Polyphyly of the *Niphargus stygius* species group (Crustacea, Amphipoda, Niphargidae) in the Southern Limestone Alps

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ABSTRACT

The *Niphargus stygius* species complex is a groundwater group of large-sized, sexually dimorphic species inhabiting mainly caves and, less frequently, wells and springs. According to the taxonomists of the last century, this species complex was supposed to be present in the whole Southern Limestone Alps of Italy as well as in peninsular Italy and Slovenia. Considering the large presumed distribution area, we tested the contrasting hypotheses of monophyly versus paraphyly of this subterranean species complex, taking in account the presence of putative cryptic species. For this reason, we sampled the type localities of all the described species in the complex present in the Italian Southern Limestone Alps and neighbouring areas, and used nuclear (28S, ITS region) and mtDNA (COI) sequences to assess their phylogenetic relationships and species richness. Phylogenetic analysis confirmed that the *Niphargus stygius* complex in the Southern Limestone Alps is not monophyletic but comprises an eastern clade (the *N. kenki* clade, present in NW Italy, northern Slovenia, and southern Austria) and two western clades (the *N. brixianus* and *N. montellianus* clades), _These two clades are not closely related to the eastern one but rather form a monophyletic group together with a widely distributed Apennine clade (*N. speziae* clade). None of these clades is closely related to typical *N. stygius*. Three different molecular species delimitation methods applied to COI and ITS recognized different number of putative species, richer for the COI marker, suggesting that each clade is a species complex in itself. Bayesian time-calibrated phylogeny revealed that most clades began to split up during Miocene and Pliocene, ruling out the effect of Pleistocene glaciations in explaining their evolutionary history and justifying the presence of several putative cryptic species.

INTRODUCTION

With more than 425 species described (Horton et al., 2021), Niphargidae are the most species-rich taxon of freshwater amphipods in the world (Väinölä et al., 2008). The family Niphargidae is

distributed in the Western Palearctic (Borko et al., 2021), including most of Europe, mainly – but not exclusively – in groundwaters south of the Pleistocene ice sheet boundary (McInerney et al. 2013; Zagmajster et al., 2014). The genus *Niphargus* was described by Schiödte (1849), and *Niphargus stygius* (Schiödte, 1847) was designated as its type species (type locality: Postojna caves in Slovenia). The taxonomists of the last century considerd this species as quite widespread. In the pioneering paper by D'Ancona (1942), most of the known species and subspecies described during the first half of the last century for the genus *Niphargus* were considered as synonyms of *N. stygius*. However, despite his exhaustive morphological study of minute morphocharacters, the conclusions of D'Ancona's (1942) monograph were considered erroneous in the following years when taxonomists identified several distinct morphotypes within the genus *Niphargus* and assigned species to subgenera or 'species groups' (Karaman S., 1952; Straškraba 1972). Unfortunately, the high number of species, the unknown variability of the morphocharacters used for species identification, and the varying quality of their descriptions scattered over several local journals (Fišer et al., 2009a), i.e., 'grey literature', prevented taxonomists from achieving satisfactory results.

The most important contribution to the assessment of 'species groups' within niphargids was the one by Straškraba (1972), reported in conclusive paper concluding the proceeding of the "1er Colloque International sur le genre *Niphargus*" held in Verona in 1972. In this article, the genus *Niphargus* s. str. was divided into 13 'species groups' plus a fourteenth group comprising 10 species 'incertae sedis'. The '*stygius*-group'encompassed several species from Slovenia and northeastern Italy, while the Apenninic species were allocated, following the suggestion of another contribution published in the same proceedings (Vigna Taglianti, 1972), in the '*speziae-romuleus-tatrensis'* group, including also the *Niphargus tatrensis* species complex distributed in central and eastern Europe. Later, following the recommendations of Straškraba (1972), the species was redescribed by Sket (1974) as the representative of its own 'group'. This picture was turned over by G. Karaman (1993), who, in his monograph on the fauna of Italy, reassigned all the different species and subspecies of the *'speziae-romuleus'* group to the single species *Niphargus stygius*. These conclusions were criticized by Stoch (1998), who considered the species present in the Southern Limestone Alps in Italy as different, albeit belonging to the well-established *'N. stygius* group'.

Recently, molecular and morphological systematic studies suggested that the classification proposed by Straškraba (1972) was not justified from a phylogenetic perspective (Trontelj et al. 2009; Fišer et al. 2009b). As regards *N. stygius*, Delić et al. (2017) used molecular taxonomy to revise the populations present in Slovenia and northwestern Croatia where S. Karaman (1952) had subdivided *N. stygius* into seven subspecies. Using uni- and multilocus delineation methods, Delić et al. (2017) showed that the group in the northern Balkan area was not monophyletic but consisted of 15 parapatric

and sympatric cryptic species, which were described using molecular diagnoses. Later, Stoch et al. (2020) revised the *N. tatrensis* species complex, demonstrating that there was no relationship between this old, monophyletic lineage and neither typical *N. stygius* nor *N. speziae* and *N. romuleus* from the Apennines, thereby rejecting the hypothesis of Vigna Taglianti (1972). Finally, *Niphargus stygius* itself was considered as a species complex (Delić et al., 2021), and at least four different lineages were recognized even within the small distribution area of this species encompassing the karstic areas of northeastern Italy and western Slovenia. No other paper has addressed the molecular taxonomy of Italian pre-Alpine species attributed to the *Niphargus stygius* group so far.

The lack of phylogenetic knowledge for wide areas as regards groundwater niphargids and the presence of cryptic and pseudocryptic species are the worst-case scenario of morphotaxonomy's inadequacy to describe biodiversity. This is not only an exclusively taxonomic problem; no ecological inforamtion on these cryptic species is in most cases available, raising conservation issues (Delić et al., 2017).

Given these premises, we present herein a molecular phylogeny of the various species inhabiting the Southern Limestone Alps in Italy and neighbouring areas of Austria and Slovenia, traditionally attributed to the *Niphargus stygius* species complex (Stoch, 1998). The studied group includes morphologically similar populations distributed from Lake Como along the Southern Limestone Alpine chain to western Slovenia and southern Austria. Our study has three major aims. First, to reconstruct the phylogeny of this 'western species group' of the *Niphargus stygius* complex so that we can test the hypothesis that it constitutes a monophyletic evolutionary unit and assess its relationships with *N. stygius* as well as morphotaxonomically similar clades. Second, to test the conjecture that the species complex comprises multiple cryptic species in this study area and apply molecular species delimitation methods to identify them. Third, to reconstruct a time-calibrated phylogeny to shed light on the origin and separation of the different species complexes in the area.

MATERIAL AND METHODS

Sampling design

Samples were collected from 129 sites (caves, wells, and springs) throughout the wide range of the so-called *Niphargus stygius* species complex as defined by Karaman (1993) and Stoch (1998) in Italy and southern Austria, with a focus on the Southern Limestone Alps where 54 sites (mainly caves) were sampled (Fig. 1 and Tab. S1). Despite their wide distribution, many species occur at very low densities exclusively in caves, where sampling of groundwater fauna is logistically demanding (Fišer & Zagmajster, 2009; Pipan & Culver, 2007), requiring a proper equipment and a good knowledge of

progression in caves using ropes and specialized techniques. All the type localities of the different species and subspecies described from Italy were sampled to build a complete reference library for taxonomy and barcoding.

Cave-dwelling specimens were collected by one of the authors (F.S.), as well as by several speleologists (mentioned in the acknowledgements). In caves, most of the species are present in pools fed by percolating waters as well as in small siphons and subterranean lakes; in some cases (like Grotta della Bigonda, Trentino) the access to the cave needed to empty the entrance siphon using an electric pump. In few cases, specimens were present in deep lakes in quite complex karstic systems reaching the saturated zone and were collected by speleodivers. Sampling was carried out using hand nets, and by positioning baited traps (bottles with chicken liver) in siphons and small lakes overnight. Spring specimens were hand-netted, or drift nets were positioned for a few hours at the spring outlet. Finally, artificial wells were sampled either using Cvetkov nets (Cvetkov, 1968) or by positioning baited traps at the bottom of the wells. The samples are deposited in the collection of the Evolutionary Biology & Ecology unit of the Université libre de Bruxelles (ULB), Belgium, stored in freezers at -20°C. Remaining samples used for morphotaxonomical analyses are stored in the first author's personal collection and in the Natural History Museum of Verona, Italy. All the information on the specimens used in the analyses, georeferenced collection sites and DNA vouchers are available in Supplementary Information, Table S1.

Additional sequences were downloaded from GenBank, where results of previous studies on the *Niphargus stygius* complex in Slovenia (Delić et al. 2017, 2021) and of the alleged similar *Niphargus tatrensis* complex in Austria and Eastern European countries (Stoch et al. 2020) are deposited. Moreover, all the sequences reported in Borko et al. (2021, supplementary material) obtained from 385 niphargid and pseudoniphargid species, complemented by those reported by Weber et al. (2021), were downloaded.

Phylogenetic and species delimitation analyses

One or two pereopods of each specimen (sometimes more if specimens were juveniles) were used for DNA extraction, and the remaining parts of each specimen were stored in 96% EtOH at -20°C at ULB. Extraction of genomic DNA was performed using the NucleoSpin@ Tissue kit by Macherey-Nagel, following the manufacturer's protocol. The eluted DNA was stored at 4°C until amplification, then long-term stored at -20°C. The following markers were PCR-amplified: (1) a fragment (between 987 and 993 bp long) of the nuclear 28S rRNA gene (Verovnik's fragment: external primers of Verovnik et al. 2005 complemented with internal primers of Flot et al. 2010) : (ii) a 658 bp fragment (Folmer's fragment: Folmer et al. 1994, using the primers of Astrin & Stüben 2008) of the

mitochondrial cytochrome *c* oxidase subunit I (COI); (3) the complete internal transcribed spacer (ITS) region (together with flanking portions of the 18S and 28S genes and including 5.8S) using the six primers described in Flot et al. (2010). A list of primers and PCR amplification protocols used in this study is available in Tab. S2. Direct sequencing was performed using the same primers as for amplification as well as internal primers (Flot et al. 2010); 28S and COI PCR products were sent for bidirectional Sanger sequencing to Genoscreen (Lille, France), while ITS products were sent to Macrogen Europe (Amsterdam, The Netherlands).

Chromatograms were inspected, assembled, and cleaned using the program Sequencher 5.4.6 (Gene Codes). Some 28S and ITS chromatograms contained double peaks, as expected in the case of length-variant heterozygotes (Flot et al. 2006); these individuals were phased using the web tool Champuru (Flot 2007, available online at https://eeg-ebe.github.io/Champuru).

The position of the *Niphargus stygius* - like species complexes of the Southern Limestone Alps and of the other species groups cited in the taxonomic literature as related to these populations (*Niphargus stygius* group and *Niphargus speziae* group) within the phylogenetic tree of Niphargidae were inferred by comparison with 219 other niphargid species using as outgroups the family Pseudoniphargidae (genera *Microniphargus* and *Pseudoniphargus*), which was assessed as the sister group of Niphargidae in our previous study (Weber et al., 2021). For this phylogenetic analysis, we screened the molecular dataset assembled by Borko et al. (2021), including 385 species sequenced for two 28S fragments (28S-I corresponding to our 28S fragment, and 28S-II), Folmer's COI fragment, the histone H3 gene, a fragment of the 18S rRNA gene, as well as partial sequences of the phosphoenolpyruvate carboxykinase (PEPCK), glutamyl-prolyl-tRNA synthetase (EPRS), heat shock protein 70 (HSP70), and arginine kinase (ArgKin) genes. Considering that (i) the dataset included several species represented only by mitochondrial COI gene sequences and (ii) to account for cryptic species, the authors relied on delimitations using a rather conservative delimitation approach (a 16% patristic distance treshold; Léfebure et al. 2006), we revised and reduced the dataset as follows: we retained only complete (or almost complete) sequences (when these sequences were related to those of the target clades, we performed the phylogenetic analyses before and after removing them, to check for any important loss of information); likewise, two markers (PEPCK and HSP70) were not used, considering they did not change the results of our analysis. All in all, 272 species were retained in the analysis (including our newly obtained sequences) (see Tab. S1).

All sequences were aligned for each marker using the E-INS-i algorithm implemented in MAFFT 7 (Katoh and Standley 2013), except COI and H3 markers that were aligned manually) and the optimal substitution model was selected for each marker using ModelFinder (Kalyaanamoorthy et al. 2017, implemented in the IQ-TREE 2 software package) according to the Bayesian Information Criterion

(Schwarz 1978). Phylogenetic relationships were reconstructed on the concatenated dataset using maximum likelihood and 1,000 ultrafast bootstrap replicates in IQ-TREE 1.6.12 (Nguyen et al. 2015); partitions were not used, considering that the same optimal substitution model (GRT+G+I) was selected for every marker.

Considering that some markers were missing in our dataset, and the addition of distantly related clades was impractical for our purposes given the complexity of the global phylogenetic tree, a subset of the COI and 28S sequences was used to build a ML tree using IQ-TREE 2 with the same modalities illustrated above. A recursive removal of single clades and poorly related species was obtained repeating the IQ-TREE analysis to check for loss of information. The reduced subset (Tab. S1) included all sequences of interest where the ITS region was carefully sequenced. A new dataset was built by concatenating the (partial)18S+ITS1+5.8S+ITS2+28S(partial) sequences. Phylogenetic trees were obtained for both the ITS region and the COI region to check for mitonuclear discordances. Considering that no discordance was detected, phylogenetic analyses were performed by concatenating the aligned rDNA long fragment with COI Folmer's fragment.

Apart the ML phylogenetic analysis, a time-calibrated Bayesian phylogeny was reconstructed on the same concatenated dataset in BEAST (Bayesian Evolutionary Analysis Sampling Trees) 2.6.1 (Bouckaert et al. 2019), following the best-fit model of evolution proposed by the bModelTest (Bouckaert and Drummond 2017) package. Substitution models and clock models were unlinked for the two partitions (ITSregion+28S-I and COI). Based on marginal likelihood (Path Sampler extension: Baele et al. 2016), a Yule speciation tree prior was used for the analyses. To account for lineage-specific rate heterogeneity, a lognormal relaxed clock (Drummond et al. 2006) was used. The coefficient of variation (CV) reported in Tracer 1.7 (Rambaut et al. 2018) employing the relaxed clock was higher than 0.1, suggesting that this clock fitted better the dataset than a strict clock (Drummond and Bouckaert 2015). Unfortunately, no fossil is known in the *Niphargus stygius* complex; for this reason, we used the calibrated phylogeny of the genus *Niphargus* assembled in a previous paper (reference). Each important split of our reduced dataset was calibrated as in Borko et al. (2021). A comparison of the results between the global tree and the reduced tree showed a negligible effect of missing data on the final phylogeny reconstruction. Four independent runs of 10,000,000 generations sampled every 1,000 steps were performed and combined using LogCombiner 2.6.1 included in the BEAST package. The stationarity of each single run was checked in Tracer 1.7 (Rambaut et al. 2018). The first 10% of the trees were discarded as burn-in and the remaining samples from the posterior distribution were summarized using TreeAnnotator in the maximum clade credibility tree.

Phylogenetic networks were built for both COI and ITS sequences using HaplowebMaker (Spöri & Flot 2020, available online at https://eeg-ebe.github.io/HaplowebMaker/).ASAP (Assemble Species by Automatic Partitioning: Puillandre et al. 2021) performed using the webserver (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with p-distances (with p<0,01), as well as mPTP (the multi-rate version of the Poisson Tree Processes: Kapli et al. 2017) using the webserver (https://mptp.h-its.org/#/tree) were used as species delimitation methods both on COI and ITS sequences.

RESULTS

Phylogenetic analysis

Phylogenetic analyses confirmed that Straškraba's (1972) *Niphargus stygius* group in Italy and bordering regions (Fig. 1) is not monophyletic (Fig. S1). The detailed ML phylogenetic analysis reported in Fig. 2, confirmed by the subsequent Bayesian analysis (see below), demonstrates the polyphyly of the species complex; at least two large clades (Fig. 2) are recognized, and neither of the two is sister group of *Niphargus stygius* or *Niphargus tatrensis* species groups. However, there were some strongly supported clades (Fig. 2 and haplonetworks in Figs. 3, 4, 5): (i) the *Niphargus julius-N. kenki* species complex (defined herein as the *Niphargus kenki* clade), distributed in the western Alps, with a disjunct distribution area (Fig. 1) intercalated by the presence of *Niphargus stygius*; (ii) an Alpine-Apenninic large clade (Fig. 1), with a rather strong bootstrap support (97%), which is composed of three monophyletic units (100% support). The three clades were: (i) the *Niphargus brixianus* clade, distributed in the western part of the Southern Limestone Alps (Lombardy and Veneto/Trentino up to the western Lessinian massif); the *Niphargus costozzae* clade, distributed in Veneto and Friuli Venezia Giulia regions, with two distinct subclades; (iii) the *Niphargus speziae* clade, present in the Apennines, outside the area covered by our study. The *N. costozzae* clade is clearly separated in two strongly supported subclades: (i) the *Niphargus costozzae* s.str. subclade, encompassing in its distribution several karstic massifs in Veneto (eastern Lessinian mountains, Berici and Euganei hills, Grappa massif, Asiago massif); and the *Niphargus montellianus* subclade, present from the conglomeratic massifs of Montello and Asiago to the western Carnic Prealps.

Molecular species delimitation methods

ASAP applied to ITS gave a very conservative putative species estimate (5), failing in separating species within the above-mentioned clades and the two subclades, whereas applying it to COI yieled a higher putative species number (20), oversplitting the *N. brixianus* clade (see Tab. S3). mPTP yielded slighly more species than ABGD when applied to ITS (7), giving a geographically coherent

separation in the *N. brixianus* clade, while lumping all the species within the *N. kenki* clade (Tab 3); when applied to COI, 13 putative species were recognized..

Time-calibrated phylogeny

The polyphyly of the *Niphargus stygius* group in Italy is well illustrated by the time-calibrated phylogeny obtained using the Bayesian analysis (Fig. 6). Results were very similar, if not identical, to those reported by the ML phylogenetic tree. Both the origin and the most ancient splits between the main recognized clades took place in the upper Miocene, i.e., around 6-7 Ma (with a confidence interval around 2.5-3 Myr). However, the split of the *N. brixianus* - *costozzae* - *speziae* clades began around 13 Ma (range 10-16 Ma). The separation of most of the minor clades took place during the Pliocene; only the most recent splits followed the vicissitudes of the Pleistocene glaciations.

DISCUSSION

The *Niphargus stygius* group in Italy

The results illustrated above allow to answer at least partially to the main questions posed in the introduction. First, the so-called *Niphargus stygius* group in Italy, following the definitions by Straškraba (1972), Vigna Taglianti (1972), and later by Karaman G. (1993) and Stoch (1998), is polyphyletic. Moreover, as already shown in a previous paper (Stoch et al. 2020), the group postulated by Vigna Taglianti (1972) for the Apennine populations (the so-called *speziae-romuleus-tatrensis* group) has no phylogenetic value, being the *N. tatrensis* clade of ancient origin and completely unrelated to the Apennine clade. A similar polyphyly was demonstrated by Delič et al. (2017) for the northwestern Balkan populations (Slovenia and NW Croatia) of the *N. stygius* group. For these reasons, hypotheses based on the morphological similarities of these different species (see Stoch, 1998, for a complete iconography), mainly based on their large size (from 20 to 40 mm, the latter one reached by *N. costozzae*), large gnathopods and stout body shape, marked sexual dimorphism of the third uropod (elongated in males), and similar length of the two branches of the first male uropod (subequal as in female or only slightly different in length), have proved to be completely inadequate characters for establishing phylogenetic relationships between different species, possibly due to convergence or retention of ancestral traits (Fišer et al. 2008). The incorrect interpretation of the value of these morphocharacters lead Karaman G. (1993) to attribute all the Italian populations belonging to the above-mentioned clades to a single, quite variable, species, *N. stygius*. Based on our results, it can be stated that the species *Niphargus stygius* is present in Italy only in the Karst region of Trieste and Gorizia, and that its probable cryptic Alpine species (Delič et al. 2021) reaches the Julian Pre-

Alps (Tab. S1, Uccea Valley). All the numerous citations of *N. stygius* in caves on the Italian peninsula, especially in the 'grey' speleological literature, must therefore be considered incorrect.
Another species which should be deleted from the Italian fauna is *Niphargus forelii*, reported for Lake Tovel (Karaman G. & Ruffo 1993) and later for the Monte Baldo caves close to Garda Lake and the western Lessinian massif (Galassi et al., 2009). The specimens collected in these sites are included in the *Niphargus brixianus* clade. Finally, from a nomenclatorial point of view, the topotypes of *Niphargus brixianus* (Caja de Valmala, Brescia) and *N. lessiniensis* (grotta A del Ponte di Veja, Lessini Mountains, Verona) are very similar and belong to the same species, hence *N. lessiniensis* can be treated from now on as a junior synonym of *Niphargus brixianus*. The same applies to the populations reported as *N. tridentinus* in GenBank (Bus Pursì, Brescia), very close to this taxon, a probable misidentification (Sket, personal communication).

Phylogeny, biogeography, and species richness in the Southern Limestone Alps

While the eastern clade (*Niphargus kenki* clade) is quite problematic to allocate within the global phylogeny of the genus *Niphargus*, the other two alpine clades (*Niphargus brixianus* and *N. costozzae*) form a monophyletic group together with the Apennine species (*N. speziae* clade). As regards the origin of the western Alpine clades, the split from the Apennine clade was calculated around 13 Ma, when the Apennine orogenesis came to an end and the possibility of colonization of the newly formed chain was realized. The low support of the node marking the separation between the *N. brixianus* and *N. speziae* clades does not allow to infer that the colonization took place from the Alpine area, although it seems a very plausible hypothesis.
A quick look at the distribution map reported in the present paper (Fig. 1) clearly shows that almost all populations of these clades inhabiting the Southern Limestone Alps are located south of the southern edge of the area occupied by glaciers during the last Quaternary glaciation (LGM, around 21,000 ya), indicating that their evolutionary history is clearly pre-Pleistocenic. The only exception is represented by the population of Lake Tovel (north of Garda Lake), which can be considered as putative glacial relict. The reconstructed time-calibrated phylogeny strongly confirms this hypothesis, as well as the monophyly of different small clades.
The results of the unilocus species delimitation methods applied in the present study (ASAP and mPTP) show a mitonuclear discordance in the number of putative species. Using the COI mitochondrial marker, estimates vary from 13 to 21, while using the nuclear rDNA ITS region, numbers are quite lower (5-7 species). This discordance is well known in niphargids (Stoch et al. 2020), especially in areas affected by glaciations. COI-based oversplitting may be quite common (Després 2019; Stoch et al. (2020) as a result of complex demographic history of lineage

divergence/fusion during the Pleistocene climatic fluctuations. Relying on the fact that most of the recent splits in the Austrian clade of the *Niphargus tatrensis* complex took place during the Pleistocene, Stoch et al. (2020) proposed to accept the species number suggested by the ITS region. However, within the paleogeographic scenario delineated in the Southern Limestone Alps, where most of the splits took place during Miocene and Pleistocene, it is legitimate to raise doubts about the validity of this argument. A possible mixture of different evolutionary events due to the advance and retirement of the glaciers during the several ice ages since the upper Pliocene may be the cause of the recent splits and isolation of some populations revealed by COI-based haplonets and species delimitation methods. However, the ancient origin of some clades supports the specific status of certain groups of populations occupying very fragmented and isolated karst areas. The large congruence between the time-calibrated phylogeny and the present geographical distribution suggests that these small clades of ancient origin may deserve specific status even if SDMs applied to the ITS region does not support this hypothesis. Moreover, the 'specific status' concept is quite a debatable argument. Recently, Delić et al. (2021) supported the idea that *Niphargus stygius* s. str. may be a complex of four cryptic species, originated during the last 1,5 My of glaciation events; ecological and physiological evidence, and the co-existence of different putative species in the same caves, supported this hypothesis. Further investigation using genome-wide information (for instance obtained from low-coverage whole-genome sequencing) appears required to test these contrasting hypotheses.

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CAPTIONS

Figure 1 - Map of the study area showing the distribution of the sampling sites of specimens of the *Niphargus stygius* group; species names are based on morphology and follow mainly Stoch (1998). The extent of karstic areas and of the glaciers during the Last Glacial Maximum (LGM) are reported as well.

Figure 2 - ML phylogenetic tree of the *Niphargus stygius* group using a concatenated set of markers (ITS region, 28S-I and COI). The *Niphargus tatrensis* clade was used as outgroup following the results of previous analyses (see Tab S1). Bootstrap values of very recent splits, that overlapped with species labels, were omitted for clarity (all either 99 or 100%)

Figure 3 - Median joining network of the COI sequences of the *Niphargus stygius* group obtained in the present study.

Figure 4 - Median joining network of the ITS sequences of the western clades (*Niphargus brixianus* and *N. costozzae* clades) obtained in the present study.

Figure 5 - Median joining network of the ITS sequences of the eastern clade (*Niphargus kenki* clade) obtained in the present study.

Figure 6 - Time-calibrated maximum clade credibility tree of the *Niphargus stygius* group derived from a BEAST analysis of concatenated ITS, 28S and COI sequences. Both the posterior probabilities of the nodes and the 95% confidence intervals of their ages are reported.

SUPPLEMENTARY MATERIAL

Figure S1 - Global maximum-likelihood phylogenetic tree of the genus *Niphargus* (using Pseudoniphargidae as outgroup) based on a concatenated dataset of 18S, 28S-I and II, H3, EPRS, and ArgKin genes.

Table S1 - List of all the sequences included in the concatenated ITS, 28S-I and COI trees used in the analysis of the *Niphargus stygius* group. Sampling sites and WGS84 decimal degree coordinates are reported as well. (NEW = GebBank accession numbers will be added upon acceptance of the manuscript).

Table S2 - List of primers used for amplification and sequencing and PCR amplification conditions.

Table S3 - Results of the molecular species delimitation analysis using ASAP and mPTP methods.

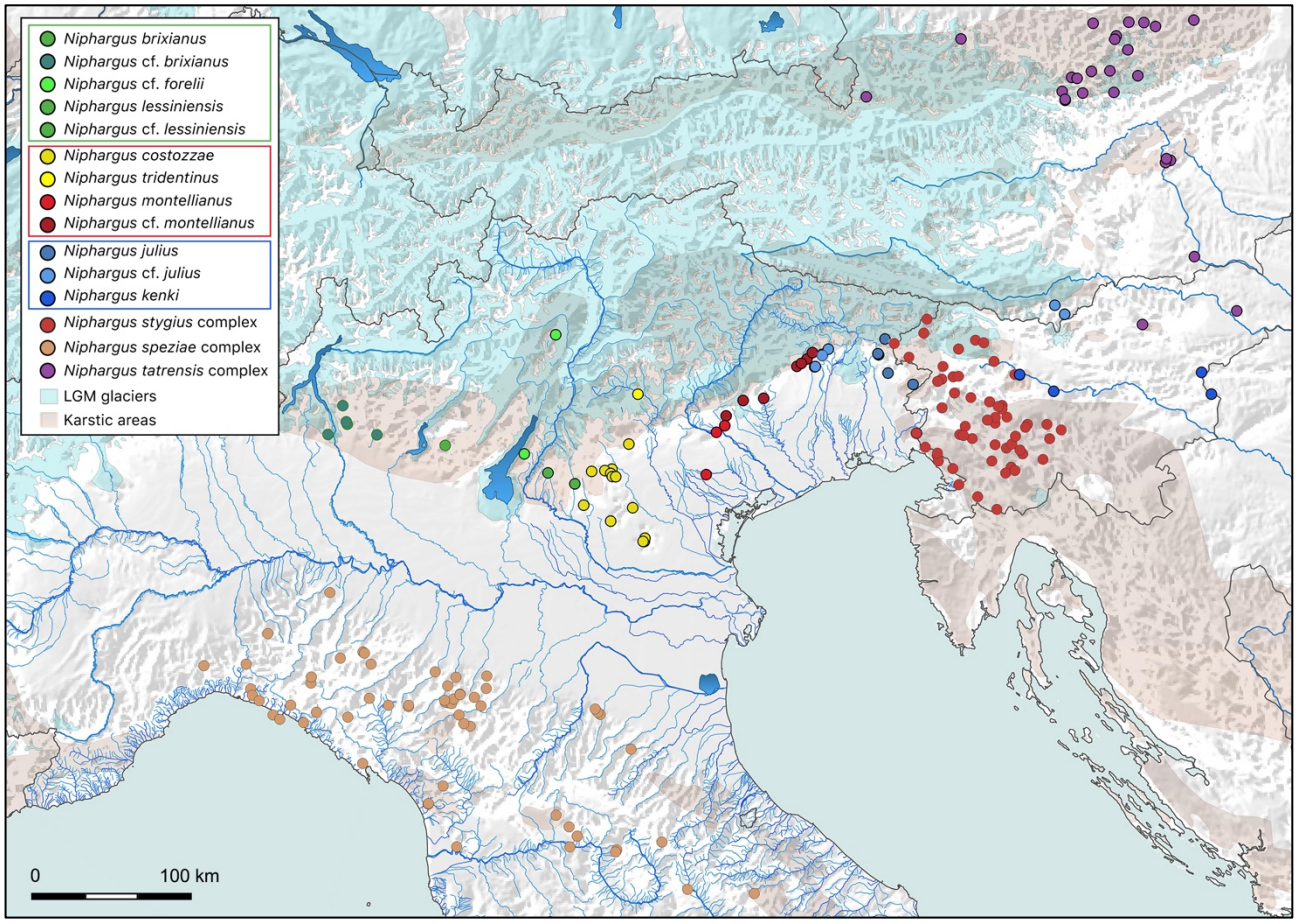

Niphargus brixianus
Niphargus cf. brixianus
Niphargus cf. forelii
Niphargus lessiniensis
Niphargus cf. lessiniensis
Niphargus costozzae
Niphargus tridentinus
Niphargus montellianus
Niphargus cf. montellianus
Niphargus julius
Niphargus cf. julius
Niphargus kenki
Niphargus stygius complex
Niphargus speziae complex
Niphargus tatrensis complex
LGM glaciers
Karstic areas
0
100 km

Figure 1

Niphargus montellianus (mm 32, topotype male)

Niphargus julius (mm 24, topotype male)

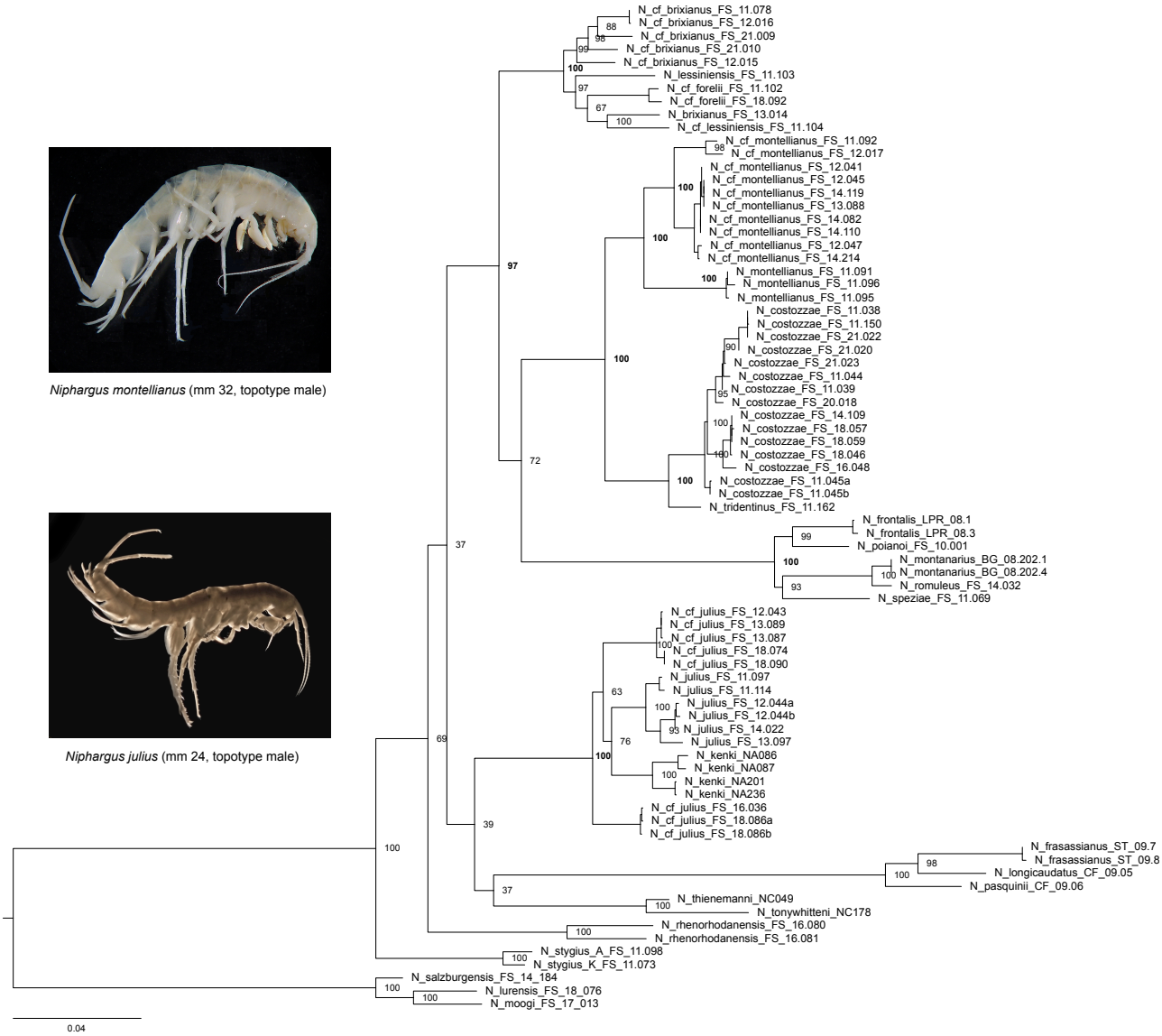
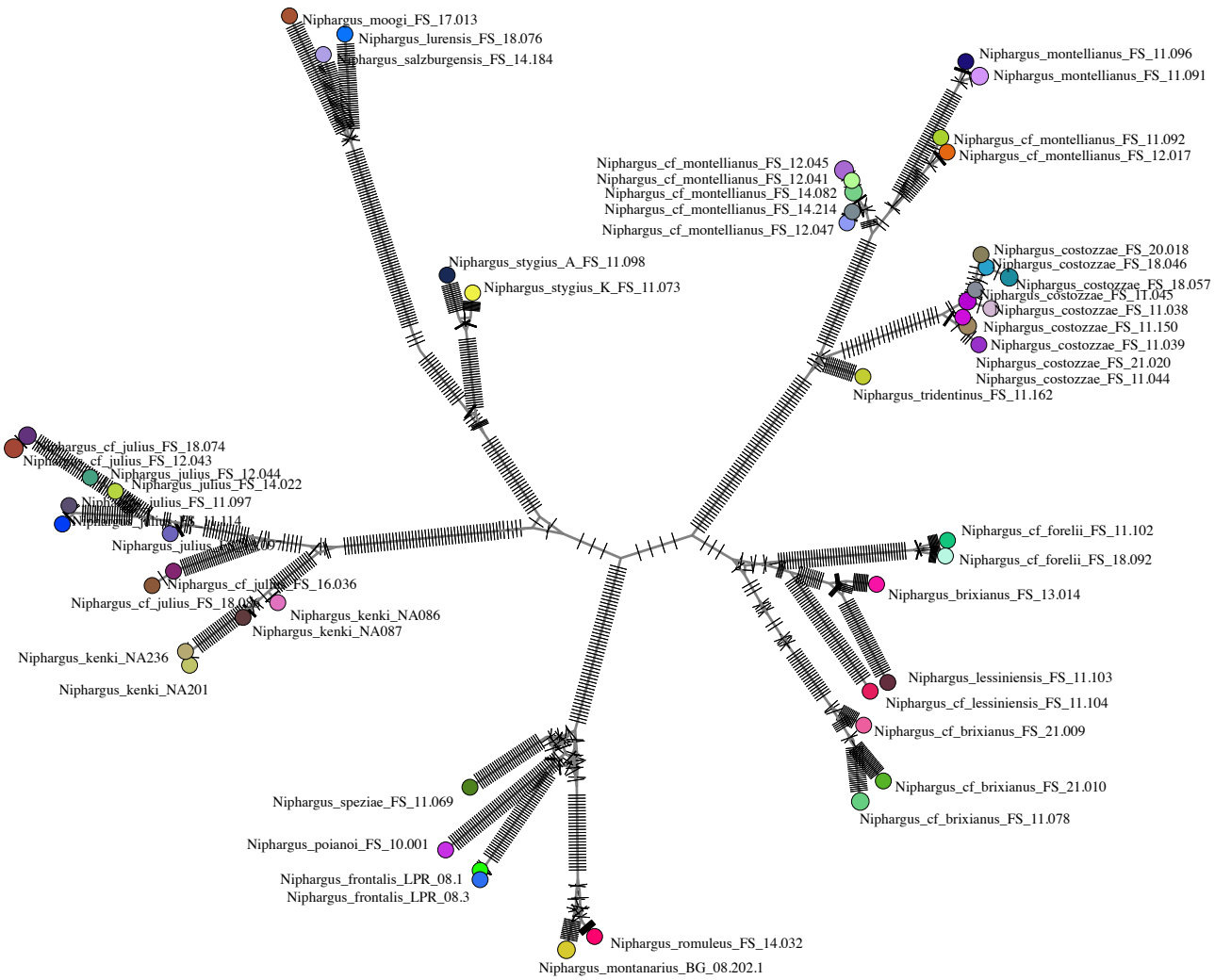


Figure 2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Niphargus_lurensis_FS_18.076
Niphargus_salzburgensis_FS_14.184
Niphargus_montellianus_FS_11.096
Niphargus_montellianus_FS_11.091
Niphargus_cf_montellianus_FS_11.092
Niphargus_cf_montellianus_FS_12.017
Niphargus_cf_montellianus_FS_12.045
Niphargus_cf_montellianus_FS_12.041
Niphargus_cf_montellianus_FS_14.082
Niphargus_cf_montellianus_FS_14.214
Niphargus_cf_montellianus_FS_12.047
Niphargus_stygius_A_FS_11.098
Niphargus_stygius_K_FS_11.073
Niphargus_costozzae_FS_20.018
Niphargus_costozzae_FS_18.046
Niphargus_costozzae_FS_18.057
Niphargus_costozzae_FS_11.045
Niphargus_costozzae_FS_11.038
Niphargus_costozzae_FS_11.150
Niphargus_costozzae_FS_11.039
Niphargus_costozzae_FS_21.020
Niphargus_costozzae_FS_11.044
Niphargus_tridentinus_FS_11.162
Niphargus_cf_julius_FS_18.074
Niphargus_cf_julius_FS_12.043
Niphargus_julius_FS_12.044
Niphargus_julius_FS_14.022
Niphargus_julius_FS_11.097
Niphargus_cf_julius_FS_16.036
Niphargus_kenki_NA086
Niphargus_kenki_NA087
Niphargus_kenki_NA236
Niphargus_kenki_NA201
Niphargus_cf_forelii_FS_11.102
Niphargus_cf_forelii_FS_18.092
Niphargus_brixianus_FS_13.014
Niphargus_lessiniensis_FS_11.103
Niphargus_cf_lessiniensis_FS_11.104
Niphargus_cf_brixianus_FS_21.009
Niphargus_cf_brixianus_FS_21.010
Niphargus_cf_brixianus_FS_11.078
Niphargus_speziae_FS_11.069
Niphargus_poianoi_FS_10.001
Niphargus_frontalis_LPR_08.1
Niphargus_frontalis_LPR_08.3
Niphargus_romuleus_FS_14.032
Niphargus_montanarius_BG_08.202.1

Figure 3

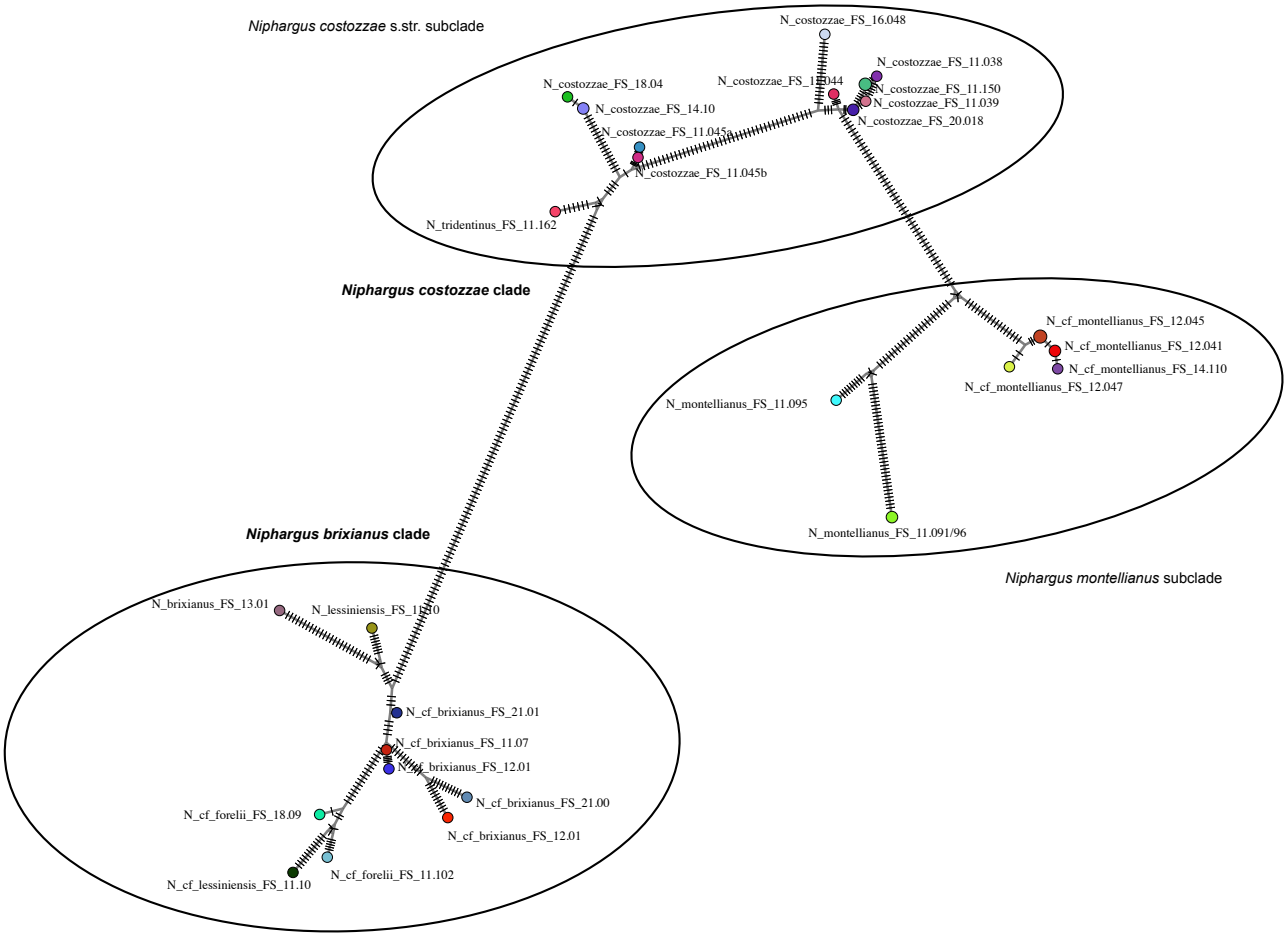


Figure 4

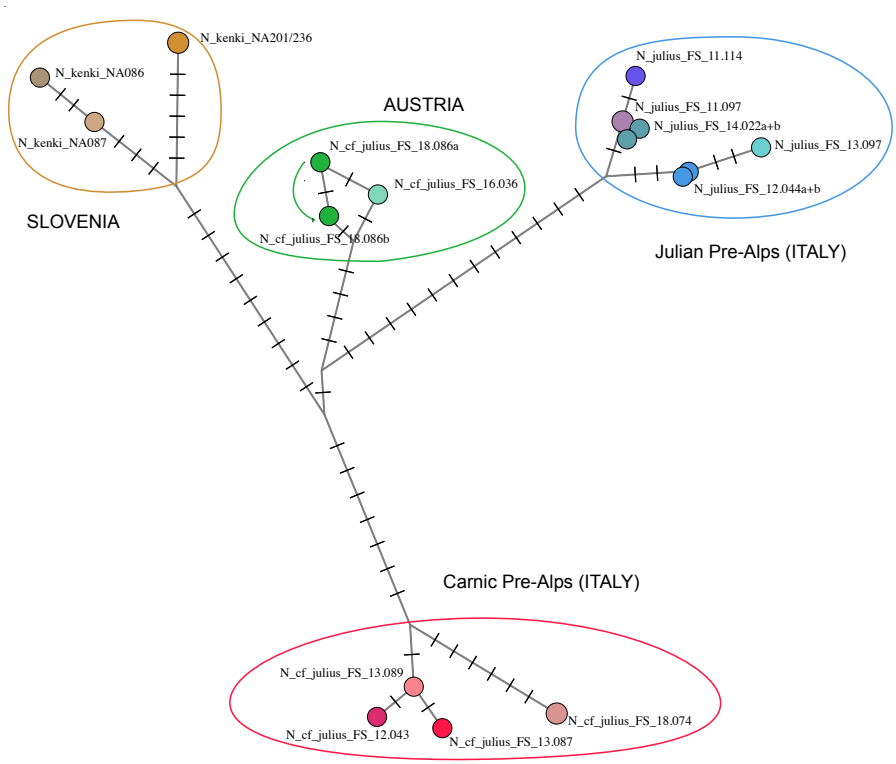
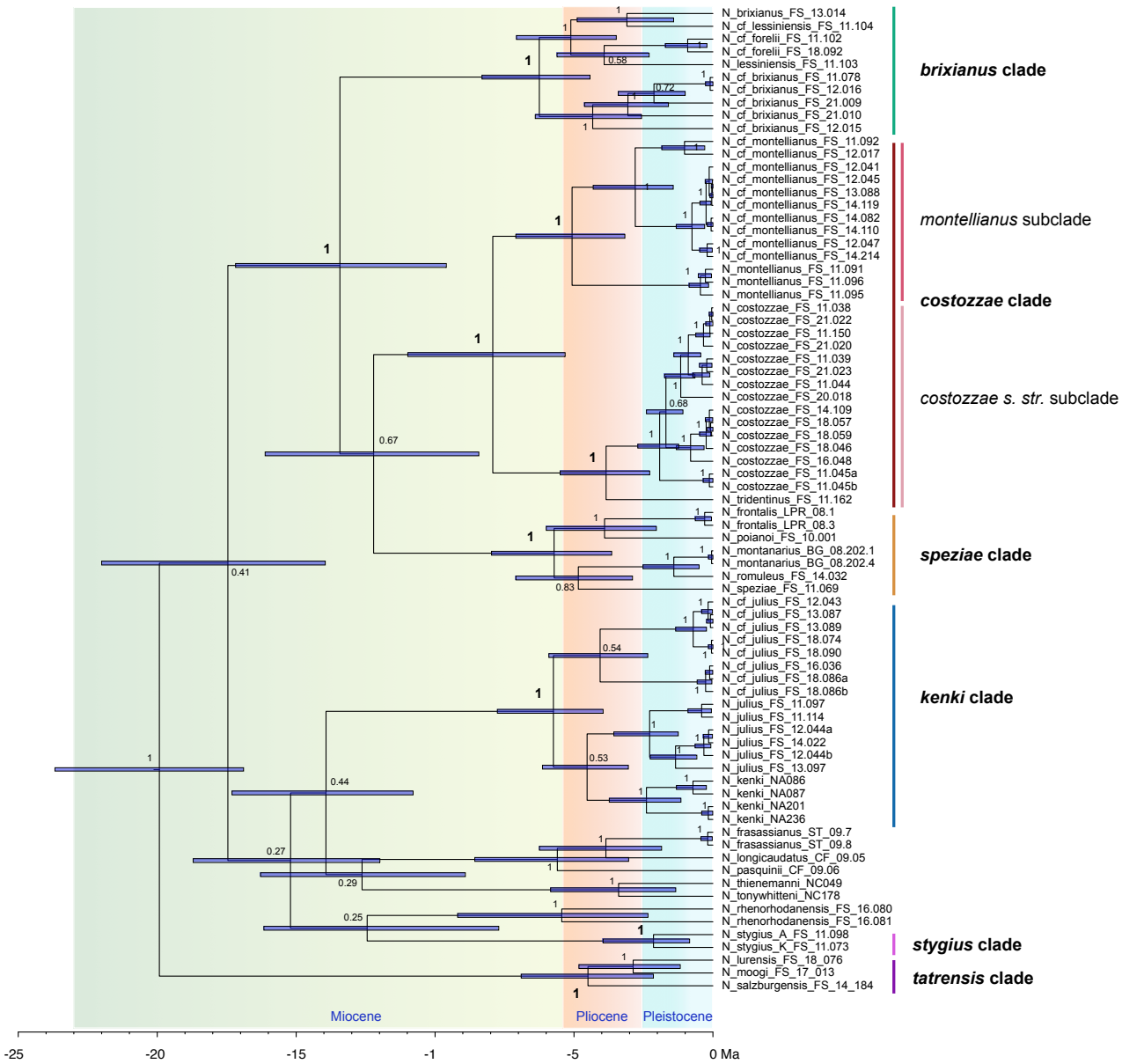


Figure 5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N_cf_forelii_FS_18.092
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N_cf_montellianus_FS_11.092
N_cf_montellianus_FS_12.017
N_cf_montellianus_FS_12.041
N_cf_montellianus_FS_12.045
N_cf_montellianus_FS_13.088
N_cf_montellianus_FS_14.119
N_cf_montellianus_FS_14.082
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N_cf_montellianus_FS_12.047
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N_montellianus_FS_11.096
N_montellianus_FS_11.095
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N_costozzae_FS_21.022
N_costozzae_FS_11.150
N_costozzae_FS_21.020
N_costozzae_FS_11.039
N_costozzae_FS_21.023
N_costozzae_FS_11.044
N_costozzae_FS_20.018
N_costozzae_FS_14.109
N_costozzae_FS_18.057
N_costozzae_FS_18.059
N_costozzae_FS_18.046
N_costozzae_FS_16.048
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N_costozzae_FS_11.045b
N_tridentinus_FS_11.162
N_frontalis_LPR_08.1
N_frontalis_LPR_08.3
N_poianoi_FS_10.001
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N_tonywhitteni_NC178
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N_rhenorhodanensis_FS_16.081
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N_stygius_K_FS_11.073
N_lurensis_FS_18_076
N_moogi_FS_17_013
N_salzburgensis_FS_14_184
brixianus clade
montellianus subclade
costozzae clade
costozzae s. str. subclade
speziae clade
kenki clade
stygius clade
tatrensis clade
Miocene
Pliocene
Pleistocene
-25
-20
-15
-1
-5
0 Ma

Figure 6