

Discriminating Danubian *Romanogobio* species by means of bar-HRM analysis

Master's Thesis

Conducted by:

Raphael Schmid, BSc

Supervised by:

Priv.-Doz. Mag. Dr. Stephan Koblmüller

To earn the academic degree:

Master of Science (MSc)

Karl-Franzens University Graz, Institute of Biology

Graz, March 2023

Inhaltsverzeichnis

ABSTRACT	3
ZUSAMMENFASSUNG	4
INTRODUCTION	5
NOMENCLATURAL ISSUES.....	5
ENDANGERMENT STATUS.....	6
BARCODING AND BAR-HRM	6
METHODS	7
GENERATION OF NEW COI SEQUENCES.....	7
PRIMER DESIGN FOR BAR-HRM.....	7
BAR-HRM	8
DATA ANALYSIS.....	8
RESULTS	9
DISCUSSION	14
BAR-HRM	16
ACKNOWLEDGEMENTS.....	18
LITERATURE.....	19
APPENDIX	24
PRIMERS	24
SAMPLE LIST.....	25

Abstract

Species of the genus *Romanogobio* are made up of small benthic fish that live both in lentic and lotic systems. Six species occur in the Danube system and they are partly listed on national Red Lists. As it is difficult to discriminate these species based on morphology, especially as juveniles, barcoding represents a promising alternative. Due to difficult identification, there is little data on the phylogeographic and population genetic structure across the whole Danube system. The main aim of this study was to establish a cheaper and faster alternative to conventional barcoding by establishing an assay for barcoding by high resolution melting analysis (bar-HRM) based on sequences of specimens across the whole Danube drainage. This method allows discrimination of species based on the melting point of a short DNA amplicon following a qPCR, saving both time and resources needed for additional steps of Sanger sequencing. Based on 150 newly generated and 183 already existing sequences of the DNA barcoding region (5'-end of the mitochondrial COI gene), a potential mini-barcode was identified and tested. Results did show discrimination of species but are not statistically supported. This could be compensated by combining this assay with another, already existing for other cypriniform species that was tested as well. In addition to that, phylogenetic and phylogeographic analyses were conducted, largely confirming current knowledge on the taxonomy of this genus, but suggesting the existence of a yet undescribed species in the Olt River, which requires further scrutiny. In strong contrast to its sister genus *Gobio*, little intraspecific distances and little phylogeographic structure was detected.

Zusammenfassung

Bei den Arten der Gattung *Romanogobio* handelt es sich um kleine benthische Gründlinge, die sowohl in stehenden, als auch in fließenden Gewässern vorkommen. Von den sechs im Donausystem vorkommenden Arten werden einige auf nationalen Roten Listen geführt. Sie sind morphologisch schwer zu unterscheiden, was nicht nur Artenschutz schwieriger macht, sondern auch zu geringem Forschungsstand zu phylogeographischen und populationsgenetischer Struktur führt. Genetisches Barcoding stellt eine vielversprechende Alternative zur morphologischen Bestimmung dar. Das Hauptziel dieser Arbeit ist die Etablierung eines Assays für Barcoding basierend auf „High Resolution Melting Analysis“ (bar-HRM). Mit dieser Methode können Arten aufgrund des Schmelzpunktes eines kurzen Amplicons nach einer qPCR unterschieden werden. Dadurch ergibt sich eine Methode, die Zeit und Ressourcen schont, da weitere Schritte wegfallen, die für Sanger-Sequenzierung notwendig sind. Basierend auf 150 neu erstellten und 183 bestehenden Sequenzen der Barcoding-Region (5'-Ende des mitochondrialen COI-Gens) wurde ein potenzieller mini-Barcode gefunden und getestet. Dabei konnten die Arten unterschieden werden, jedoch nur mit einer gewissen Unsicherheit und ohne statistische Absicherung. Das kann allerdings durch Hinzunahme eines bereits für andere Karpfenartige existierenden Assays, der ebenfalls getestet wurde, kompensiert werden. Zusätzlich wurden die Daten phylogenetisch analysiert, wodurch neue Erkenntnisse zu dieser Gattung entstanden sind. Die Ergebnisse stützen bisheriges Wissen über die Taxonomie dieser Gattung, weisen allerdings auch auf eine noch unbeschriebene Art in der Olt hin. Hierfür ist allerdings weitere Forschung notwendig. In starkem Gegensatz zu ihrer Schwesterngattung *Gobio* weisen *Romanogobio*-Arten geringe intraspezifische Distanz und wenig phylogeographische Struktur auf.

Introduction

The genus *Romanogobio* represents a sister group to the genus *Gobio* (Tang *et al.*, 2011) and is treated as member of the Gobionidae (Stout *et al.*, 2016). Both genera can be found in the Danube basin (Kottelat and Freyhof, 2007). In total, 18 *Romanogobio* species are currently recognized (Naseka, 1996, 2001; Naseka and Bogutskaya, 1998; Naseka and Freyhof, 2004; Kottelat and Freyhof, 2007) in Europe. Additionally, *Romanogobio skywalkeri* was described only recently (Friedrich *et al.*, 2018). Currently, six valid species occur in the Danube drainage (Kottelat and Freyhof, 2007; Friedrich *et al.*, 2018). *Romanogobio vladykovii*, *R. uranoscopus* and *R. carpathorossicus* can be found in large parts of the Danube drainage, whereas *R. skywalkeri* is restricted to the upper Mur in Austria, *R. kesslerii* to the middle and lower Danube drainage (IUCN, 2010) and *R. antipai* to the lower Danube drainage (Kottelat and Freyhof, 2007).

They are small benthic fishes occurring in both lentic and lotic waters but are mainly found in rheophilic rivers. Preferred habitats are over sandy bottom for *Romanogobio kesslerii*, *antipai* and *carpathorossicus*, while *R. uranoscopus* prefers stony bottoms (Kottelat and Freyhof, 2007) and *R. skywalkeri* prefers gravel banks (Friedrich *et al.*, 2018). Spawning usually takes place in shallow water (Kottelat and Freyhof, 2007).

Due to their similar size and similarity in overall morphology, correct identification on a species level is difficult in adult specimens, but even harder for juvenile specimens.

Nomenclatural issues

While Kottelat and Freyhof (2007) consider *Romanogobio kesslerii* to be present in upper parts of the Danube, Friedrich *et al.* (2018) use *R. carpathorossicus* for those specimens instead. *Romanogobio carpathorossicus* was originally described as a subspecies of *R. kesslerii*, but molecular data of Friedrich *et al.* (2018) suggests it can be seen as a separate species. Therefore, even though Eschmayer's catalogue (Fricke *et al.*, 2023) lists *Romanogobio carpathorossicus* as subspecies of *R. kesslerii*, it will be treated as a species on its own.

Romanogobio banaticus is listed as valid species in Eschmayer's catalogue of Fishes, but as Friedrich *et al.* (2018) point out, *R. carpathorossicus* was the first available name for this species that was seen as subspecies of *R. kesslerii* as well. Therefore, sequences recognized as *Romanogobio banaticus* will be treated as *R. carpathorossicus* in this thesis.

Endangerment status

Although most of the species are not endangered across their whole distribution range according to the IUCN (accessed on March 13th, 2023), they are endangered in different areas of their distribution. For example, *Romanogobio uranoscopus* is listed as threatened by extinction and *R. vladykovi* is on the watchlist for potential future endangerment in Bavaria (Effenberger *et al.*, 2021). Regarding Austria, *Romanogobio carpathorossicus* (as *Gobio kesslerii*) is listed as endangered, *R. uranoscopus* (listed as *Gobio uranoscopus*) as critically endangered (Wolfram and Mikschi, 2007). Additionally, *Romanogobio kessleri*, *vladykovi* and *uranoscopus* are listed on the annex II of the Habitats Directive (Umweltbundesamt.at, accessed on March 13th, 2023). Concerning the Habitats Directive, Ratschan *et al.* (2021) argue that *Romanogobio skywalkeri* should be treated similar to *R. carpathorossicus* and *uranoscopus*. *R. antipai* was declared extinct in 2008 (IUCN, 2008) and rediscovered in 2016 (Bogutskaya *et al.*, 2019), which suggests it should be in need of protection as well.

These listings in combination with difficult morphological determination suggest that genetic barcoding may play a vital role in conservation efforts as it presents a fast and simple approach to identify species.

Barcoding and bar-HRM

In recent years, DNA-barcoding using cytochrome oxidase subunit 1 (Hebert, Ratnasingham and de Waard, 2003) was widely established for genetic determination of animals. For example, the Austrian Barcode of Life (ABOL) initiative (Szucsich, 2015) established references for a wide range of taxa in Austria. DNA barcoding presents a viable method to identify species for conservational issues (Krishnamurthy and Francis, 2012). An alternative method to conventional DNA-barcoding is High Resolution Melting analysis based on barcodes (bar-HRM) when specimens could be determined to a certain taxonomic level (e.g. family or genus, depending on assay). Like conventional barcoding, a selected region in the genome is amplified with PCR. With a fluorescent dye that intercalates with double-stranded DNA (in this case the PCR-product), the change of fluorescent signal during stepwise increase of temperature is measured (Wittwer, 2009). When the melting curve of this DNA fragment is known for a species, assignment of the melting curves of unidentified specimens to a species can be done (Zangl *et al.*, 2020). The great advantage to conventional barcoding is that it is more time-efficient and cost-saving (Behrens-Chapuis *et al.*, 2018).

Methods

In order to have a representative overview of the Danube system, COI-sequences of available specimens of the Danube drainage were mined from BOLDsystems and new sequences were generated with 200 fin clips provided by Péter Takács. In addition to that, Jörg Freyhof, Matthias Geiger and Martina Topić provided several unpublished sequences. A list of sequences used in this thesis can be found in Table 3 in the appendix, an overview of the sample sites in Figure 1. Unfortunately, no genetic resource of *Romanogobio antipai* was available for this thesis, therefore it was not included in the analysis.

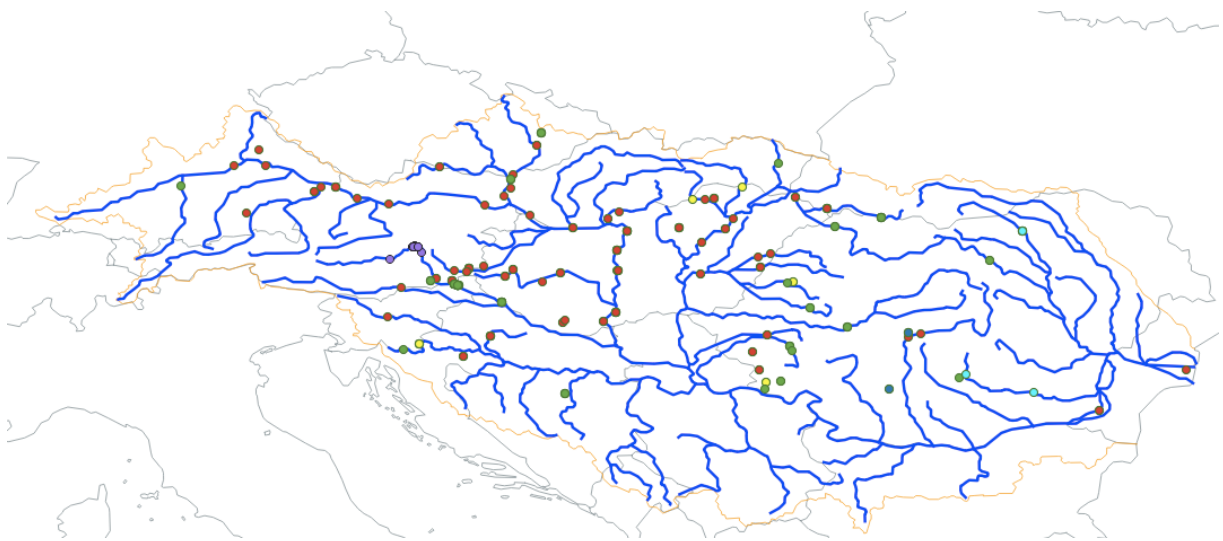


Figure 1: Sample sites of used sequences. Light blue dots: *Romanogobio kesslerii*, dark blue dots: *Romanogobio sp.Olt*, green dots: *Romanogobio uranoscopus*, yellow dots: *Romanogobio carpathorossicus*, red dots: *Romanogobio vladkovii* and violet dots: *Romanogobio skywalkerii*. Species assignment based on COI-based barcoding.

Generation of new COI sequences

Fin clips were stored at -20°C in 70% Ethanol. DNA extraction followed (Richlen and Barber, 2005). PCR followed (Koblmüller *et al.*, 2011) with 4 µl of DNA extract instead of 1 µl, annealing temperature of 49°C and as primer pair C_FishF1t1-C_FishR1t1 (Ivanova *et al.*, 2007). Purification of PCR-products and chain termination sequencing followed (Duftner, Koblmüller and Sturmbauer, 2005). Subsequent purification of DNA fragments was done with Sephadex™ G-50 (Amersham Biosciences) and visualization was done on a 3500xl capillary sequencer (ABI).

Primer Design for bar-HRM

As Behrens-Chapuis *et al.* (2018) already provide a bar-HRM pipeline for cyprinid fish, the possibility to use the same amplicon for discriminating *Romanogobio* species was checked by

comparing it to the COI sequences in the used alignment. As there seemed to be sufficiently low intraspecific diversity and at least one difference between each species pair, this amplicon was considered to be usable for bar-HRM analysis.

Additionally, other amplicons in the suitable length range of 100 to 150 bp were searched for by using primer blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), then the suitability of the results was compared to existing sequences and the best suited amplicon was chosen for further analysis. As result a suitable fragment of 127bp of length, spanning from position 225 to position 351 in the COI region (named Rom-9 in the course of this thesis) was identified. By using PCR and cycle sequencing in the way mentioned above, successful amplification and species determination was tested for both primer pairs. The sequence of the used primers can be found in Table 2 in the appendix. Both assays investigated in this thesis rest upon the assumption that specimens are known to belong to the genus *Romanogobio*.

Bar-HRM

Bar-HRM analysis was run in a Rotor-Gene 3000 thermal cycler (Corbett Research, Mortlake, New South Wales, Australia).

The reactions for the bar HRM were done with 10 µl of total volume as well, following the same composition as the PCR-reactions for the COI-sequences, with the alteration of substituting 0.2 µl of double distilled water with EvaGreen® Fluorescent DNA stain. PCR conditions and subsequent optical measurements followed Zangl *et al.* (2020). Samples were chosen randomly from the most commonly occurring haplotypes when possible.

Data analysis

Generated sequences were edited and aligned with MEGA 11 (Tamura, Stecher and Kumar, 2021). Species verification based on COI of newly generated sequences were checked using BLAST (Altschul *et al.*, 1990) on GenBank. In case of deviation from previous morphological determination the nucleic ribosomal protein S7 (RPS7) was amplified, sequenced, and blasted as well, following the protocol above and using primer pair S7univL and S7univP (Mendel *et al.*, 2008). In case of several ambiguous determinations at one sample point, RPS7 of only one specimen was checked.

With MEGA 11 the best fitting model of evolution was evaluated. This model was then applied for inferring pairwise genetic distance and a Neighbour-Joining tree, also in MEGA 11. For thorough analysis a phylogenetic tree based on Bayesian Inference was computed with the BEAST (v.2.7.1) package (Bouckaert *et al.*, 2019) with JC69 as substitution model and Yule

Model as prior. Effective sample sizes (ESS) exceeded 200 for all parameters. Trees were edited with FigTree (Andrew Rambaut, 2006).

Haplotype networks were calculated in POPART (Leigh and Bryant, 2015) using TCS (Clement, Posada and Crandal, 2000). For haplotype networks, only 535 bp of the COI gene were used to avoid missing regions.

Statistical analysis of the bar-HRM analysis was carried out with R version 4.2.2 using the R-code provided by Hannes Oberreiter (https://github.com/HannesOberreiter/melt_graz; Zangl *et al.*, 2020).

Results

Of the 200 fin clips provided by Péter Takács, a total of 150 usable sequences could be obtained, recognizing only sequences with a length over 500 bp as such. Of these 150 sequences 120 could be determined as *Romanogobio* specimens. For specimens where morphological identification differed from barcoding results, RPS7 confirmed the barcoding results.

Table 1: Mean intraspecific distances, maximum intraspecific distances and mean distances to nearest neighbor. All distances were calculated with K2P (G=0.19)

	Mean intraspecific distances [%]	Maximum intraspecific distance [%]	Mean distance to nearest neighbor [%]
<i>R. carpathorossicus</i>	0.93	1.37	7.19 (<i>R. sp. Olt</i>)
<i>R. kesslerii</i>	0.23	0.47	9.09 (<i>R. carpathorossicus</i>)
<i>R. skywalkeri</i>	0.20	0.79	11.94 (<i>R. vladykovi</i>)
<i>R. sp.nov. OLT</i>	0.90	1.46	7.19 (<i>R. carpathorossicus</i>)
<i>R. uranoscopus</i>	0.36	1.14	7.07 (<i>R. vladykovi</i>)
<i>R. vladykovi</i>	0.46	2.11	7.07 (<i>R. uranoscopus</i>)

Table 1 illustrates that there is nothing unexpected about the phylogeny of this genus, beside the specimens of the Olt River, which are therefore referred to as *Romanogobio* sp.Olt. They separate quite unambiguous from *Romanogobio carpathorossicus* and their cluster also shows higher intraspecific diversity compared to the other species. It becomes apparent that *R.*

vladykovi shows a higher maximal intraspecific distance than *R. carpathorossicus*, but lower mean differences.

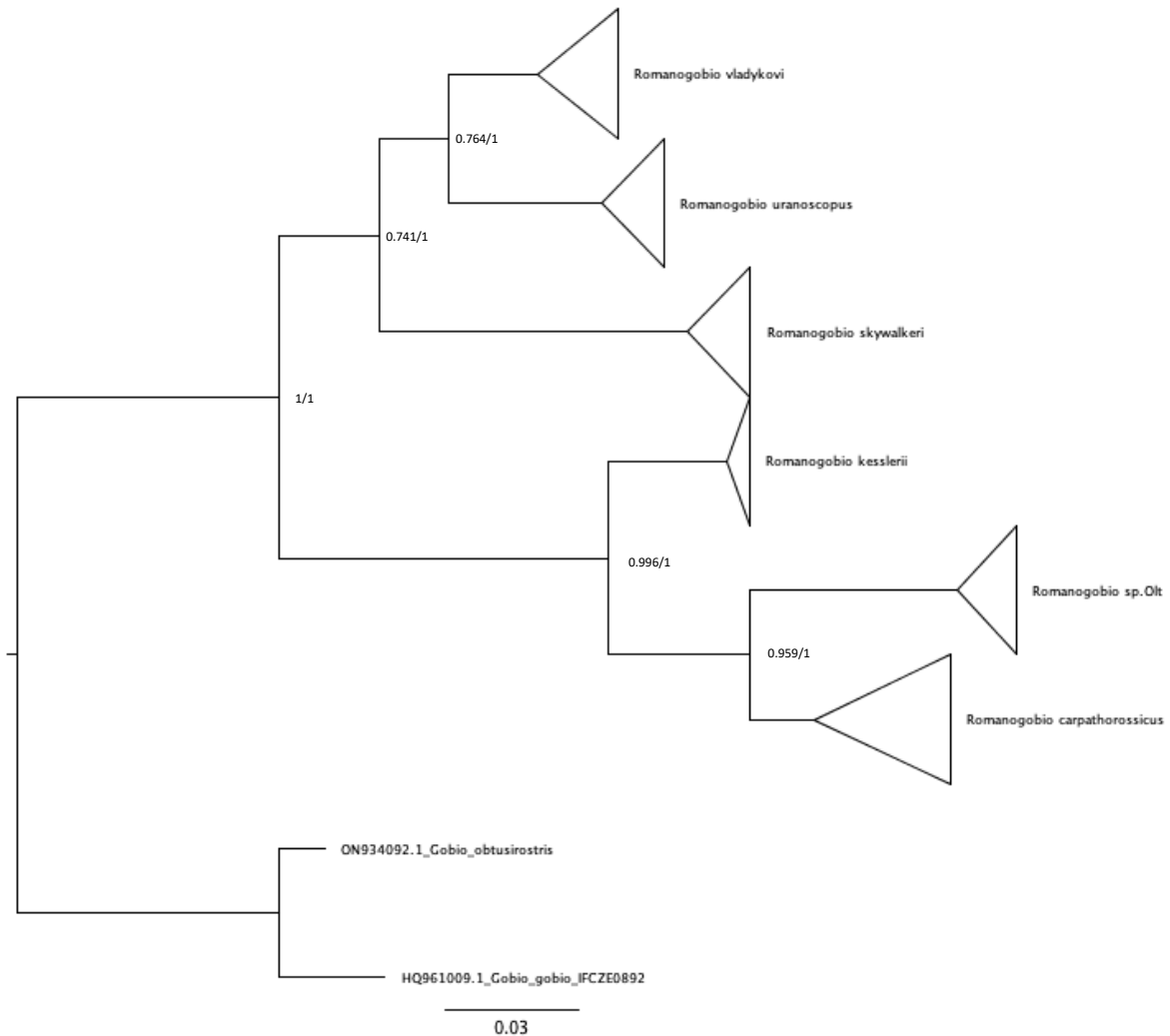


Figure 2: Neighbour-Joining Tree (K2P, g=0.19) of *Romanogobio*. branches of single species are compressed. Bootstrap of NJ-tree and Posterior Probabilities of BI-tree are shown in this order.

Bayesian inference resulted in a tree with the same topology and similar branch lengths as the Neighbor-joining tree. Therefore, only the latter is illustrated in Figure 3 with added posterior probabilities. Looking at genus level and comparing species branches, all nodes show bootstrap values above 70 and posterior probabilities of 1, showing strong statistical support for this tree.

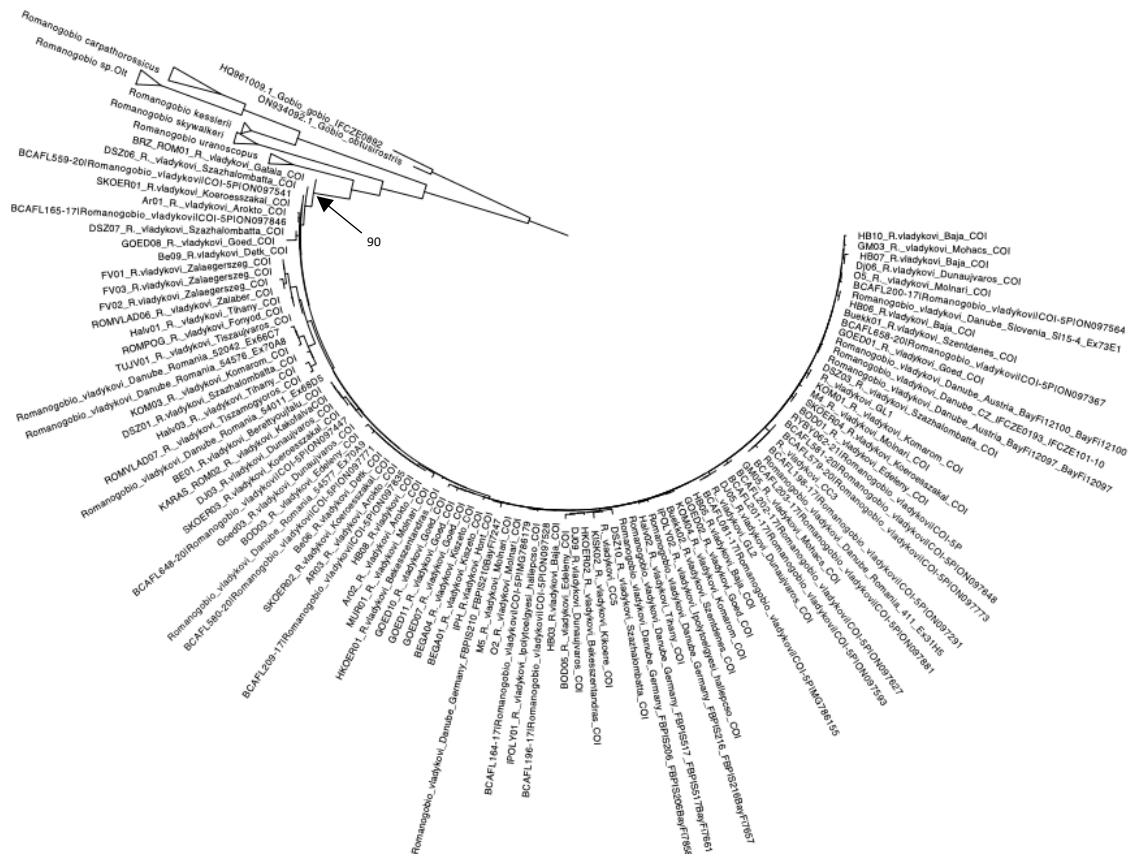


Figure 3: NJ tree (K2P, $G=0.19$) with focus on *R. vladkovi*. Only one specimen per haplotype is depicted and only bootstrap values above 80 are shown.

As an example for intraspecific diversity, Figure 3 illustrates the phylogenetic relationships inside *Romanogobio vladkovi*. Only one node shows a bootstrap value above 80, indicating poor statistical support. For better illustration of haplotype distribution, a haplotype network is shown in Figure 4. Even though little structure can be observed, no clear pattern in the geographical distribution of haplotypes is recognizable. For example, the haplotype represented by RYBY062-22 can be found in Austria, Croatia, Czechia, Hungary, and Romania, similar can be said about other haplotypes.

Figure 5 depicts a haplotype network of *Romanogobio carpathorossicus* with similar results. The majority of samples can be clustered into two haplotypes that occur across the whole drainage.

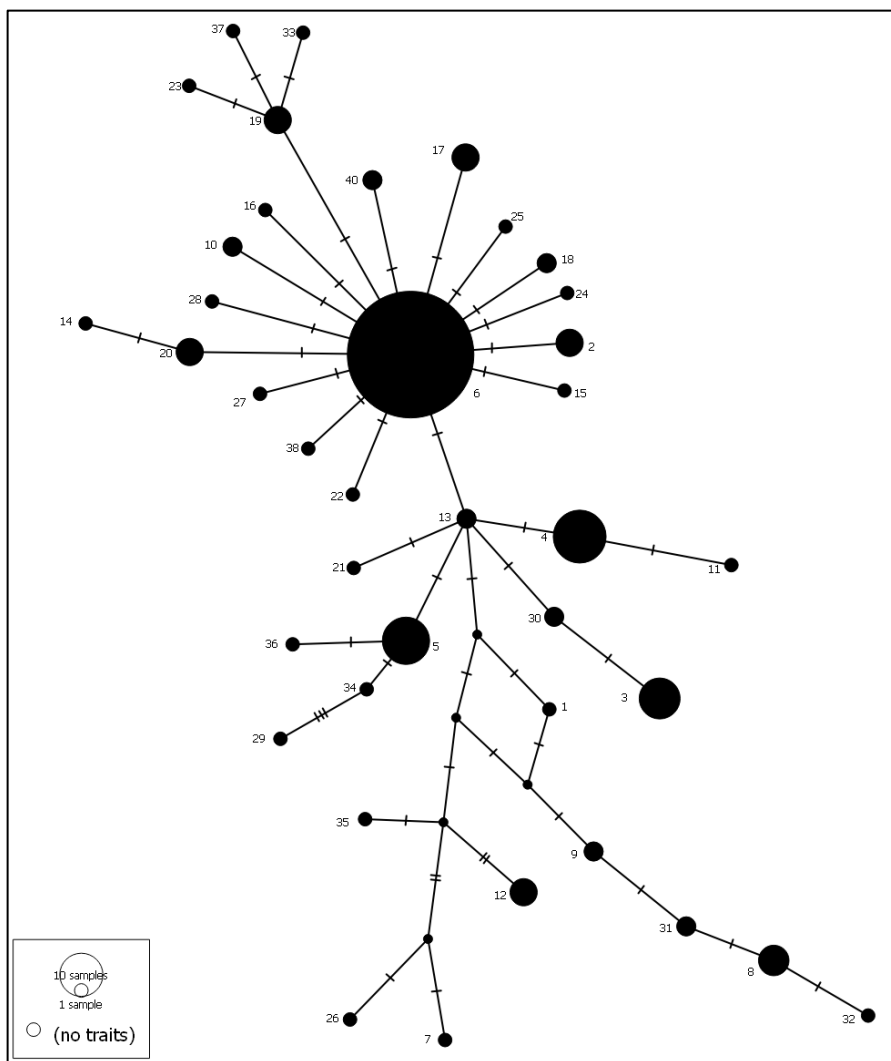


Figure 4: Haplotype network of *Romanogobio vladkovi*. Haplotype numbers can be found in Table 3 in the appendix.

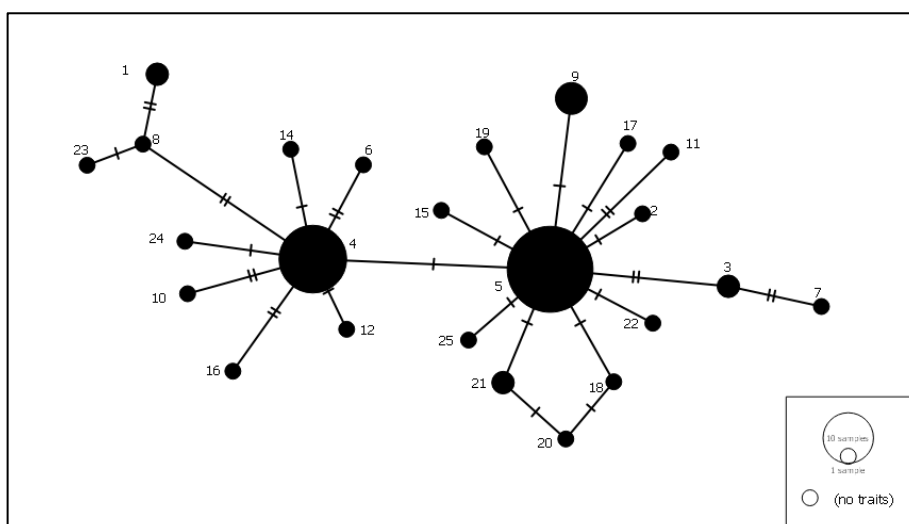


Figure 5: Haplotype network of *Romanogobio carpathorossicus*. Haplotype numbers can be found in Table 3 in the appendix.

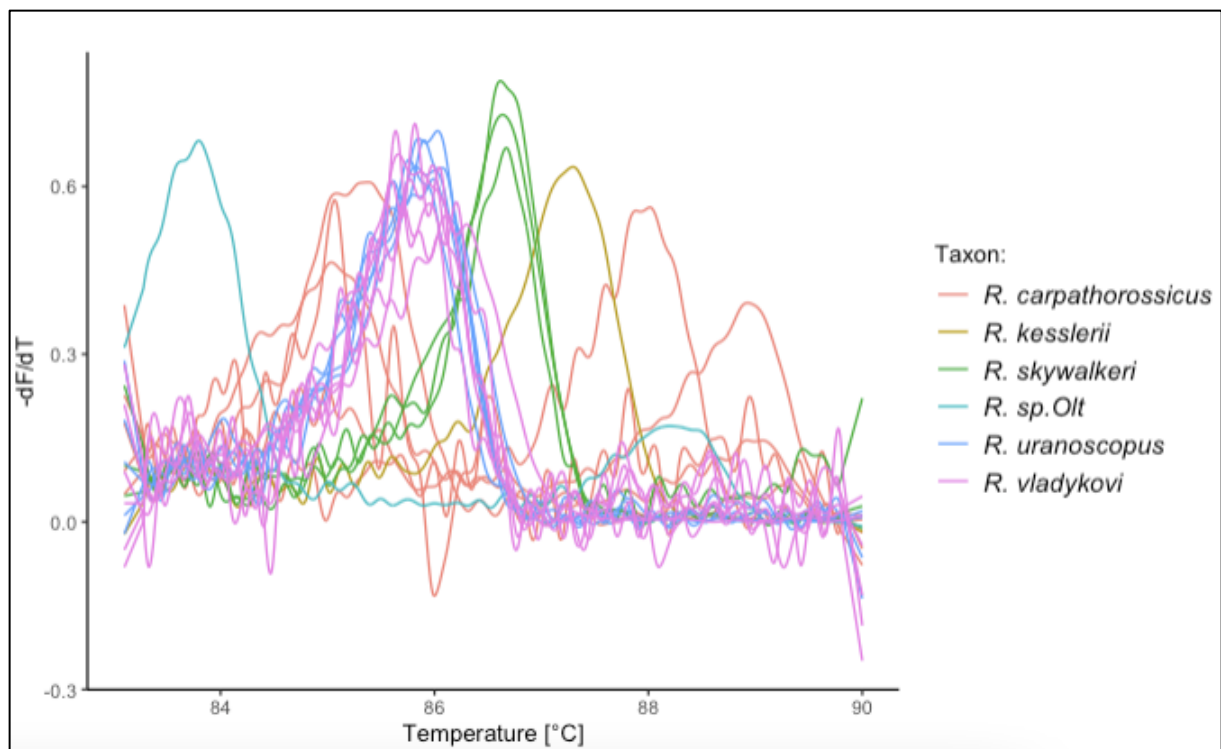


Figure 6: Results of the bar HRM using the Cypr fragment (Behrens-Chapuis *et al.* 2018).

Results of the bar-HRM-pipeline provided by Behrens-Chapuis *et al.*, 2018 show clear separation of all species from each other except from *R. vladykovi* and *R. uranoscopus*. It appears that specimens of *Romanogobio carpathorossicus* show two peaks both at the beginning and the end of the range of species.

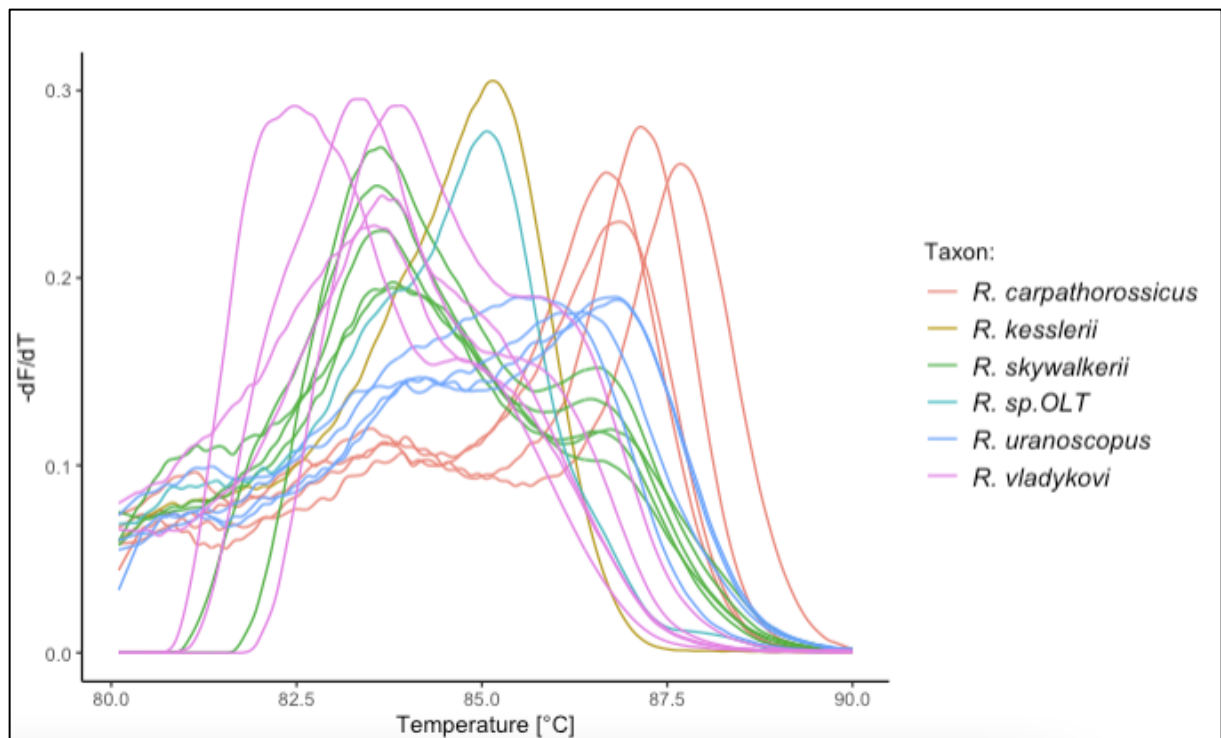


Figure 7: Results of the bar-HRM analysis using the Rom-9-fragment.

Results of the bar-HRM analysis with Rom-9 were smoothed by means of moving average. Figure 7 indicates that species discrimination is possible. *Romanogobio vladykovi* and *R. skywalkeri* have their highest peak close to each other and some specimens are overlapping. Still, they can be clearly separated when taking shoulders (the shoulder of *Romanogobio skywalkeri* appears at a higher temperature than the shoulder of *R. vladykovi*) into account. This can also be seen in Figure 8A. *Romanogobio kesslerii* and the potential new species in the Olt River share an almost identical peak, but discrimination should still be given due to a shoulder of *Romanogobio* sp. Olt at a lower temperature than its peak. *R. uranoscopus* and *R. carpathorossicus* display peaks at higher temperatures that show an overlap as well. Both derivative functions show peaks at lower temperatures as well but do that at a similar temperature. Still, both species can be discriminated from each other when looking at the derivative functions, as *Romanogobio uranoscopus* shows lower peaks with relatively high shoulders compared to *R. carpathorossicus*. Figure 8B illustrates the difference between the melting curves of *Romanogobio carpathorossicus* and *uranoscopus* with a flatter curve for the latter.

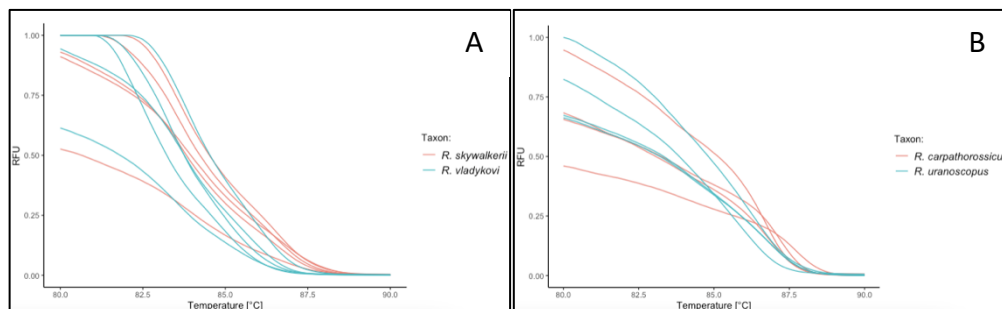


Figure 8: A compares the melting curves of *Romanogobio vladykovi* and *skywalkeri*, B shows the different melting curves of *Romanogobio carpathorossicus* and *uranoscopus*.

Discussion

The results in Figure 2: Neighbour-Joining Tree (K2P, $g=0.19$) of *Romanogobio*. branches of single species are compressed. Bootstrap of NJ-tree and Posterior Probabilities of BI-tree are shown in this order., depicting the relationships between the species, are similar to the phylogeny of Austrian *Romanogobio* specimens (Zangl *et al.*, 2022), yet show differences to the phylogeny provided by Friedrich *et al.* (2018). The use of other *Romanogobio* species in Friedrich *et al.* (2018) probably affected the topology of the tree. Referring to the nomenclatural issues in the introduction, this thesis supports *Romanogobio carpathorossicus* to be a species on its own. It appears as sister group to *Romanogobio kesslerii* but shows a clear barcoding gap. The same can be stated for *Romanogobio carpathorossicus* and *R. sp.Olt*.

This also implies, that the distribution of *Romanogobio kesslerii* is restricted to the lower part of the Danube drainage and specimens identified as *R. kesslerii* in middle and upper parts of the system can be assumed to be *R. carpathorossicus*.

Looking at intraspecific distances, relatively low diversity in *Romanogobio kesslerii* and *R. skywalkerii* can be explained by the restricted distribution area. While *Romanogobio skywalkerii* is restricted to the upper Mur River in Austria, *R. kesslerii* only occurs in the lower Danube river. Mean intraspecific distance is higher in *Romanogobio skywalkerii* compared to *Romanogobio vladykovi*, but the maximum intraspecific distances act vice versa. The reason for that may lie in different sample sizes.

Comparing the maximum intraspecific distances of these two species to Zangl *et al.* (2022), *Romanogobio carpathorossicus* shows a higher value there as well, suggesting higher diversity in this species.

Comparing the maximum intraspecific distances to Zangl *et al.* (2022) in general, the distances turn out to be higher in this thesis. Comparing the mean intraspecific distances to Knebelsberger *et al.*, (2015), who recognized a mean intraspecific distance of 0.26% (with means ranging from 0 to 2.95%), it is clearly higher than the average for *Romanogobio carpathorossicus*, *R. sp.Olt* and slightly higher for *R. vladykovi*, but always inside the range. Tsoupas *et al.* (2022) also recognize relatively low mean intraspecific distances (0.29%). A possible reason for higher values in this thesis can be the wider distribution of samples, as scale may have an effect on intraspecific distances (Papadopoulou *et al.*, 2008).

Relating mean intraspecific distances to (Geiger *et al.*, 2014), mean distances (0.59% on average in a range from 0 to 12.5) are higher in *Romanogobio carpathorossicus* and *Romanogobio sp. Olt* as well, but lower in all other species. It can be concluded, that species of *Romanogobio* tend to have lower mean intraspecific distances except for *Romanogobio carpathorossicus* and *Romanogobio sp.Olt*.

The sister genus of *Romanogobio*, *Gobio*, displays a hybrid zone in Austria, which is similar to the suture zone for the European Perch (*Perca fluviatilis*) in the upper Danube that is described by Nesbø *et al.* (1999). As there is little phylogeographic structure detectable in *Romanogobio* species, it is unlikely that a similar zone for those species exist in this area. A possible explanation for that might be that *Romanogobio* species occurred in only one of the glacial refugia Nesbø *et al.* (1999) respectively Banareescu (1992) suggested. The overlap of the Danube system with the refugium in Eastern Europe would suggest, that it is more probable

that most *Romanogobio* species endured there and not in the Alps. This does not seem to be the case for *Romanogobio skywalkeri*, which probably endured in the upper Mur as a relict species. (Friedrich *et al.*, 2015)

Specimens of *Romanogobio* sp. from the Olt River need further investigation, as their sequences clearly clustered apart from its nearest neighbor, *Romanogobio carpathorossicus*. This suggests cryptic diversity in *Romanogobio carpathorossicus* and therefore the existence of a yet undescribed species. The distance to *Romanogobio carpathorossicus* is comparable to the distance between *R. vladykovi* and *R. uranoscopus*, supporting the hypothesis that another species is present. Additionally, the description of *Romanogobio skywalkeri* in the near past might be a hint for a lack in research on this group and the possibility for more species.

Though only 4 specimens of the potentially new species in the Olt River are included in this thesis, maximum intraspecific distances are second highest after *Romanogobio vladykovi*, raising even more questions. For any conclusions, more research in that area would be necessary.

Bar-HRM

It can be concluded that the pipeline provided by Behrens-Chapuis (2018) is a viable possibility to separate most of the species from each other. The DNA-fragment proposed by this thesis does not have the ability to discriminate the species solely with the highest peaks but in combination with the shape of the derivative function. It is noteworthy, that *Romanogobio carpathorossicus* shows peaks at completely different temperatures, which still can be clearly discriminated from the other species.

Unfortunately, only one specimen of *R. kesslerii* and *R. sp.Olt* could be used in the analysis, giving room to uncertainty – especially as the potential species in the Olt shows high diversity compared to other *Romanogobio*-species. In addition to that, the rediscovered *R. antipai* is completely missing for this study.

Theoretically, Rom-9 would be sufficient to discriminate *Romanogobio* species, yet further research is needed to confirm the findings of this thesis. As the results of this assay are not statistically supported, it may be the most reasonable option to combine the use of both assays. The Cypr fragment allows clear discrimination for all species, except for *Romanogobio vladykovi* and *uranoscopus*. This disadvantage could be compensated for with the Rom-9 fragment. In any case, using both methods could be assuring the quality of the results. The

main advantage of bar-HRM over conventional barcoding is time- and cost-efficiency, which would still be achieved even when two separate runs are conducted for each specimen.

In conclusion, species discrimination should be possible with the proposed method, despite some ambiguities. To provide all the benefits of this method, further research needs to be done. There might be the possibility to optimize bar-HRM for this fragment, but most probably the search for another mini-barcode outside of COI would be the best solution. COI was the obvious first choice to search for a suitable fragment because of the huge amount of already existing data, but the most suitable regions were presented in this thesis. As bar-HRM might be a key element for future monitoring and conservation actions of *Romanogobio* species, further research would be crucial.

Acknowledgements

I sincerely want to thank all colleagues that helped me through the process of writing this thesis, either in the lab or through stimulating talks. Thanks to Lukas, Nadine, Jacky, Sylvia, Martina, Tamara, Thomas, and Chrissi. I also want to thank Lena and my family for their love and everlasting support. Last, but not least, special thanks to Stephan for the amazing opportunity, continuous support and enduring patience.

Literature

Altschul, S.F. *et al.* (1990) 'Basic local alignment search tool', *Journal of Molecular Biology*, 215(3), pp. 403–410. Available at: [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).

Andrew Rambaut (2006) 'FigTree'. Institute of Evolutionary Biology, University of Edinburgh.

Banarescu, P. (1992) *Distribution and dispersal of freshwater animals in North America and Eurasia*. Wiesbaden: AULA

Behrens-Chapuis, S. *et al.* (2018) 'Discriminating European cyprinid specimens by barcode high-resolution melting analysis (Bar-HRM)—A cost efficient and faster way for specimen assignment?', *Fisheries Research*, 204, pp. 61–73. Available at: <https://doi.org/10.1016/j.fishres.2018.02.007>.

Bogutskaya, N.G. *et al.* (2019) 'A recent record of *Romanogobio antipai* (Actinopterygii, Cyprinidae, Gobioninae) from the Danube River in Bulgaria', *ZooKeys*, 825, pp. 105–122. Available at: <https://doi.org/10.3897/zookeys.825.32434>.

Bouckaert, R. *et al.* (2019) 'BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis', *PLOS Computational Biology*. Edited by M. Perteza, 15(4), e1006650. Available at: <https://doi.org/10.1371/journal.pcbi.1006650>.

Clement, M., Posada, D. and Crandal, K. (2000) 'TCS: a computer program to estimating gene genealogies.', *Molecular Ecology*, 9, pp. 1657–1659.

Duftner, N., Koblmüller, S. and Sturmbauer, C. (2005) 'Evolutionary Relationships of the Limnochromini, a Tribe of Benthic Deepwater Cichlid Fish Endemic to Lake Tanganyika, East Africa', *Journal of Molecular Evolution*, 60(3), pp. 277–289. Available at: <https://doi.org/10.1007/s00239-004-0017-8>.

Effenberger, M. *et al.* (2021) *Rote Liste und Gesamtartenliste Bayern – Fische und Rundmäuler*. Edited by Bayerisches Landesamt für Umwelt.

Fricke, R., Eschmeyer, W., and Van der Laan, R. (2023) 'Eschmeyer's Catalog of Fishes: Genera, Species, References'. Available at: <http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp> (Accessed: 13 March 2023).

Friedrich, T. *et al.* (2015) 'Eine neue, unbeschriebene Gründlingsart der Gattung *Romanogobio* in der Oberen Mur - Eine erste Beschreibung anhand morphologischer Merkmale und DNA-Barcodes', *Österreichs Fischerei*, 68, pp. 91–99.

Friedrich, T. *et al.* (2018) '*Romanogobio skywalkeri*, a new gudgeon (Teleostei: Gobionidae) from the upper Mur River, Austria', *Zootaxa*, 4403(2), pp. 336-350. Available at: <https://doi.org/10.11646/zootaxa.4403.2.6>.

Geiger, M.F. *et al.* (2014) 'Spatial heterogeneity in the Mediterranean Biodiversity Hotspot affects barcoding accuracy of its freshwater fishes', *Molecular Ecology Resources*, 14(6), pp. 1210–1221. Available at: <https://doi.org/10.1111/1755-0998.12257>.

Hebert, P.D.N., Ratnasingham, S. and de Waard, J.R. (2003) 'Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species', *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 270(1512), pp. 313-321. Available at: <https://doi.org/10.1098/rsbl.2003.0025>.

IUCN (2008) '*Romanogobio antipai*: Freyhof, J. & Kottelat, M.: The IUCN Red List of Threatened Species 2008: e.T135636A4167651'. International Union for Conservation of Nature. Available at: <https://doi.org/10.2305/IUCN.UK.2008.RLTS.T135636A4167651.en>.

IUCN (2010) '*Romanogobio kesslerii*: Freyhof, J.: The IUCN Red List of Threatened Species 2011: e.T9297A97803482'. International Union for Conservation of Nature. Available at: <https://doi.org/10.2305/IUCN.UK.2008.RLTS.T9297A12980164.en>.

Ivanova, N.V. *et al.* (2007) 'Universal primer cocktails for fish DNA barcoding: BARCODING', *Molecular Ecology Notes*, 7(4), pp. 544–548. Available at: <https://doi.org/10.1111/j.1471-8286.2007.01748.x>.

Knebelsberger, T. *et al.* (2015) 'Molecular diversity of Germany's freshwater fishes and lampreys assessed by DNA barcoding', *Molecular Ecology Resources*, 15(3), pp. 562–572. Available at: <https://doi.org/10.1111/1755-0998.12322>.

Kottelat, M. and Freyhof, J. (2007) *Handbook of european freshwater fishes*. Cornol: Publications Kottelat.

Krishnamurthy, K. and Francis, R.A. (2012) 'A critical review on the utility of DNA barcoding in biodiversity conservation', *Biodiversity and Conservation*, 21(8), pp. 1901–1919. Available at: <https://doi.org/10.1007/s10531-012-0306-2>.

Leigh, J.W. and Bryant, D. (2015) 'POPART: full-feature software for haplotype network construction', *Methods in Ecology and Evolution*. Edited by S. Nakagawa, 6(9), pp. 1110–1116. Available at: <https://doi.org/10.1111/2041-210X.12410>.

Mendel, J. *et al.* (2008) 'Molecular phylogeny of the genus *Gobio* Cuvier, 1816 (Teleostei: Cyprinidae) and its contribution to taxonomy', *Molecular Phylogenetics and Evolution*, 47(3), pp. 1061–1075. Available at: <https://doi.org/10.1016/j.ympev.2008.03.005>.

Naseka, A.M. (1996) 'Comparative study on the vertebral column in the Gobioninae (Cyprinidae, Pisces) with special reference to its systematics.', *Publicaciones Especiales Instituto Espanol de Oceanografia*, 21, pp. 149–167.

Naseka, A.M. (2001) 'Contribution to the knowledge of infraspecific structure of whitefin gudgeon *Romanogobio alpinus* (Lukasch, 1933)(Cyprinidae: Gobioninae), with a description of a new subspecies, *R. alpinus tanaiticus*, from the Don drainage.', *Proceedings of the Zoological Institute*, 287, pp. 99–119.

Naseka, A.M. and Bogutskaya, N.G. (1998) 'A new gudgeon species *Romanogobio pentatrichus* (Gobioninae, Cyprinidae) from the basin of the Kuban River', *Journal of Ichthyology*, 38(3), pp. 219–227.

Naseka, A.M. and Freyhof, J. (2004) 'Romanogobio parvus, a new gudgeon from River Kubn, southern Russia (Cyprinidae, Gobioninae).', *Ichthyological Exploration of Freshwater*, 15(1), pp. 17–24.

Nesbo, C.L. *et al.* (1999) 'Genetic divergence and phylogeographic relationships among European perch (*Perca fluviatilis*) populations reflect glacial refugia and postglacial colonization', *Molecular Ecology*, 8(9), pp. 1387–1404. Available at: <https://doi.org/10.1046/j.1365-294x.1999.00699.x>.

Papadopoulou, A. *et al.* (2008) 'Speciation and DNA barcodes: testing the effects of dispersal on the formation of discrete sequence clusters', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1506), pp. 2987–2996. Available at: <https://doi.org/10.1098/rstb.2008.0066>.

Richlen, M.L. and Barber, P.H. (2005) 'A technique for the rapid extraction of microalgal DNA from single live and preserved cells', *Molecular Ecology Notes*, 5(3), pp. 688–691. Available at: <https://doi.org/10.1111/j.1471-8286.2005.01032.x>.

Ratschan, C., Friedrich, T. and Freyhof, J. (2021) 'Handelt es sich beim in der Mur endemischen Smaragdgressling (*Romanogobio skywalkeri*) um ein Schutzgut nach Anhang II der FFH-Richtlinie?', *Österreichs Fischerei*, 74(7), pp. 192–199.

Szucsich, N.U. (2015) 'ABOL - DNA-Barcoding als Impuls für die Biodiversitätsforschung in Österreich', *Acta ZooBot Austria*, 152, pp. 157–160.

Tamura, K., Stecher, G. and Kumar, S. (2021) 'MEGA11: Molecular Evolutionary Genetics Analysis Version 11', *Molecular Biology and Evolution*. Edited by F.U. Battistuzzi, 38(7), pp. 3022–3027. Available at: <https://doi.org/10.1093/molbev/msab120>.

Tang, K.L. *et al.* (2011) 'Phylogeny of the gudgeons (Teleostei: Cyprinidae: Gobioninae)', *Molecular Phylogenetics and Evolution*, 61(1), pp. 103–124. Available at: <https://doi.org/10.1016/j.ympev.2011.05.022>.

Tsoupas, A. *et al.* (2022) 'DNA barcoding identification of Greek freshwater fishes', *PLOS ONE*. Edited by Z. Peng, 17(1), p. e0263118. Available at: <https://doi.org/10.1371/journal.pone.0263118>.

Wittwer, C.T. (2009) 'High-resolution DNA melting analysis: advancements and limitations', *Human Mutation*, 30(6), pp. 857–859. Available at: <https://doi.org/10.1002/humu.20951>.

Wolfram, G.; Mikschi, E. (2007) 'Rote Liste der Fische (Pisces) Österreichs', in Zulka, K. P. (ed.) *Rote Liste gefährdeter Tiere Österreichs. Checklisten, Gefährdungsanalysen, Handlungsbedarf. Teil 2: Kriechtiere, Lurche, Fische, Nachtfalter, Weichtiere*. Wien, Böhlau (Grüne Reihe des Bundesministeriums für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft), pp. 61–198.

Zangl, L., *et al.* (2020) 'Austrian gudgeons of the genus *Gobio* (Teleostei: Gobionidae): A mixture of divergent lineages', *Journal of Zoological Systematics and Evolutionary Research*, 58(1), pp. 327–340. Available at: <https://doi.org/10.1111/jzs.12340>.

Zangl, L., *et al.* (2020) 'Discriminating larvae of two syntopic *Cychramus* species (Coleoptera, Nitidulidae) by means of bar-HRM analysis', *Molecular Biology Reports*, 47(10), pp. 8251–8257. Available at: <https://doi.org/10.1007/s11033-020-05786-9>.

Zangl, L. *et al.* (2022) 'A comprehensive DNA barcode inventory of Austria's fish species', *PLOS ONE*. Edited by S.D. Fugmann, 17(6), e0268694. Available at: <https://doi.org/10.1371/journal.pone.0268694>.

Appendix

Primers

Table 2: Primers used for PCR, sequencing and HRM-analysis.

Gene (area)	T _a	Primer	Sequence (5'-3')	Reference
COI	49°	F1t1	TGTA AACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC	(Ivanova <i>et al.</i> , 2007)
	C	(VF_t1 & FishF2_t1)	TGTA AACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC	
		R1t1	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA	(Ivanova <i>et al.</i> , 2007)
		(FishR2_t1 & FR1d_t1)	CAGGAAACAGCTATGACACCTCAGGGTGTCGAARAAYCARAA	
RPS7	49°	S7univL	ACAATTGTAAGTCGGAGATG	(Mendel <i>et al.</i> , 2008)
	C	S7univP	CCCACAAAATAAGATATTAGG	(Mendel <i>et al.</i> , 2008)
COI (5 – 146)	49°	Cypr-F	TATCTYGTATTTGGTGCCTG	(Behrens-Chapuis <i>et al.</i> , 2018)
	C	Cypr-R	TTATTACRAAGGCGTGGG	(Behrens-Chapuis <i>et al.</i> , 2018)
COI(225-332)	64°	Rom_F9	TGGCATTCCCGCGGATAAATA	
	C	Rom_R9	GTCTACCCGCCACTTCTGG	

Sample list

Table 3: List of samples used in the analyses assorted by species and haplotype number.

BOLD-ID	Genbank-Acc.-No.	Other ID	Longitude (N)	Latitude (E)	SPECIES	Haplotype number	Country
BCAFL106-17	MG786152		46,678	16,002	Romanogobio carpathorossicus	1	Austria
BCAFL572-20	ON097310		46,684	15,989	Romanogobio carpathorossicus	1	Austria
BCAFL108-17	MG786184		46,678	16,002	Romanogobio carpathorossicus	2	Austria
BCAFL159-17	MG786158		46,763	15,477	Romanogobio carpathorossicus	3	Austria
BCAFL279-18	MG786169		46,763	15,477	Romanogobio carpathorossicus	3	Austria
		Ex05G6	45,604	15,277	Romanogobio carpathorossicus	4	Slovenia
		Ex65H4	45,906	23,220	Romanogobio carpathorossicus	4	Romania
		Ex65H5	45,906	23,220	Romanogobio carpathorossicus	4	Romania
		KBO3	45,064	15,741	Romanogobio carpathorossicus	4	Croatia
		Nera Rom01	44,902	21,988	Romanogobio carpathorossicus	4	Romania
		ROM09	48,107	22,831	Romanogobio carpathorossicus	4	Hungary
		ROM16	48,107	22,831	Romanogobio carpathorossicus	4	Hungary
		SAJ01	48,282	20,347	Romanogobio carpathorossicus	4	Hungary
BCAFL197-17	MN017080		46,707	15,919	Romanogobio carpathorossicus	4	Austria
BCAFL261-17	ON097689		46,684	15,989	Romanogobio carpathorossicus	4	Austria
BCAFL266-17	ON097788		46,684	15,989	Romanogobio carpathorossicus	4	Austria
BCAFL570-20	ON097639		46,684	15,989	Romanogobio carpathorossicus	4	Austria
BCAFL578-20	ON097649		46,684	15,989	Romanogobio carpathorossicus	4	Austria
IFCZE910-11			49,506	17,532	Romanogobio carpathorossicus	4	Czech Republic
IFCZE912-11			49,506	17,532	Romanogobio carpathorossicus	4	Czech Republic
IFCZE917-11			48,647	16,970	Romanogobio carpathorossicus	4	Czech Republic

	Ex01B7	46,684	15,988	Romanogobio carpathorossicus	5	Austria
	Ex05G3	45,604	15,277	Romanogobio carpathorossicus	5	Slovenia
	Ex05G4	45,604	15,277	Romanogobio carpathorossicus	5	Slovenia
	Ex05G5	45,604	15,277	Romanogobio carpathorossicus	5	Slovenia
	Ex05G7	45,604	15,277	Romanogobio carpathorossicus	5	Slovenia
	Ex41C1	44,677	17,990	Romanogobio carpathorossicus	5	BiH
	Ex41C2	44,677	17,990	Romanogobio carpathorossicus	5	BiH
	Ex65G2	45,546	22,154	Romanogobio carpathorossicus	5	Romania
	Ex68H5	47,783	22,980	Romanogobio carpathorossicus	5	Romania
	Ex68H6	47,783	22,980	Romanogobio carpathorossicus	5	Romania
	Ex70C5	47,933	23,854	Romanogobio carpathorossicus	5	Romania
	Ex70C6	47,933	23,854	Romanogobio carpathorossicus	5	Romania
	Ex70C7	47,933	23,854	Romanogobio carpathorossicus	5	Romania
	Ex70C8	47,933	23,854	Romanogobio carpathorossicus	5	Romania
	Ex70E4	46,759	22,214	Romanogobio carpathorossicus	5	Romania
	FKG03	46,277	22,535	Romanogobio carpathorossicus	5	Romania
	FKG05	46,277	22,535	Romanogobio carpathorossicus	5	Romania
	HER07	48,511	21,271	Romanogobio carpathorossicus	5	Hungary
	Mur07	46,374	16,817	Romanogobio carpathorossicus	5	Hungary
	O18	45,239	17,764	Romanogobio carpathorossicus	5	Croatia
	ROM08	48,107	22,831	Romanogobio carpathorossicus	5	Hungary
	ROM10	48,107	22,831	Romanogobio carpathorossicus	5	Hungary
	Romkes01	48,317	22,248	Romanogobio carpathorossicus	5	Hungary
BayFi12096	BayFi12096	46,990	16,193	Romanogobio carpathorossicus	5	Austria
BayFi12098	BayFi12098	46,990	16,193	Romanogobio carpathorossicus	5	Austria
BayFi12106	BayFi12106	46,990	16,193	Romanogobio carpathorossicus	5	Austria
BCAFL260-17	ON097562	46,684	15,989	Romanogobio carpathorossicus	5	Austria
BCAFL263-17	ON097468	46,684	15,989	Romanogobio carpathorossicus	5	Austria

BCAFL557-20	ON097850		46,707	15,919	Romanogobio carpathorossicus	5	Austria
BCAFL573-20	ON097636		46,684	15,989	Romanogobio carpathorossicus	5	Austria
BCAFL577-20	ON097411		46,684	15,989	Romanogobio carpathorossicus	5	Austria
IFCZE913-11			49,506	17,532	Romanogobio carpathorossicus	5	Czech Republic
BCAFL571-20	ON097704		46,684	15,989	Romanogobio carpathorossicus	7	Austria
BCAFL574-20	ON097697		46,684	15,989	Romanogobio carpathorossicus	8	Austria
		Ex65G3	45,546	22,154	Romanogobio carpathorossicus	9	Romania
		Ex68E8	45,546	22,154	Romanogobio carpathorossicus	9	Romania
		Ex68E9	45,546	22,154	Romanogobio carpathorossicus	9	Romania
BCAFL651-20	ON097781		46,684	15,989	Romanogobio carpathorossicus	9	Austria
BCAFL654-20	ON097899		46,684	15,989	Romanogobio carpathorossicus	10	Austria
		HB01	46,178	18,918	Romanogobio carpathorossicus	11	Hungary
		Mur02	46,374	16,817	Romanogobio carpathorossicus	12	Hungary
		Mur08	46,374	16,817	Romanogobio carpathorossicus	13	Hungary
		KB5	45,064	15,741	Romanogobio carpathorossicus	14	Croatia
		ROM21	48,107	22,831	Romanogobio carpathorossicus	15	Hungary
BayFi12101	BayFi12101		46,990	16,193	Romanogobio carpathorossicus	16	Austria
BayFi12105	BayFi12105		46,990	16,193	Romanogobio carpathorossicus	17	Austria
		Ex41C4	44,677	17,990	Romanogobio carpathorossicus	18	BiH
		Ex65E6	44,886	21,708	Romanogobio carpathorossicus	19	Romania
		Ex65E7	44,886	21,708	Romanogobio carpathorossicus	20	Romania
		Ex70D5	47,933	23,854	Romanogobio carpathorossicus	21	Romania
		RomKes02	48,317	22,248	Romanogobio carpathorossicus	21	Hungary
		Ex70E3	46,759	22,214	Romanogobio carpathorossicus	22	Romania
RYBY074-21			48,954	21,947	Romanogobio carpathorossicus	23	Slovakia
RYBY149-22			48,048	18,669	Romanogobio carpathorossicus	24	Slovakia
		Ex69D12	45,031	25,420	Romanogobio kesslerii		Romania

		ExHSX2017H7	47,699	26,460	Romanogobio kesslerii	Romania
		ExHSX2017H8	47,699	26,460	Romanogobio kesslerii	Romania
		IG05	44,687	26,671	Romanogobio kesslerii	Romania
		Ex01B4	46,593	14,036	Romanogobio skywalker	Austria
BCAFL297-18	MG786160		47,403	15,176	Romanogobio skywalker	Austria
ANGBF53159-19	MG751105		47,290	15,326	Romanogobio skywalker	Austria
BCAFL280-18	MG786142		47,402	15,240	Romanogobio skywalker	Austria
BCAFL156-17	MG786143		47,403	15,249	Romanogobio skywalker	Austria
BCAFL283-18	MG786145		47,402	15,240	Romanogobio skywalker	Austria
BCAFL288-18	MG786146		47,402	15,240	Romanogobio skywalker	Austria
BCAFL301-18	MG786147		47,402	15,240	Romanogobio skywalker	Austria
BCAFL300-18	MG786151		47,402	15,240	Romanogobio skywalker	Austria
BCAFL287-18	MG786154		47,402	15,240	Romanogobio skywalker	Austria
BCAFL299-18	MG786156		47,171	14,731	Romanogobio skywalker	Austria
BCAFL292-18	MG786159		47,402	15,240	Romanogobio skywalker	Austria
BCAFL289-18	MG786161		47,402	15,240	Romanogobio skywalker	Austria
BCAFL281-18	MG786162		47,402	15,240	Romanogobio skywalker	Austria
BCAFL284-18	MG786163		47,402	15,240	Romanogobio skywalker	Austria
BCAFL286-18	MG786168		47,402	15,240	Romanogobio skywalker	Austria
BCAFL290-18	MG786171		47,402	15,240	Romanogobio skywalker	Austria
BCAFL295-18	MG786172		47,395	15,142	Romanogobio skywalker	Austria
BCAFL294-18	MG786173		47,413	15,204	Romanogobio skywalker	Austria
BCAFL293-18	MG786176		47,413	15,204	Romanogobio skywalker	Austria
BCAFL302-18	MG786177		47,402	15,240	Romanogobio skywalker	Austria
BCAFL282-18	MG786178		47,402	15,240	Romanogobio skywalker	Austria
BCAFL285-18	MG786181		47,402	15,240	Romanogobio skywalker	Austria
BCAFL157-17	MG786182		47,403	15,249	Romanogobio skywalker	Austria
BCAFL296-18	MG786183		47,395	15,142	Romanogobio skywalker	Austria

BCAFL291-18	MG786186	47,402	15,240	Romanogobio skywalker	Austria
BCAFL298-18	MG786187	47,403	15,176	Romanogobio skywalker	Austria
BCAFL429-20	ON097560	47,406	15,258	Romanogobio skywalker	Austria
BCAFL127-17		47,406	15,258	Romanogobio skywalker	Austria
	Ex65E1	45,802	24,353	Romanogobio sp. Olt	Romania
	Ex65E2	45,802	24,353	Romanogobio sp. Olt	Romania
	Ex68H10	44,752	23,998	Romanogobio sp. Olt	Romania
	Ex68H9	44,752	23,998	Romanogobio sp. Olt	Romania
	Ex28E2	44,750	21,683	Romanogobio uranoscopus	Romania
	Ex41C5	44,677	17,990	Romanogobio uranoscopus	BiH
	Ex41C6	44,677	17,990	Romanogobio uranoscopus	BiH
	Ex65B12	44,963	25,296	Romanogobio uranoscopus	Romania
	Ex65G1	45,546	22,154	Romanogobio uranoscopus	Romania
	Ex65G12	45,906	23,220	Romanogobio uranoscopus	Romania
	Ex65H1	45,906	23,220	Romanogobio uranoscopus	Romania
	Ex66B3	47,144	25,864	Romanogobio uranoscopus	Romania
	Ex66B4	47,144	25,864	Romanogobio uranoscopus	Romania
	Ex66C6	47,699	26,460	Romanogobio uranoscopus	Romania
	Ex68H7	46,734	22,099	Romanogobio uranoscopus	Romania
	Ex70C12	47,933	23,854	Romanogobio uranoscopus	Romania
	Ex70D1	47,933	23,854	Romanogobio uranoscopus	Romania
	Ex70D12	47,783	22,980	Romanogobio uranoscopus	Romania
	Ex70D2	47,933	23,854	Romanogobio uranoscopus	Romania
	Ex70D3	47,933	23,854	Romanogobio uranoscopus	Romania
	FKG01	46,277	22,535	Romanogobio uranoscopus	Romania
	Mur03	46,374	16,817	Romanogobio uranoscopus	Hungary
	Romura Nera				
	3	44,902	21,988	Romanogobio uranoscopus	Romania

		Temes				
		uranoscop 1	45,478	22,184	Romanogobio uranoscopus	Romania
		Ur VRT	45,497	14,992	Romanogobio uranoscopus	Slovenia
FBPIS211-10	HM392089		48,550	10,870	Romanogobio uranoscopus	Germany
FBPIS212-10	HM392090		48,550	10,870	Romanogobio uranoscopus	Germany
FBPIS213-10	HM392091		48,730	10,910	Romanogobio uranoscopus	Germany
FBPIS384-11	KM373663		48,534	10,867	Romanogobio uranoscopus	Germany
FBPIS382-11	KM373679		48,534	10,867	Romanogobio uranoscopus	Germany
FBPIS383-11	KM373685		48,534	10,867	Romanogobio uranoscopus	Germany
BCAFL103-17	MG786153		46,678	16,002	Romanogobio uranoscopus	Austria
BCAFL104-17	MG786180		46,678	16,002	Romanogobio uranoscopus	Austria
BCAFL650-20	ON097324		46,684	15,989	Romanogobio uranoscopus	Austria
BCAFL262-17	ON097406		46,684	15,989	Romanogobio uranoscopus	Austria
BCAFL652-20	ON097529		46,684	15,989	Romanogobio uranoscopus	Austria
BCAFL105-17	ON097644		46,678	16,002	Romanogobio uranoscopus	Austria
BCAFL259-17	ON097665		46,684	15,989	Romanogobio uranoscopus	Austria
BCAFL264-17	ON097731		46,684	15,989	Romanogobio uranoscopus	Austria
BCAFL265-17	ON097802		46,684	15,989	Romanogobio uranoscopus	Austria
RYBY075-21			48,954	21,947	Romanogobio uranoscopus	Slovakia
FBPIS385-11			48,534	10,867	Romanogobio uranoscopus	Slovakia
		Tujv01	47,923	21,092	Romanogobio vladykovi	1 Hungary
		Bod01	48,296	20,740	Romanogobio vladykovi	2 Hungary
		Bod04	48,296	20,740	Romanogobio vladykovi	2 Hungary
		Ex68D6	45,802	24,353	Romanogobio vladykovi	2 Romania
		SKÖR04	47,011	21,607	Romanogobio vladykovi	2 Hungary
		BE01	47,209	21,564	Romanogobio vladykovi	3 Hungary
		DSZ05	47,329	18,942	Romanogobio vladykovi	3 Hungary
		HB09	46,178	18,918	Romanogobio vladykovi	3 Hungary

		Karas Rom 01	45,109	21,581	Romanogobio vladykovi	3	Romania
		Karas Rom 02	45,109	21,581	Romanogobio vladykovi	3	Romania
		SKÖR03	47,011	21,607	Romanogobio vladykovi	3	Hungary
IFCZE096-10	HQ960508		48,889	15,649	Romanogobio vladykovi	3	Czech Republic
IFCZE097-10	HQ960509		48,889	15,649	Romanogobio vladykovi	3	Czech Republic
		DJ03	46,960	18,955	Romanogobio vladykovi	3	Hungary
		Ar02	47,731	20,964	Romanogobio vladykovi	4	Hungary
		Ar03	47,731	20,964	Romanogobio vladykovi	4	Hungary
		Be04	47,745	20,093	Romanogobio vladykovi	4	Hungary
		Be08	47,745	20,093	Romanogobio vladykovi	4	Hungary
		Be10	47,745	20,093	Romanogobio vladykovi	4	Hungary
		Bod03	48,296	20,740	Romanogobio vladykovi	4	Hungary
		CC2	45,749	16,598	Romanogobio vladykovi	4	Croatia
		DSZ09	47,329	18,942	Romanogobio vladykovi	4	Hungary
		Ex31H6	44,367	27,900	Romanogobio vladykovi	4	Romania
		HB08	46,178	18,918	Romanogobio vladykovi	4	Hungary
		SKÖR02	47,011	21,607	Romanogobio vladykovi	4	Hungary
BCAFL209-17	ON097835		46,707	15,919	Romanogobio vladykovi	4	Austria
BCAFL580-20	ON097771		46,684	15,989	Romanogobio vladykovi	4	Austria
RYBY150-22			47,995	17,332	Romanogobio vladykovi	4	Slovakia
		Ar01	47,731	20,964	Romanogobio vladykovi	5	Hungary
		Be09	47,745	20,093	Romanogobio vladykovi	5	Hungary
		CC6	45,749	16,598	Romanogobio vladykovi	5	Croatia
		Ex01B1	46,684	15,987	Romanogobio vladykovi	5	Austria
		Saj07	48,281	20,584	Romanogobio vladykovi	5	Hungary
		SKÖR01	47,011	21,607	Romanogobio vladykovi	5	Hungary

BCAFL160-17	MG786164	46,767	15,889	Romanogobio vladykovi	5	Austria
BCAFL163-17	MG786185	46,767	15,889	Romanogobio vladykovi	5	Austria
BCAFL165-17	ON097846	46,767	15,889	Romanogobio vladykovi	5	Austria
BCAFL199-17	ON097426	46,707	15,919	Romanogobio vladykovi	5	Austria
BCAFL559-20	ON097541	46,707	15,919	Romanogobio vladykovi	5	Austria
	BE02	47,277	21,802	Romanogobio vladykovi	6	Hungary
	Be05	47,745	20,093	Romanogobio vladykovi	6	Hungary
	Béga 04	45,764	21,732	Romanogobio vladykovi	6	Romania
	BRZ ROM03	45,441	21,454	Romanogobio vladykovi	6	Romania
	Bükk01	45,992	17,939	Romanogobio vladykovi	6	Hungary
	Bükk02	45,992	17,939	Romanogobio vladykovi	6	Hungary
	Bükk03	46,043	17,978	Romanogobio vladykovi	6	Hungary
	CC4	45,749	16,598	Romanogobio vladykovi	6	Croatia
	CC7	45,749	16,598	Romanogobio vladykovi	6	Croatia
	Dj02	46,960	18,955	Romanogobio vladykovi	6	Hungary
	DJ06	46,960	18,955	Romanogobio vladykovi	6	Hungary
	DJ10	46,960	18,956	Romanogobio vladykovi	6	Hungary
	DSZ03	47,329	18,942	Romanogobio vladykovi	6	Hungary
	DSZ04	47,329	18,942	Romanogobio vladykovi	6	Hungary
	DSZ06	47,329	18,942	Romanogobio vladykovi	6	Hungary
	DSZ08	47,329	18,942	Romanogobio vladykovi	6	Hungary
	Ex01A7	46,954	15,919	Romanogobio vladykovi	6	Austria
	Ex01B2	48,508	13,456	Romanogobio vladykovi	6	Austria
	Ex01B3	46,638	14,945	Romanogobio vladykovi	6	Austria
	Ex68D3	45,792	24,578	Romanogobio vladykovi	6	Romania
	Ex69G12	45,726	24,342	Romanogobio vladykovi	6	Romania
	GL3	45,359	16,099	Romanogobio vladykovi	6	Croatia
	GM03	46,023	18,684	Romanogobio vladykovi	6	Hungary

	GM04	46,023	18,684	Romanogobio vladykovi	6 Hungary
	GM05	46,023	18,684	Romanogobio vladykovi	6 Hungary
	Göd01	47,691	19,127	Romanogobio vladykovi	6 Hungary
	Göd02	47,691	19,127	Romanogobio vladykovi	6 Hungary
	Göd04	47,691	19,127	Romanogobio vladykovi	6 Hungary
	Göd07	47,691	19,127	Romanogobio vladykovi	6 Hungary
	Göd09	47,691	19,127	Romanogobio vladykovi	6 Hungary
	Göd12	47,691	19,127	Romanogobio vladykovi	6 Hungary
	Halv 02	46,917	17,894	Romanogobio vladykovi	6 Hungary
	HB03	46,178	18,918	Romanogobio vladykovi	6 Hungary
	HB05	46,178	18,918	Romanogobio vladykovi	6 Hungary
	IPH01	48,055	18,986	Romanogobio vladykovi	6 Hungary
	lpoly01	47,932	18,771	Romanogobio vladykovi	6 Hungary
	lpoly02	47,932	18,771	Romanogobio vladykovi	6 Hungary
	Jrb2	46,930	16,154	Romanogobio vladykovi	6 Austria
	JRb4	46,930	16,154	Romanogobio vladykovi	6 Austria
	Kisk01	47,485	20,515	Romanogobio vladykovi	6 Hungary
	KOM04	47,748	18,131	Romanogobio vladykovi	6 Hungary
	M5	46,374	16,817	Romanogobio vladykovi	6 Hungary
	O2	46,374	16,817	Romanogobio vladykovi	6 Hungary
	O5	46,374	16,817	Romanogobio vladykovi	6 Hungary
	OR2	45,239	17,780	Romanogobio vladykovi	6 Croatia
	Pin08	47,036	16,473	Romanogobio vladykovi	6 Hungary
	ROM01	48,107	22,831	Romanogobio vladykovi	6 Hungary
	ROM06	48,107	22,831	Romanogobio vladykovi	6 Hungary
	RomVlad05	46,977	17,024	Romanogobio vladykovi	6 Hungary
ANGBF53158-19	MG751104	48,350	16,844	Romanogobio vladykovi	6 Austria
BayFi12097	BayFi12097	46,990	16,193	Romanogobio vladykovi	6 Austria

BayFi12099	BayFi12099	46,990	16,193	Romanogobio vladykovi	6 Austria
BayFi12100	BayFi12100	46,990	16,193	Romanogobio vladykovi	6 Austria
BCAFL032-17	MG786157	46,805	15,588	Romanogobio vladykovi	6 Austria
BCAFL073-17	MG786148	48,189	14,716	Romanogobio vladykovi	6 Austria
BCAFL075-17	MG786175	48,507	13,731	Romanogobio vladykovi	6 Austria
BCAFL080-17	MG786167	48,507	13,731	Romanogobio vladykovi	6 Austria
BCAFL087-17	MG786166	46,805	15,588	Romanogobio vladykovi	6 Austria
BCAFL130-17	-	-		Romanogobio vladykovi	6 Austria
BCAFL161-17	MG786149	46,767	15,889	Romanogobio vladykovi	6 Austria
BCAFL162-17	MG786144	46,767	15,889	Romanogobio vladykovi	6 Austria
BCAFL164-17	MG786179	46,767	15,889	Romanogobio vladykovi	6 Austria
BCAFL196-17	ON097528	46,707	15,919	Romanogobio vladykovi	6 Austria
BCAFL200-17	ON097564	46,707	15,919	Romanogobio vladykovi	6 Austria
BCAFL303-18	MG786170	48,507	13,731	Romanogobio vladykovi	6 Austria
BCAFL304-18	MG786174	48,179	16,483	Romanogobio vladykovi	6 Austria
BCAFL558-20	ON097446	46,707	15,919	Romanogobio vladykovi	6 Austria
BCAFL568-20	ON097349	46,707	15,919	Romanogobio vladykovi	6 Austria
BCAFL575-20	ON097841	46,684	15,989	Romanogobio vladykovi	6 Austria
BCAFL576-20	ON097350	46,684	15,989	Romanogobio vladykovi	6 Austria
BCAFL581-20	ON097648	46,684	15,989	Romanogobio vladykovi	6 Austria
BCAFL658-20	ON097367	46,692	15,990	Romanogobio vladykovi	6 Austria
FBPIS-125-10	HM902136 (suppressed)	48,310	14,131	Romanogobio vladykovi	6 Germany
FBPIS206-10	HM392084 (suppressed)	-	-	Romanogobio vladykovi	6 Germany
FBPIS207-10	HM392085	48,510	13,720	Romanogobio vladykovi	6 Germany
FBPIS214-10	HM392092	48,916	12,427	Romanogobio vladykovi	6 Germany
FBPIS215-10	HM392093	48,900	11,840	Romanogobio vladykovi	6 Germany
FBPIS218-10	HM392096	48,037	12,080	Romanogobio vladykovi	6 Germany
FBPIS517-11	-	48,417	13,334	Romanogobio vladykovi	6 Germany

IFCZE093-10	HQ960505		48,647	16,970	Romanogobio vladykovi	6	Czech Republic
IFCZE094-10	HQ960506		48,647	16,970	Romanogobio vladykovi	6	Czech Republic
IFCZE095-10	HQ960507		48,647	16,970	Romanogobio vladykovi	6	Czech Republic
IFCZE101-10	HQ960513		49,277	17,452	Romanogobio vladykovi	6	Czech Republic
IFCZE102-10	HQ960514		49,277	17,452	Romanogobio vladykovi	6	Czech Republic
IFCZE603-10	HQ960996		48,741	17,027	Romanogobio vladykovi	6	Romania
RYBY062-21		JFDNA1029	45,117	29,500	Romanogobio vladykovi	6	Slovakia
		RomVlad07	48,493	16,969	Romanogobio vladykovi	7	Hungary
		FV05	48,317	22,248	Romanogobio vladykovi	8	Hungary
		M1	46,844	16,870	Romanogobio vladykovi	8	Hungary
		M2	46,374	16,817	Romanogobio vladykovi	8	Hungary
		RomPog	46,374	16,817	Romanogobio vladykovi	8	Hungary
		RomVlad04	46,753	17,567	Romanogobio vladykovi	8	Hungary
		RomVlad06	46,977	17,024	Romanogobio vladykovi	8	Hungary
		Halv 01	46,977	17,024	Romanogobio vladykovi	8	Hungary
		Ex73E1	46,917	17,894	Romanogobio vladykovi	9	Hungary
		HB06	46,091	14,695	Romanogobio vladykovi	10	Slovenia
		Ex70A9	46,178	18,918	Romanogobio vladykovi	10	Hungary
		Ex66C7	47,699	26,460	Romanogobio vladykovi	11	Romania
		Ex66C8	47,699	26,460	Romanogobio vladykovi	12	Romania
		Ex70A8	47,699	26,460	Romanogobio vladykovi	12	Romania
		KOM03	47,748	18,131	Romanogobio vladykovi	12	Hungary

FBPIS216-10	HM392094	Ex68D5	45,802	24,353	Romanogobio vladykovi	13	Romania
		HKÖR01	46,891	20,494	Romanogobio vladykovi	13	Hungary
		Ex31H5	44,367	27,900	Romanogobio vladykovi	14	Romania
FBPIS210-10	HM392088	GL2	48,430	13,334	Romanogobio vladykovi	15	Germany
		DJ05	45,359	16,099	Romanogobio vladykovi	16	Croatia
		KOM05	49,197	12,297	Romanogobio vladykovi	16	Germany
BCAFL081-17	MG786155	CC1	46,960	18,955	Romanogobio vladykovi	17	Hungary
		CC5	47,748	18,131	Romanogobio vladykovi	17	Hungary
		DJ09	48,507	13,731	Romanogobio vladykovi	17	Austria
		HKÖR02	45,359	16,099	Romanogobio vladykovi	18	Croatia
		cc3	47,748	18,131	Romanogobio vladykovi	18	Hungary
		Mur01	45,749	16,598	Romanogobio vladykovi	19	Croatia
		M4	45,749	16,598	Romanogobio vladykovi	19	Croatia
		Kisk02	46,960	18,956	Romanogobio vladykovi	19	Hungary
		HB10	46,891	20,494	Romanogobio vladykovi	19	Hungary
		HB07	45,749	16,598	Romanogobio vladykovi	20	Croatia
BCAFL198-17	ON097291	Halv 03	46,707	15,919	Romanogobio vladykovi	20	Austria
BCAFL203-17	ON097881	Göd11	46,707	15,919	Romanogobio vladykovi	20	Austria
BCAFL579-20	ON097773	Göd10	46,684	15,989	Romanogobio vladykovi	20	Austria
		Göd08	46,374	16,817	Romanogobio vladykovi	21	Hungary
			46,374	16,817	Romanogobio vladykovi	22	Hungary
			47,485	20,515	Romanogobio vladykovi	23	Hungary
			46,178	18,918	Romanogobio vladykovi	24	Hungary
			46,178	18,918	Romanogobio vladykovi	25	Hungary
			46,917	17,894	Romanogobio vladykovi	26	Hungary
			47,691	19,127	Romanogobio vladykovi	27	Hungary
			47,691	19,127	Romanogobio vladykovi	28	Hungary
			47,691	19,127	Romanogobio vladykovi	29	Hungary

BCAFL648-20	ON097447	Göd03	47,691	19,127	Romanogobio vladykovi	30	Hungary
			46,678	16,002	Romanogobio vladykovi	30	Austria
		FV01	46,844	16,870	Romanogobio vladykovi	31	Hungary
		FV03	46,844	16,870	Romanogobio vladykovi	31	Hungary
		FV02	46,844	16,870	Romanogobio vladykovi	32	Hungary
		DSZ10	47,329	18,942	Romanogobio vladykovi	33	Hungary
		DSZ07	47,329	18,942	Romanogobio vladykovi	34	Hungary
		DSZ01	47,329	18,942	Romanogobio vladykovi	35	Hungary
		BRZ ROM01	45,441	21,454	Romanogobio vladykovi	36	Romania
		Bod05	48,296	20,740	Romanogobio vladykovi	37	Hungary
		Béga 01	45,764	21,732	Romanogobio vladykovi	38	Romania
BCAFL202-17	ON097627	Be06	47,745	20,093	Romanogobio vladykovi	39	Hungary
			46,707	15,919	Romanogobio vladykovi	39	Austria
BCAFL201-17	ON097593		46,707	15,919	Romanogobio vladykovi	40	Austria
