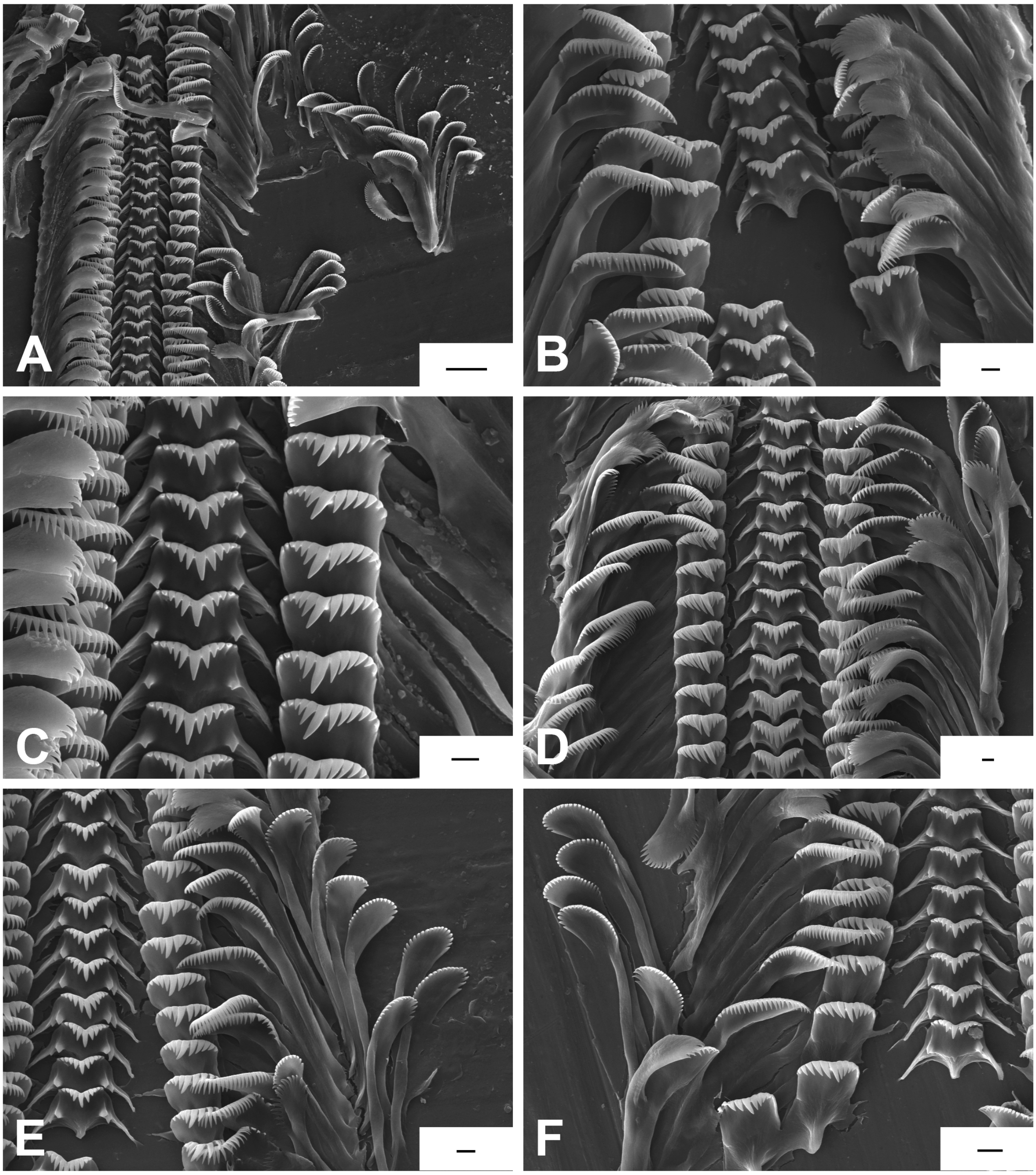

**Fig. 15.** Radulae (SEM micrographs). **A**–**C**. *H. lozekiana* sp. nov. **D**–**F**. *H. steffeki* sp. nov. **D**. Type locality. **E**–**F**. Spring Krivina (locality 74 from Table 1). Scale bars: A = 10 µm; B–E = 2 µm; F = 3 µm.

**Table 4.** Morphometrics of the new species. Abbreviations: AH = aperture height; AW = aperture width; CV = coefficient of variation corrected for unequal sample sizes; max = maximum; min = minimum; SD = standard deviation; SH = shell height; SW = shell width; W = number of whorls (to the nearest eighth). Measurements in mm.

| Species | SH | SW | AH | AW | SH/SW | AH/AW | W |
|---|---|---|---|---|---|---|---|
| *H. lozekiana* sp. nov.<br>Kalinovský<br>N = 16 | | | | | | | |
| Holotype | 0.65 | 1.36 | 0.60 | 0.54 | 0.48 | 1.12 | 2.375 |
| min | 0.58 | 1.10 | 0.49 | 0.47 | 0.47 | 0.94 | 2.125 |
| max | 1.03 | 1.58 | 0.66 | 0.64 | 0.69 | 1.12 | 2.750 |
| mean | 0.73 | 1.35 | 0.58 | 0.55 | 0.52 | 1.04 | 2.432 |
| median | 0.68 | 1.34 | 0.60 | 0.54 | 0.49 | 1.04 | 2.375 |
| SD | 0.12 | 0.14 | 0.05 | 0.06 | 0.07 | 0.05 | 0.197 |
| CV | 16.87 | 10.27 | 8.75 | 10.22 | 13.31 | 4.44 | 8.802 |
| *H. steffeki* sp. nov.<br>Donji<br>N = 20 | | | | | | | |
| Holotype | 0.92 | 1.63 | 0.72 | 0.68 | 0.57 | 1.07 | 2.625 |
| min | 0.69 | 1.40 | 0.58 | 0.54 | 0.45 | 1.00 | 2.250 |
| max | 1.08 | 1.77 | 0.74 | 0.69 | 0.72 | 1.15 | 2.875 |
| mean | 0.89 | 1.56 | 0.68 | 0.62 | 0.57 | 1.09 | 2.575 |
| median | 0.88 | 1.54 | 0.69 | 0.64 | 0.56 | 1.10 | 2.625 |
| SD | 0.11 | 0.10 | 0.05 | 0.05 | 0.07 | 0.03 | 0.148 |
| CV | 12.38 | 6.73 | 7.18 | 7.70 | 12.87 | 3.14 | 5.837 |
| *H. steffeki* sp. nov.<br>Dabarska Pećina<br>N = 20 | | | | | | | |
| min | 0.52 | 1.34 | 0.51 | 0.48 | 0.36 | 1.02 | 2.375 |
| max | 0.69 | 1.54 | 0.61 | 0.55 | 0.47 | 1.13 | 2.625 |
| mean | 0.61 | 1.45 | 0.55 | 0.52 | 0.42 | 1.07 | 2.544 |
| median | 0.61 | 1.47 | 0.56 | 0.52 | 0.43 | 1.06 | 2.500 |
| SD | 0.04 | 0.06 | 0.03 | 0.02 | 0.03 | 0.03 | 0.073 |
| CV | 6.52 | 4.07 | 4.66 | 3.76 | 6.41 | 2.50 | 2.921 |
| *H. steffeki* sp. nov.<br>Krivina<br>N = 5 | | | | | | | |
| min | 0.56 | 1.06 | 0.47 | 0.43 | 0.49 | 1.04 | 2.125 |
| max | 0.76 | 1.27 | 0.57 | 0.52 | 0.59 | 1.13 | 2.375 |
| mean | 0.64 | 1.18 | 0.52 | 0.47 | 0.54 | 1.08 | 2.250 |
| median | 0.62 | 1.21 | 0.53 | 0.48 | 0.54 | 1.08 | 2.250 |
| SD | 0.07 | 0.10 | 0.05 | 0.04 | 0.07 | 0.06 | 0.177 |
| CV | 11.88 | 8.55 | 9.39 | 7.98 | 8.47 | 2.97 | 6.320 |

### Description

Shell (Figs 12D–E, 13C–D, 14B; Table 4). Valvatiform, maximum height measured at type locality 1.08 mm, maximum width 1.77 mm, with up to 2.875 whorls, on average twice as wide as high, considerably smaller in Krivina and Dabarska Pećina, there also flatter, final part of last whorl may be detached, fairly variable in shape with a coefficient of variation of 12.87% for the height/width ratio at the type locality, less pronounced at the other two localities where measurements have been taken; embryonic shell with 1.1–1.2 whorls, first 0.75 whorls faintly wrinkled, then smooth; aperture almost round only slightly higher than wide, ortho- or prosocline, lip continuous, not thickened; umbilicus wide.

Operculum (Fig. 19). Thin, chitinous, orange, almost circular with central nucleus, the latter without peg or peg-like modification.

External features. Epidermis without pigment, no eye spots visible, tentacles without particular ciliation.

Mantle cavity. 9–12 gill filaments (N = 2), osphradium short, oval, behind middle of gill.

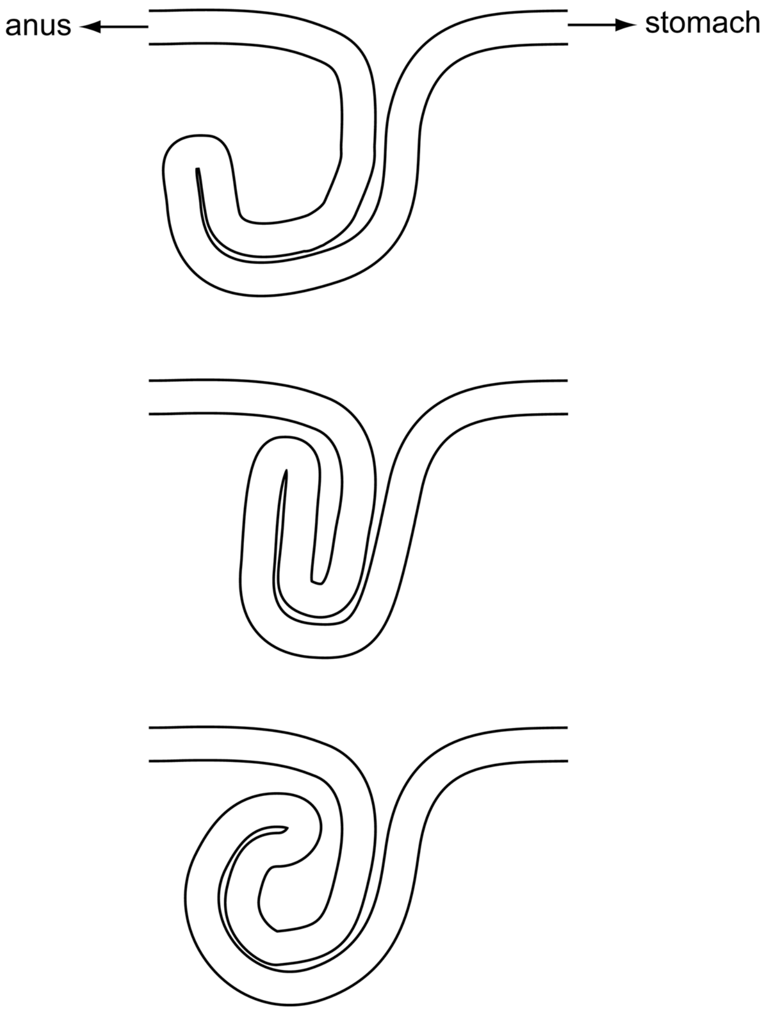

**Fig. 16.** Schematic representations of intestinal loop configurations.

Digestive system (Figs 15D–F, 16). Radula with formula R 5-6 1 5-6/1 1, L 5 1 5, M1 22-24, M2 17-19 (Donji Kamengrad, N = 1), R 5 1 5/1-2 1-2, L 4-5 1 5, M1 22-26, M2 19-23 (spring Krivina, N = 2); intestine forming a long loop in roof of mantle cavity, the loop is additionally coiled, its appearance seems to vary depending on the presence and number of fecal pellets.

Female genitalia. Not observed; all live collected specimens were male.

Male genitalia (Fig. 17C–E). Testis starting at least one whorl below apex, comprising ca 0.25 whorls, reaching stomach; prostate kidney shaped; penis massive, trapezoidal, with rounded lobe on left side, small, brown, probably chitinous stylet anterior right near penial opening.

### Habitat

The type locality is a small well in a meadow in a dry valley west of Donji Kamengrad associated to a nearby karst spring at the north side of the valley. The Dabarska Pećina is a large river cave, one of the two main sources of the Dabar River (left tributary of the Sana River), draining the karst waters from the plateaus above the Jezernica basin through Bobijaško oko Estavella and numerous sinkholes. The other localities are rather smaller permanent karst springs and wells associated to small alluvial stripes with forested surroundings. These localities suggest a habitat preference for the phreatic rhizosphere as discussed for *H. kissdalmae*. However, the live specimens were also found in sediment on the bottom of the cave which indicates also a wider subterranean distribution including open cave conduits in a similar

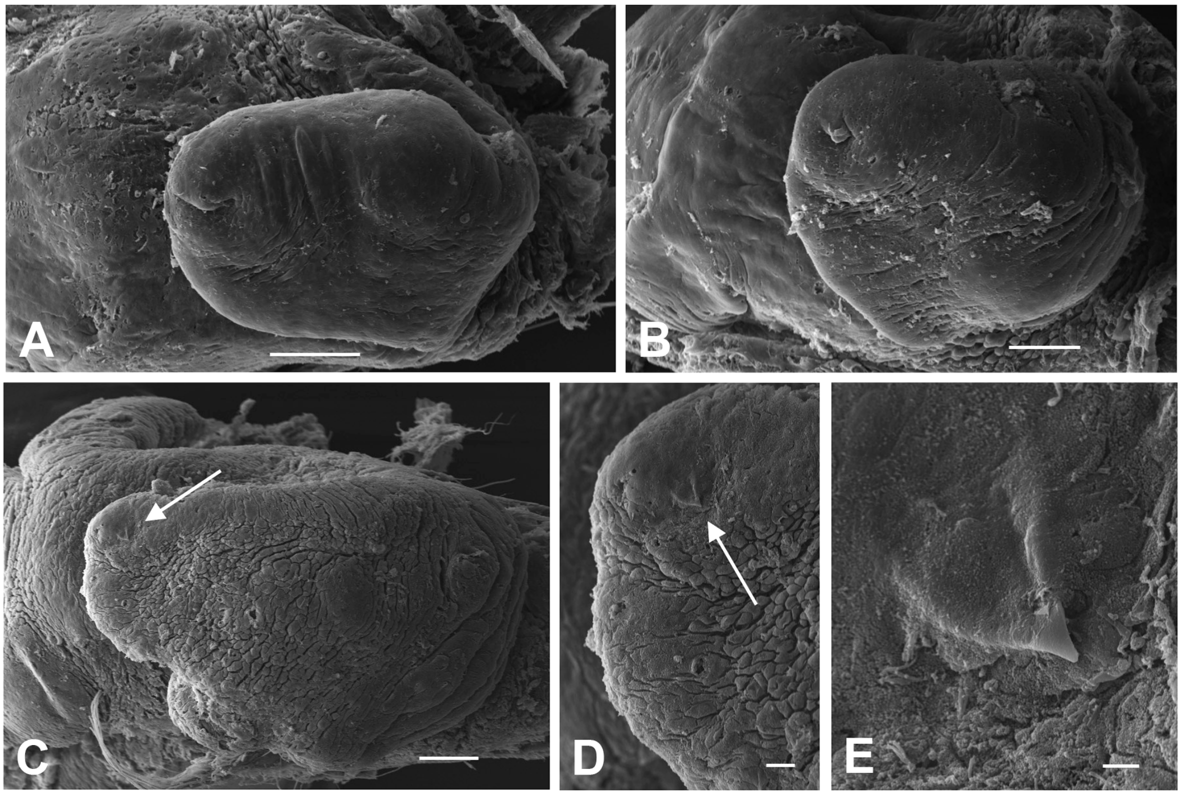

**Fig. 17.** Penes (SEM micrographs). **A–B**. *H. lozekiana* sp. nov. (different specimens). **C–E**. *H. steffeki* sp. nov. (one specimen). Arrow points at penial stylet, which is withdrawn in A and B. Scale bars: A–C = 50 µm; D = 10 µm; E = 2 µm.

way as suggested for the species of *Hauffenia* from Slovenia, Italy and Croatia (Bole 1970; Bole & Velkovrh 1987; Rysiewska *et al.* 2016).

### Distribution

Known only from the type locality and four nearby localities at Bosanska Krajina west and northwest of Sanski Most.

### Conservation

The new species is known from only five locations and it is very probable that the area of occupancy is smaller than 20 km$^2$. At this stage, we could not identify any plausible threats so that *H. steffeki* sp. nov. is assessed as Near Threatened (NT; IUCN Standards and Petitions Committee 2019).

### Remarks

*Hauffenia steffeki* sp. nov. extends the distribution of the genus further south and is genetically/phylogenetically distinct from all other species for which sequence information is available. It is likely that the species has a wider distribution in springs draining to and in the alluvium of the Sana River up to its source. In the type locality, it is syntopic with *Lanzaia bosnica* Bole, 1970 and in Dabarska Pećina with stygobiotic gastropods including *Dabriana bosniaca* Radoman, 1974, *Lanzaia bosnica* Bole, 1970 and *Paladilhiopsis blihensis* (Glöer & Grego, 2015) (Glöer & Grego 2015).

## Discussion

The phylogenetic analyses based on different DNA markers and their combinations did not converge to an unambiguous topology, node support remained insignificant for many splits. However, a few important insights into the evolution of *Hauffenia* can safely be inferred. 1) The genus probably had its origin in the Middle or Late Miocene; 2) most speciation events occurred in the Pliocene; 3) within species differentiation is young and happened during the Pleistocene; and 4) there appear to be two

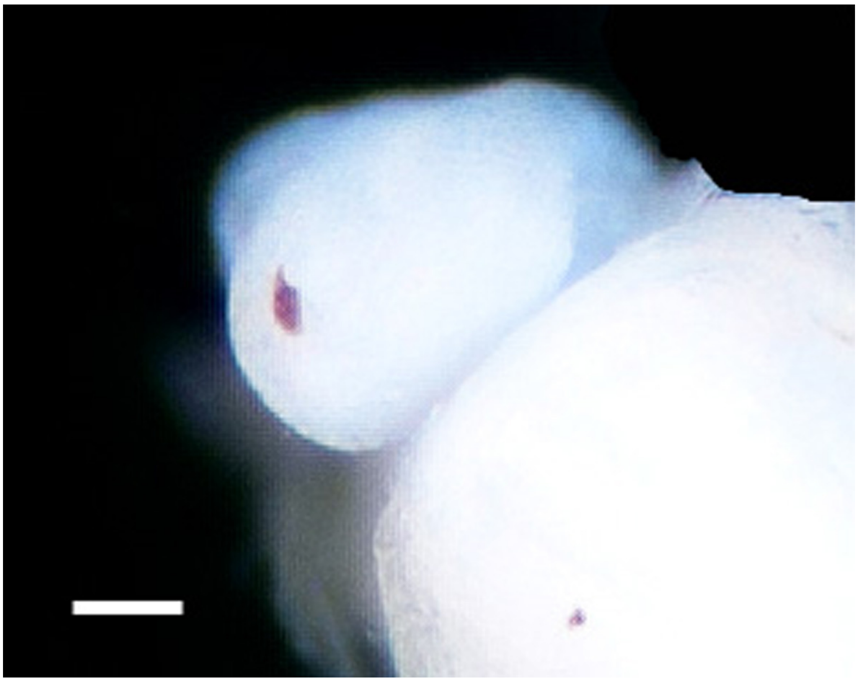

**Fig. 18.** Penis of *H. lozekiana* sp. nov. Frontal view showing stylet. Scale bar = 50 µm.

major groups, a radiation on the Balkans and into NE Italy, and a northern group extending from central Austria to Hungary and Slovakia. It is unclear, though, whether these groups are sister clades or whether the former is paraphyletic with respect to the latter.

Based on the time tree (Fig. 9) – theoretically considered superior to conventional analyses requiring an outgroup for rooting by some authors (Drummond *et al.* 2007) – and paleogeographic considerations as well as considering the above caveats, we developed the following – admittedly speculative – scenario for the evolution of *Hauffenia* (Fig. 20). *Hauffenia* probably had a Dinaric origin and spread northwards in the Upper Miocene about 10 Ma, when the Pannonian Sea had formed as an isolated continental lake, a remnant of the Paratethys, after the closure of the straits towards Bavaria, Đerdap Valley, Preševo Valley, and Slovenia (Kázmér 1990; Magyar *et al.* 1999). Especially closing the Slovenian strait (Ivančič *et al.* 2018) opened the dispersal path towards what are now Slovenia and NE Italy. Similar to the extant species of the region [*H. erythropomatia*, *H. media* Bole, 1961, *H. michleri*, *H. subcarinata* Bole & Velkovrh, 1987, *H. subpiscinalis* (Kuščer, 1932), *H. tellinii* (Pollonera, 1898), *H. wagneri* (Kuščer, 1928)], the ancestral populations likely dwelled in karstic and cave conduits (Falniowski & Szarowska 2015). The Pannonian Sea gradually shrunk in surface and the salinity dropped due to discharge of larger rivers and alluvial deposits from the north. The sea turned into a freshwater lake (Uhrin 2011). Ancestral populations of *Hauffenia* now possibly adapted to and spread into alluvial interstices with penetrating tree roots. The hyporheic zone with its phreatic rhizosphere thus provided dispersal possibilities for colonizing the extensive alluvia at the NW banks of the Pannonian Lake (Sümeghy 1955; Somogyi 1961; Borsy 1989). This way, *Hauffenia* successfully colonized the Danube valley as well as the north-eastern Alps in what is now eastern Austria about 8 Ma. As the Pannonian Lake further contracted to become the Slavonian Lake (Paludina Lake) 5.5 Ma (Magyar *et al.* 1999; Rundić *et al.* 2016), more rivers drained the NE part of the Pannonian Basin opening further dispersal paths along Paleo-Ipeľ (Ipoly), Paleo-Slaná (Sajó) and Paleo-Bódva Rivers (Mezősi 2015) towards the now Slovak/Aggtelek Karst limestone formation founding eventually *H. kissdalmae.* The ancestor of *H. lozekiana* sp. nov. probably diverged

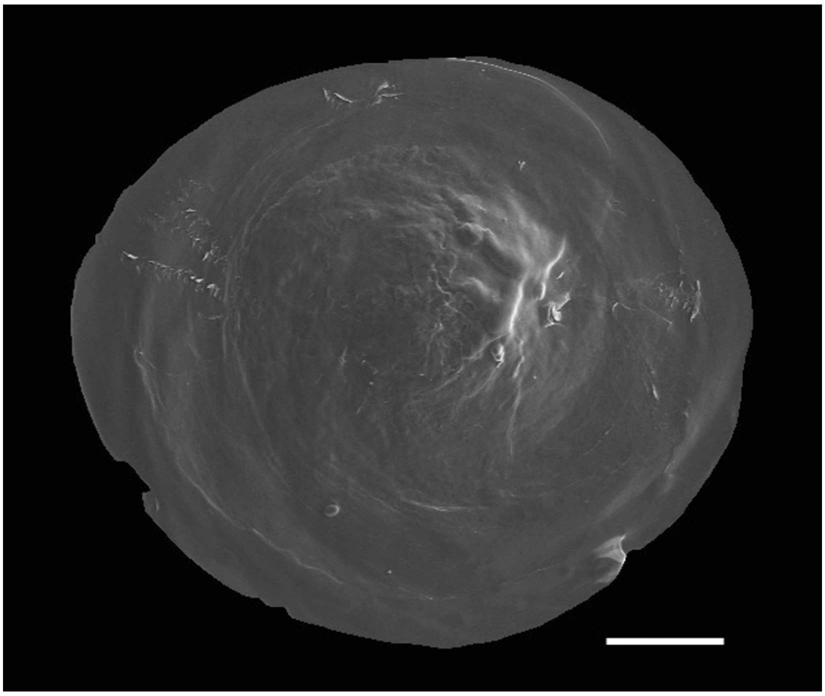

**Fig. 19.** Operculum of *H. steffeki* sp. nov., from type locality; internal side. Scale bar = 100 µm.

from the lineage moving northwards towards today's Austria through the Paleo-Danube and Paleo-Hron River alluvium about 4.5 Ma. About 2 Ma the ancestral population of *H. kissdalmae* colonized the Paleo-Ipeľ (Ipoly) alluvium and the eastern foothills of the Börzsöny Mountains (today, the Ipeľ River is situated in the northern and western foothills) and further dispersed through the Rimava River Basin to the Slaná (Sajó) Valley at about 1.5 Ma. Dispersal into the Hron Valley up to Nemecká was only enabled later. After the elevation of the Western Pannonian Basin the Paleo-Danube significantly changed its direction and intersected into the Paleo-Ipeľ (Ipoly) Valley by the "Visegrad Break-Through" (Visegrádi áttörés) (Gábris 1994; Karátson 2014), hence connected the alluvia of Hron and Ipeľ at about 2 Ma. The Hron Valley fully opened for colonization about 0.7–1 Ma when the Paleo-Ipeľ River changed its flow direction towards the northern and western foothills of the Börzsöny Mountains, coming to its present-day position adjacent to the Hron River (Mike 1991) and the alluvial cross-connection of both rivers became more intensive. At that time (about 1 Ma), the ancestral populations of *H. lozekiana* sp. nov. dwelling in the Hron Valley were probably already declining and fragmented due to neovolcanic activity over the past few Ma. Several lava flows reached the Hron valley and likely influenced the water

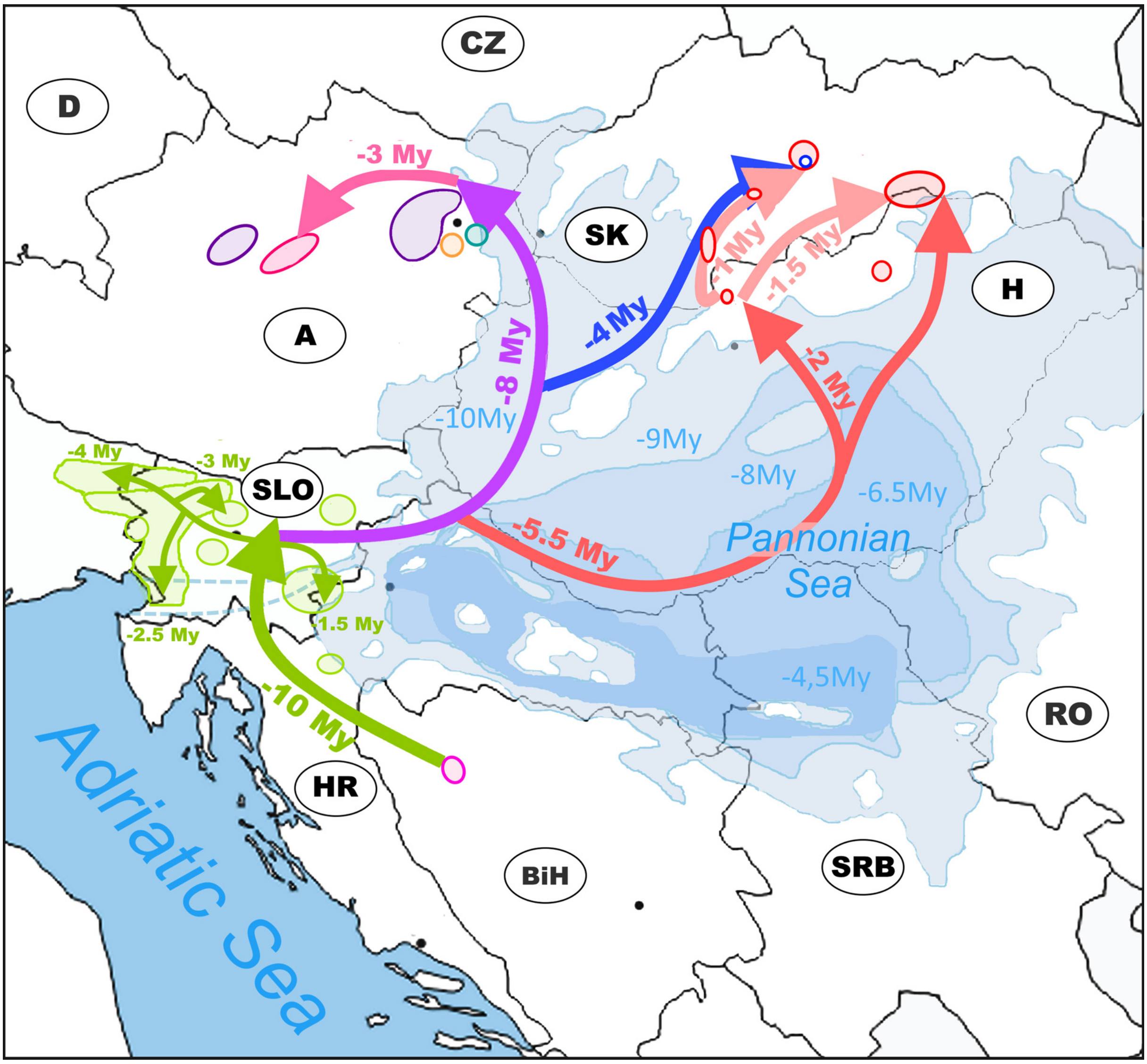

**Fig. 20.** Scenario of evolutionary diversification of *Hauffenia* Pollonera, 1898.

quality (Šimon 2000). Thus the more recent invasion of *H. kissdalmae* into the Hron Valley resulted in a successful colonization of former *H. lozekiana* sp. nov. habitats. The only known extant population of *H. lozekiana* sp. nov. has certainly to be seen as a relict.

The Miocene origin coincides with the oldest fossils that have been attributed to *Hauffenia*, although their ages seem to predate the above scenario (in chronological order): *H. mandici* Neubauer & Harzhauser, 2014, early Langhian (Badenian), Rein Basin in the eastern foothills of the Alps of Austria (Harzhauser *et al.* 2014); *H. sarmatica* (Papp, 1954), *Hauffenia* sp. 1 and *Hauffenia* sp. 2, Vienna Basin and Weinviertel in eastern Austria (Papp 1954; Reischütz 2000); *Hauffenia* sp., Tortonian (Pannonian), Kötese at Lake Balaton in Hungary (Bandel 2010), ?*Hauffenia* sp., upper Tortonian (Maeotian), Ukraine (Anistratenko & Anistratenko 2009). However, as already mentioned, fossil hydrobiids can rarely be attributed to an extant genus with certainty due to their poverty in shell characters and the high degree of convergence. Most of the above authors discussed these caveats. At least for the Austrian and Hungarian findings the allocation to *Hauffenia* may be supported by the fact that today no other valvatiform genus occurs there. But ultimately, our knowledge will not go beyond the coincidence of the occurrence of *Hauffenia*-like Miocene fossils in the range of contemporaneous species and the temporal inferences based on our time-tree. Regarding the slight temporal mismatch of fossil ages and our above evolutionary scenario we have also to recall that the time estimates in the tree come with considerable highest posterior density intervals, i.e., uncertainties (Fig. 9).

The extension of the occurrence of *H. wienerwaldensis* also west of the range of *H. kerschneri* was surprising (Haase 1992, 1993) but well substantiated by genetic and morphological (operculum, penis) data. Whether the range of *H. wienerwaldensis* is continuous – e.g., along the southern tributaries of the Danube – or disjunct, i.e., interrupted by *H. kerschneri* (see also Haase 1993 for further localities toward the east) is premature to judge. Clearly, *Hauffenia* has to be targeted in Austria in a similar systematic and thorough fashion as was done for *H. kissdalmae*. This would also include recollecting *H. danubialis* (Haase, 1993) and *H. nesemanni* Reischütz & Reischütz, 2006 in order to confirm their status as separate species (type localities given in Fig. 3). The penial morphology of the former appears to be unique, indeed (Haase 1993). However, *H. nesemanni* has only been described based on shell morphology and is known from a single locality in the southern Vienna Basin (Reischütz & Reischütz 2006). Looking at the morphological variation encountered within both here newly described species (Figs 12–13) at least questions whether shell morphology is diagnostic in *Hauffenia*. Clearly, anatomical and DNA sequence data are needed here.

Our analyses confirm Falniowski & Szarowska (2015) and Rysiewska *et al.* (2017) that *H. michleri* is a valid species and should not be synonymized with *H. tellinii* as proposed by Bodon *et al.* (2001). Both sequences KT236156 and KY087861 are still listed as *H. tellinii* in GenBank. The possibility that the two sequences attributed to *H. erythropomatia* represent in fact two different species has already been suggested by Rysiewska *et al.* (2017) because of their genetic disparity reflected in the disjunct positions in the phylogenetic trees (Fig. 9; Supp. file 1).

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## Supplementary files

**Supp. file 1**. Phylogenetic trees. Outgroup taxa were pruned off. Specimens identified by two-digit locality code in bold, four digit individual DNA code, and orographic unit (see Table 1) or GenBank accession number; posterior probabilities or bootstrap support values at nodes; scale bars in substitutions per site; the sequence of *H. tellinii* (Pollonera, 1898) was generated by Ponder *et al.* (2008). https://doi.org/10.5852/ejt.2021.775.1555.5329

**Supp. file 2**. Reference alignment of COI for identification of diagnostic molecular characters in FASTA format. Known species abbreviated by first three letters of epitheton followed by GenBank accession number. New sequences contain four digit individual code (see Table 1). https://doi.org/10.5852/ejt.2021.775.1555.5331

**Supp. file 3**. Uncorrected genetic distances (p-distances) between species of *Hauffenia* Pollonera, 1898 based on COI. Known species abbreviated by first three letters of epitheton followed by GenBank accession number. New sequences contain four digit individual code (see Table 1).
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