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Multiple old differentiation centres around the Alps and complex range expansion patterns in a semi-aquatic insect: the phylogeny and biogeography of the *Wormaldia occipitalis* species complex (Trichoptera)

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Abstract

The understanding of cryptic species complexes with their often highly interesting biogeographical patterns is still a crucial aspect in evolutionary biology and related disciplines. Trichoptera are a group of insects particularly rich in unresolved groups. One example is the *Wormaldia occipitalis* species complex in which morphological studies suggest remarkable patterns of differentiation. In order to determine genetic differentiation and phylogenetic structure, one mitochondrial (COI) and two nuclear markers (CAD, wingless) were analysed for the *W. occipitalis* species complex around the Alps and northwards to Germany. The morphology-defined differentiation pattern was also observed at the genetic level. The morphologically well distinguishable groups *W. occipitalis* and *W. subterranea* were identified as two genetically distant monophyletic groups with about 10 % genetic divergence of the mitochondrial marker. These two taxa likely split during the Mio-Pliocene transition. Genetic analyses revealed four subgroups within *W. occipitalis* and three within *W. subterranea*. Several possible postglacial dispersal and differentiation processes are proposed. Thereby, *W. occipitalis* from the western Alps and individuals of *W. subterranea* from the eastern Alps spread towards Central Europe after the Last Glacial Maximum. Today, both species groups are sympatric and partly syntopic in the recolonised area in western Germany but apparently allopatric in their centres of origin around the Alps. The high genetic differentiation, lack of detectable genetic evidence for hybridisation, their syntopic distribution and the morphological distinctness indicate that *W. occipitalis* and *W. subterranea* are two distinct species. The genetically determined subgroups might represent subspecies.

Keywords

Alps, caddisflies, extra-Mediterranean refugia, Last Glacial Maximum, mid-Pleistocene Transition, range dynamics, speciation

1. Introduction

Since their formation in the Cretaceous and Cenozoic (Pfiffner 2015), the Alps as a geographical part of Europe had and still have a great impact on the European flora and fauna. Especially since the Miocene, and even more since the Pleistocene, the Alps represent an important biogeographical element (Hewitt 2000; Hewitt 2004; Jiménez-Moreno et al. 2008). Geological changes and climatic oscillations during these time periods led to

range expansions and regressions of many species, that in turn are associated with diversification processes (Graf & Vitecek 2016; Hewitt 2000; Schmitt 2009). In addition, the Alps had great importance for providing extra-Mediterranean glacial refugia and were one origin for (re) colonisations to Central Europe (Engelhardt et al. 2011; Malicky 2006; Schmitt 2020; Schmitt & Varga 2012). In particular, cold-tolerant and (semi-)aquatic species have been found to survive glacial periods in extra-Mediterranean refugia within periglacial regions (Gum et al. 2005; Malicky 1983; Pauls et al. 2006; Schröder et al. 2021). For instance, Malicky (1983) already proposed Pleistocene survival of caddisflies in Central Europe. Due to his reasoning, these insects were able to survive glacial pe-

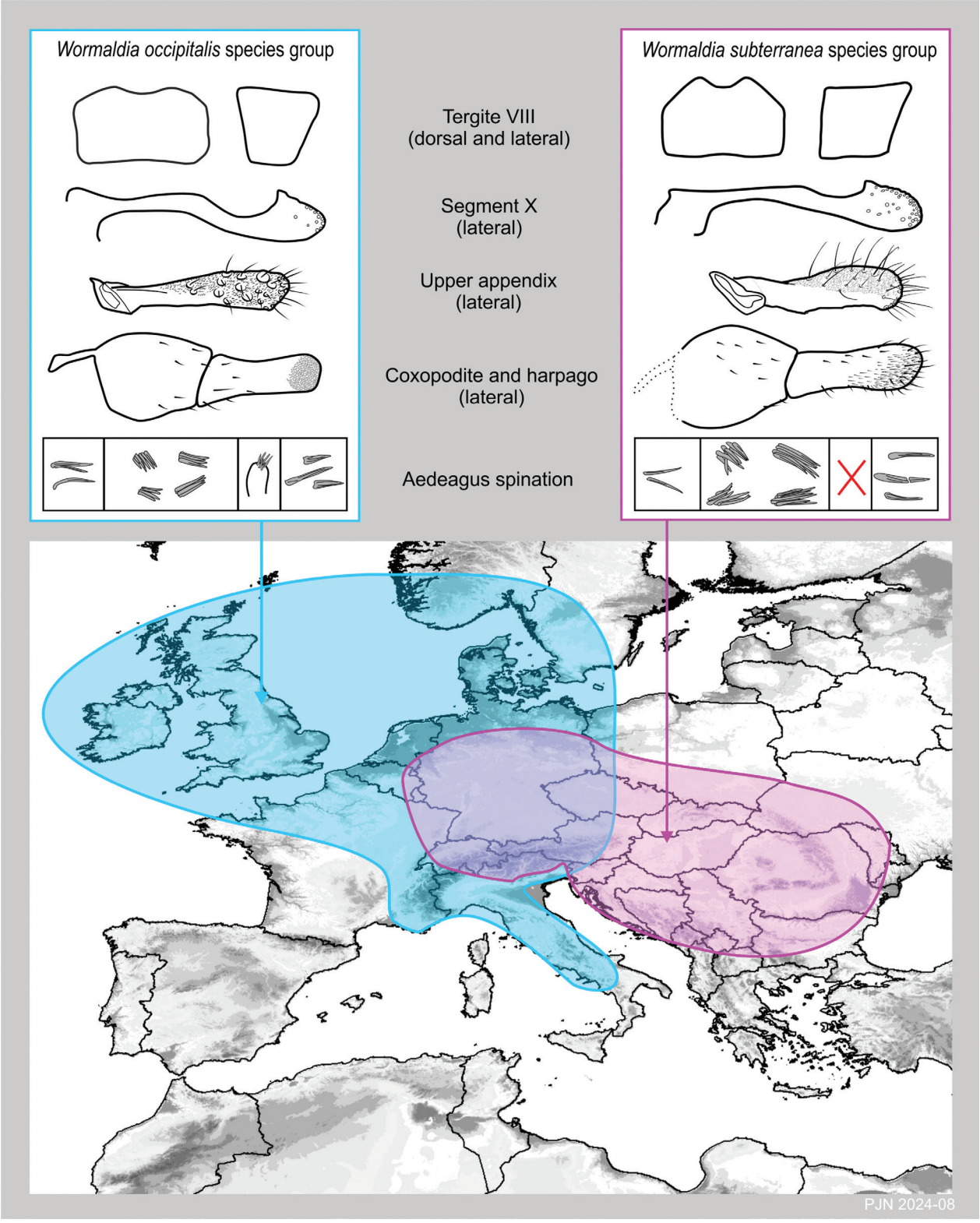

Figure 1. Distribution area and morphological differentiation characters of the male genitalia of the *Wormaldia occipitalis* (light blue) and *W. subterranea* (pink) species group.

riods in permanently flowing waters whose temperature never dropped far below 0 °C. Species that survived glacial cycles in these refuges, he called 'Dinodal' (Malicky 1983). Over time, dinodal species have been identified not only in Trichoptera (Engelhardt et al. 2011; Macher et al. 2015; Pauls et al. 2006) but also in other taxa (Gum et al. 2005; Schröder et al. 2021; Theissinger et al. 2013).

Genetic diversity within species is usually higher in populations that presently exist in the area of former refugia than in populations that occupy areas colonised during postglacial expansion (Petit et al. 2003; Schmitt & Varga 2012). In addition, species distributed in montane regions and on sky islands or species with a limited dispersal capability often have high intraspecific differentiation (Branch et al. 2017; Knowles 2001; Pauls et al. 2006). High intraspecific differentiation was also found in dinodal caddisflies (Engelhardt et al. 2011; Macher et al. 2015; Pauls et al. 2006). The reasons behind this could be the generally reduced dispersal ability of caddisflies (Curry & Baird 2015; Schröder et al. 2021) which takes place mainly during the adult stage (Collier & Smith 1997; Wichard & Wagner 2015). Furthermore, the adults often stay at their breeding waters after hatching and possess a short live span compared to other insects (Curry & Baird 2015; Neu 2015). All this can impact intraspecific diversity and promote differentiation (Delić et al. 2021; Knowles 2001).

High intraspecific diversity and differentiation are also assumed within the trichopteran *Wormaldia occipitalis* (Neu 2015; Oláh et al. 2019). Since the first description of the species in 1834 by François Jules Pictet, different morphological types and subspecies have been reported (Neu 2015). Accordingly, some studies interpreted *Wormaldia occipitals* (Pictet 1834) as a species complex, and morphological studies of the *W. occipitalis* species complex even suggest that the different groups/subspecies may represent independent species (Kimmins 1953; Neu 2015; Oláh et al. 2019). Within the *W. occipitalis* species complex, two species groups can be distinguished morphologically: *W. occipitalis* and *W. subterranea* (Radovanovic 1932), which also includes additional morphological subgroups (Neu 2015).

So far, only the male individuals of the *W. occipitalis* species complex can be differentiated morphologically; this is not yet possible for females and larvae. The two species groups *W. occipitalis* and *W. subterranea* can be differentiated in the male genitalia by the shape of tergite VIII and segment X, the shape of the upper appendages of segment IX and the spines of the aedeagus (Fig. 1; File S1 [Figs S1, S2]). In *W. occipitalis* tergite VIII is only slightly incised distally in dorsal view and rounded in lateral view. In addition, the upper appendix has a crater-like epithelial structure in lateral view and is cut off obliquely at the end. The distal end of segment X is short and rounded. In contrast, the distal margin of tergite VIII is deeply notched and thus forms two distinct tips in *W. subterranea*. The outer surface of the upper appendix is finely structured, and the appendage end is rounded. Furthermore, the distal end of segment X has a distally extended head end. In addition to morphology, statistical evaluations show clearly different peaks in flight times (File S1 [Fig. S3]) and overlapping but not congruent distribution areas of *W. occipitalis* and *W. subterranea* (Neu 2015; Neu et al. 2018).

In addition to these distinct groups, there are also individual groups who display characteristics of both *W. occipitalis* and *W. subterranea* (Neu 2015; Neu et al. 2018). While the subgroup *W. o. occipitalis* displays solely characteristics consistent with those given above for *W. occipitalis*, the morphologically described form *W. occipitalis* x *subterranea* shows intermediate characteristics of these two species groups in a balanced proportion. *W. occipitalis meridionalis* displays the majority of characteristics associated with *W. o. occipitalis*, while also exhibiting a few characteristics typically observed in *W. subterranea*.

In this context, we assume that (i) the differentiation within the species complex *W. occipitalis* also exists at the genetic level and that (ii) the species groups *W. occipitalis* / *subterranea*, which behave like parapatric species at the southern margin of the Alps, colonised Central Europe from the western and eastern Alpine edge, respectively. In addition, we hypothesise that (iii) the differentiation of the two taxonomic groups meeting in Central Europe is the strongest in the complex and that the species groups *W. occipitalis* and *W. subterranea* are two separated species. To examine the genetic differentiation and phylogeographic structure, two mitochondrial gene fragments (COI5-P, COI3-P) representing one gene (COI) and two nuclear genes (CAD, wingless) were analysed for representatives of the *W. occipitalis* / *subterranea* species group. Subsequently, several analyses including a haplotype network and Bayesian analyses were performed to test the above erected hypotheses.

2. Material and methods

2.1. Sampling and specimens

In total, 82 morphologically identified individuals of the *W. occipitalis* species complex (File S1 [Table S1]) were included in this study. Sequences for all four analysed gene fragments were available for 36 of these samples, mitochondrial information for 39 samples. All samples were collected from 1994 to 2021 in eight European countries (Fig. 2; File S1 [Table S1]). The samples were collected mostly by net sampling, occasionally with other methods such as light or malaise traps. Afterwards, the samples were temporary stored in 65 % ethanol until morphological determination and after that finally stored in 85 % ethanol at room temperature.

Morphological determination of the samples was mostly based on the morphology of the male genitalia. Some females could be determined by the co-caught males. Individuals for which no clear morphological determination to a group could be made were labelled as *Wormaldia* sp. Only adult individuals were included for this study.

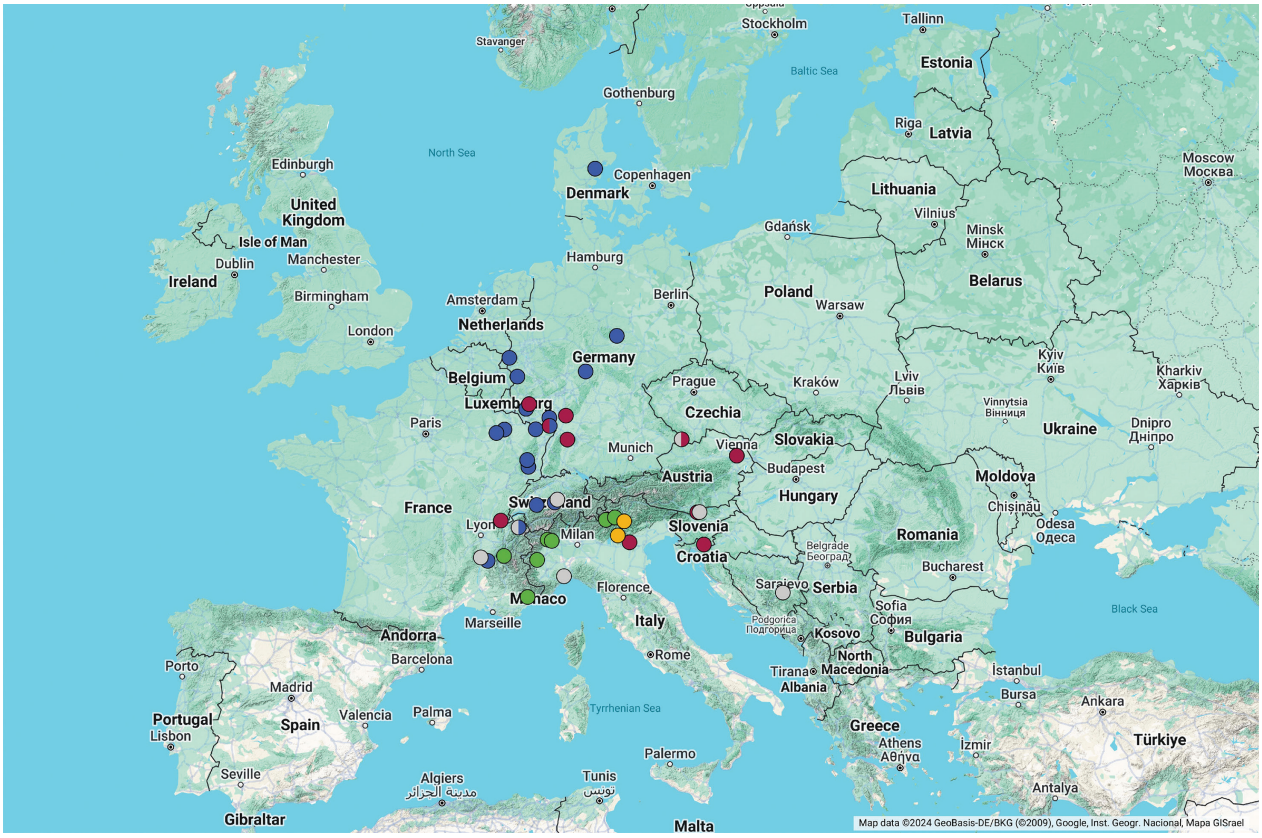

Figure 2. Map of the sampling localities of the *Wormaldia occipitalis* species complex. Individuals are colour-coded by their morphological determination. Blue – *W. occipitalis occipitalis*, green – *W. occipitalis meridionalis*, gold – *W. occipitalis* x *subterranea*, red – *W. subterranea*, grey – *W.* sp.

2.2. Molecular methods

DNA was first extracted from the whole body using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek) following the "DNA Extraction and Purification from Tissue" protocol with minor modifications (File S1 [Table S2]). In later samples, the abdomen was removed prior to extraction because of the potential risk of damaging determination-relevant body parts and the risk of contamination from the digestive system. DNA concentration for all samples was measured with fluorescence using the DeNovix Fluorescence Assay following the DeNovix dsDNA Broad Range protocol (File S1 [Table S1]).

Four gene fragments were amplified: two fragments of the mitochondrial gene cytochrome-c-oxidase subunit I, COI5-P (658 bp) and COI3-P (551 bp), and the nuclear genes wingless (wingless, 473 bp) and nuclear rRNA and carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD, 828 bp). The total volume of each PCR reaction was 15 µL. This contained 7.5 µL of Qiagen Master Mix, 1 µL (COI) to 1.5 µL (wingless and CAD) of primers, 2-4 µL of DNA and made up with water. The amplification of COI5-P was performed with the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) (Folmer et al. 1994) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994) or LepF1 (5'-ATTCAACCAATCATAAAGATATTGG-3') (Hebert et al. 2004) and LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') (Hebert et al. 2004) using the following PCR program: 5' 95°C, 38× (30" 95°C, 90" 49°C, 60" 72°C) 30' 68°C. COI3-P was amplified using the primers Jerry (5'-CAACATTTATTTTGATTTTTTGG-3′) (Simon et al. 1994) and S20 (5′-GGGAAAAAGGTTAAATTTACTCC-3′) (Pauls et al. 2003) and the following PCR program: 5' 95°C, 35× (60" 95°C, 90" 47°C, 90" 72°C) 30' 68°C. The primer Wingnut1a (5'-GAAATGCGNCARGARTGYAA-3') (Pauls et al. 2008) and Wingnut3 (5'-ACYTCRCARCACCARTGRAA-3') (Pauls et al. 2008) were used for the amplification of the nuclear gene wingless, 743nf-ino (5'-GGIGTIACIACIGCITGYTTYGARCC-3') (Johanson & Malm 2010) and 1028r-ino (5'-TTRTTIGGIARYTGICCICCCAT-3') (Johanson & Malm 2010) were used for CAD. The used PCR program of both nuclear gene fragments was 5' 95°C, 40× (45" 95°C, 90" 58°C-0,2°C/Cycle, 90" 72°C) 30' 68°C. The success of the PCR was checked by electrophoresis using 1.5 % agarose gels. PCR products were purified and prepared for sequencing using the corresponding PCR primers. The actual Sanger sequencing of the sense and antisense strands was done by Macrogen Europe (Amsterdam, The Netherlands).

2.3. Phylogeographic analyses

The sequences were edited with GENEIOUS v9.1.8 (Dotmatics 2017), imported and manually aligned in

BIOEDIT v7.2.5 (Hall 1999). Analyses were performed in R v4.2.3 (R Core Team 2023) and BEAST v2.6.7 (Bouckaert et al. 2014). The combined dataset only included samples with all four sequences available (File S1 [Table S1]). For the mitochondrial dataset, only samples with sequences available for both COI fragments were included.

For the phylogenetic analyses, first the number of haplotypes, haplotype and nucleotide diversity as well as segregating sites were calculated using the R-pages 'pegas' (Paradis 2010) and 'ape' (Paradis & Schliep 2019). Pairwise distances were calculated using TrN as the best-fitting substitution model using the R-packages 'ape' (Paradis & Schliep 2019) and 'phangorn' (Schliep 2011) and visualised in a heatmap with dendrogram. A mismatch analysis and tests of neutral mutation hypothesis with Tajima's *D* for the *W. occipitalis* species complex, species groups and subgroups were performed using the 'pegas' and 'adegenet' packages (Jombart 2008; Paradis 2010). Furthermore, a principal component analysis (PCA) was done using the package 'stats' (R Core Team 2023). A TCS haplotype network was constructed using the mitochondrial dataset of the samples in R using the package 'pegas' (Paradis 2010).

A Maximum Likelihood tree with bootstrap values was reconstructed each for the combined, mitochondrial and nuclear dataset in R using the 'phangorn' package (Schliep et al. 2016). The best-suited substitution models were selected by R based on the lowest Bayesian information criterion (BIC). For the mitochondrial TrN+I, for the nuclear HKY+I and for the combined dataset HKY+G(4)+I was used as substitution models. In BEAST, a Bayesian tree of the combined dataset was constructed. The substitution model for each gene fragment was selected based on the lowest BIC calculated in JMODELTEST v2.1.9 (Darriba et al. 2012; Guindon & Gascuel 2003). The used model was HKY+G+I for COI5-P, HKY+I for COI3-P, K80 for wingless and HKY for CAD. For the molecular clock, the settings of the relaxed clock were log normal for each gene fragment, and a clock rate of 0.0177 for the mitochondrial gene fragments and 0.00177 for the nuclear genes was used (Papadopoulou et al. 2010). For the tree priors, Yule model was used for all genes. The MCMC was set to 30 million generations saving trees every 3,000 generations. The convergence and stationarity was checked with TRACER v1.7.2 (Rambaut et al. 2018). The BEAST package LOGCOMBINER v2.6.7. was used to combine the separate tree-files from the gene fragments. A consensus tree with burning percentage of 10 and posterior probability limit of 0.5 was computed using TREEANNOTATOR v2.6.7 of the BEAST package. The consensus tree was visualized in FIGTREE v.1.4.4 (Rambaut 2018). The node ages were calculated by BEAST using the same settings described for the Bayesian tree. For this purpose, two calculations per node were performed: one calculation with the samples that had the greatest and one that had the least genetic distance in the haplotype network for the respective groups. The average of these two node ages was calculated.

3. Results

The combined dataset of the four sequenced fragments had 23 haplotypes with a haplotype diversity of 0.965 and a nucleotide diversity of about 0.025 (File S1 [Tables S3, S4]). 20 of these 23 haplotypes were found in the species group *W. occipitalis* and three in *W. subterranea*. In *W. occipitalis*, haplotype diversity and nucleotide diversity were 0.971 and 0.008, respectively, and in *W. subterranean* 0.607 and 0.010, respectively. The extended mitochondrial dataset also had 23 haplotypes; haplotype and nucleotide diversity were 0.960 and 0.044, respectively.

Pairwise genetic distances showed two distinct groups within the *W. occipitalis* species complex (Fig. 3): the first group included samples of the morphologically defined group *W. occipitalis*, the second samples of the morphological *W. subterranea* group. *W. occipitalis* x *subterranea* was clearly recognised as a subgroup of *W. occipitalis*. Pairwise distances between these two groups were 0.055–0.060 for the combined dataset (File S1 [Table S5]) and 0.093–0.105 for the mitochondrial dataset (File S1 [Fig. S4]).

Further subgroups were identified within these two groups. The *W. subterranea* group showed three distinct subgroups with pairwise genetic distances of 0.016 to 0.020 (only mtCOI: 0.030–0.035). Within the *W. occipitalis* group, four different subgroups were identified with a genetic distance range of 0.007–0.014 (mtCOI: 0.014–0.028). These (sub)groups were also detected by PCA (File S1 [Fig. S5]). The first principal component on the x-axis distinguished the two main groups *W. occipitalis* and *W. subterranea*. The species' subgroups are segregated from each other by the second principal component on the y-axis, with three subgroups being differentiated each.

The TCS haplotype network of the mitochondrial gene COI also showed a clear distinction between *W. occipitalis* and *W. subterranea* in line with the morphological determination as well as the differentiation of these into multiple subgroups. In this context, the morphologically undefined individuals were clearly genetically assigned (Fig. 4). The *W. subterranea* group consisted of three haplotypes, which are strongly separated from each other. *W.* sp. samples from Bosnia and Herzegovina (XVII) were genetically assigned to *W. subterranea*. The subgroup containing all the morphological *W. o. occipitalis* specimens, supplemented by two *W. o. meridionalis* and two *W.* sp. specimens, showed a star-like structure with one dominant haplotype (V), which included samples from different regions in Germany and France. Seven satellite haplotypes containing samples from Germany, France, and Denmark derived from the main haplotype with five or less mutation steps between them. A similar structure is observed in one subgroup including several morphological determined *W. o. meridionalis* individuals: five haplotypes are derived directly or indirectly from the central haplotype (VII). Here, the highest detected number of mutations is between the haplotypes VII and X with nine mutational steps. The morphologically de-

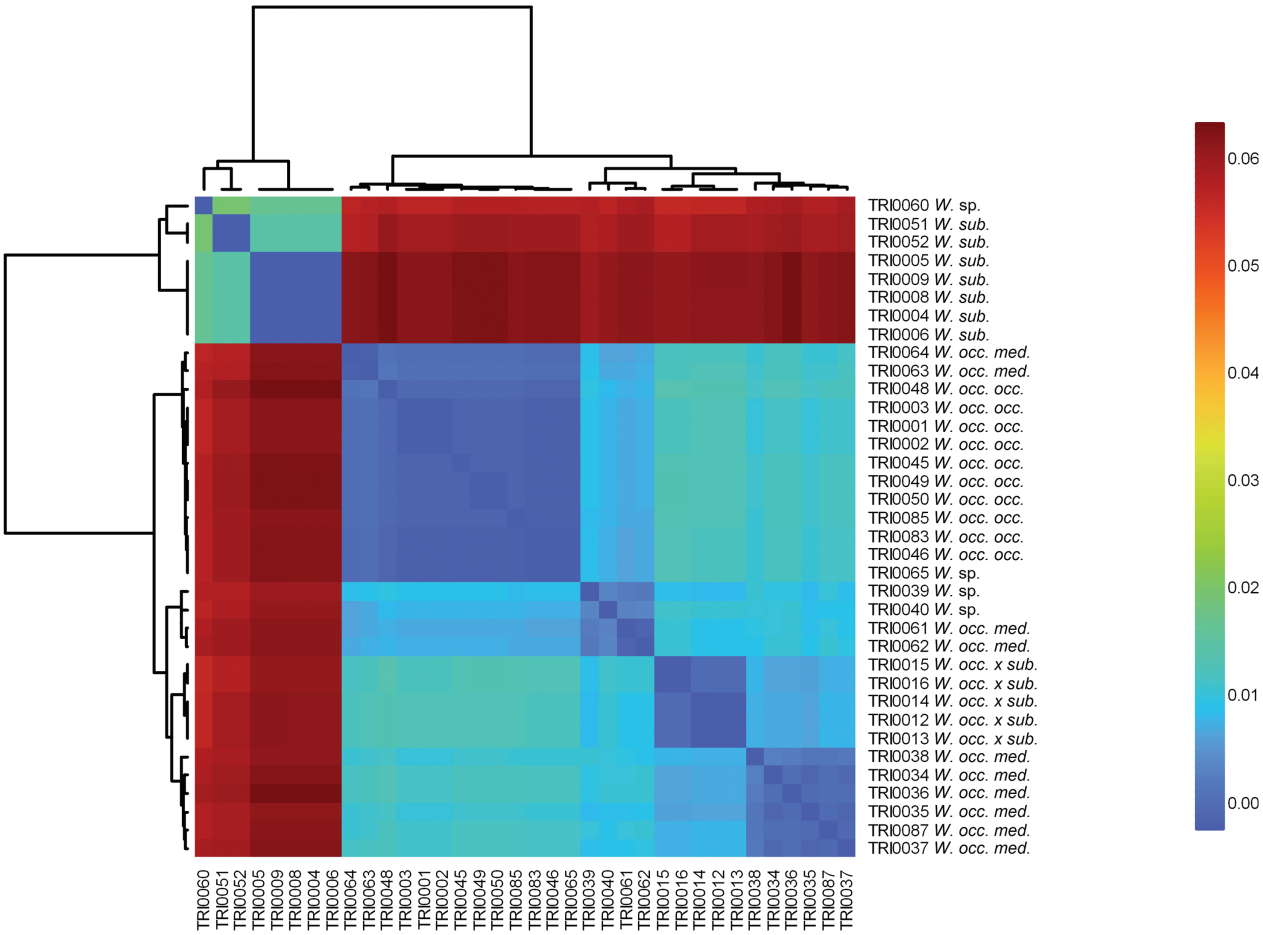

Figure 3. Heatmap presenting the pairwise genetic distances of the combined dataset of the *Wormaldia occipitalis* species complex, including one mitochondrial and two nuclear genes, with dendrogram. The colour codes of the genetic distances are shown in the legend. *W.* occ. occ. – *W. occipitalis occipitalis*, *W.* occ. *med.* – *W. occipitalis meridionalis*, *W.* occ. x *sub.* – *W. occipitalis* x *subterranea*, *W. sub.* – *W. subterranea*, *W.* sp. – *W.* sp.

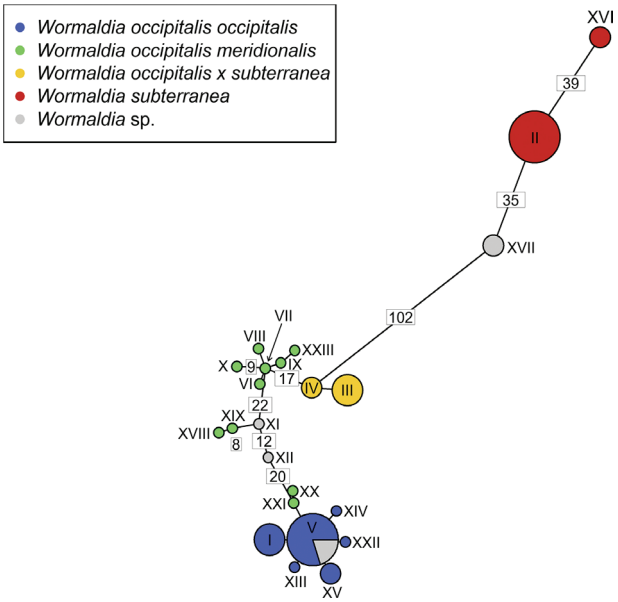

Figure 4. TCS haplotype network based on the mitochondrial gene COI of the *Wormaldia occipitalis* species complex. Mutations from six steps onwards are shown by numbers on the links. Mutational steps up to five steps are not shown. Each morphologically determined group is presented by one colour. The corresponding colour codes are given in the legend.

termined group *W. occipitalis* x *subterranea*, which was again clearly recognised within the *W. occipitalis* species group, and the second group containing *W. occipitalis meridionalis* specimens did not show a single dominant or central haplotype.

The Maximum Likelihood analysis of the combined and mitochondrial dataset showed a similar phylogenetic structure of the *W. occipitalis* species complex (Fig 5A; File S1 [Fig. S7A]). *W. occipitalis* and *W. subterranea* were identified as distinct groups with *W. subterranea* showing three and *W. occipitalis* four subgroups. Within the *W. subterranea* group, the German and Austrian samples (i.e., *W. subterranea* group 1), as well as the French samples (i.e., *W. subterranea* group 2) formed a monophyletic clade each. As in the haplotype network, samples from Bosnia and Herzegovina, determined as *W.* sp., were genetically clearly assigned to *W. subterranea* and formed the third subgroup (*W. subterranea* 3). The morphologically determined *W. o. occipitalis* specimens, along with one to two morphologically undefined and two morphologically determined *W. o. meridionalis* individuals, formed a genetic group (i.e., *W. occipitalis* group 1), as did the *W. occipitalis* x *subterranea* individuals (i.e., *W. occipitalis* group 3). The remaining two genetic groups consisted of either only individuals morphologically de-

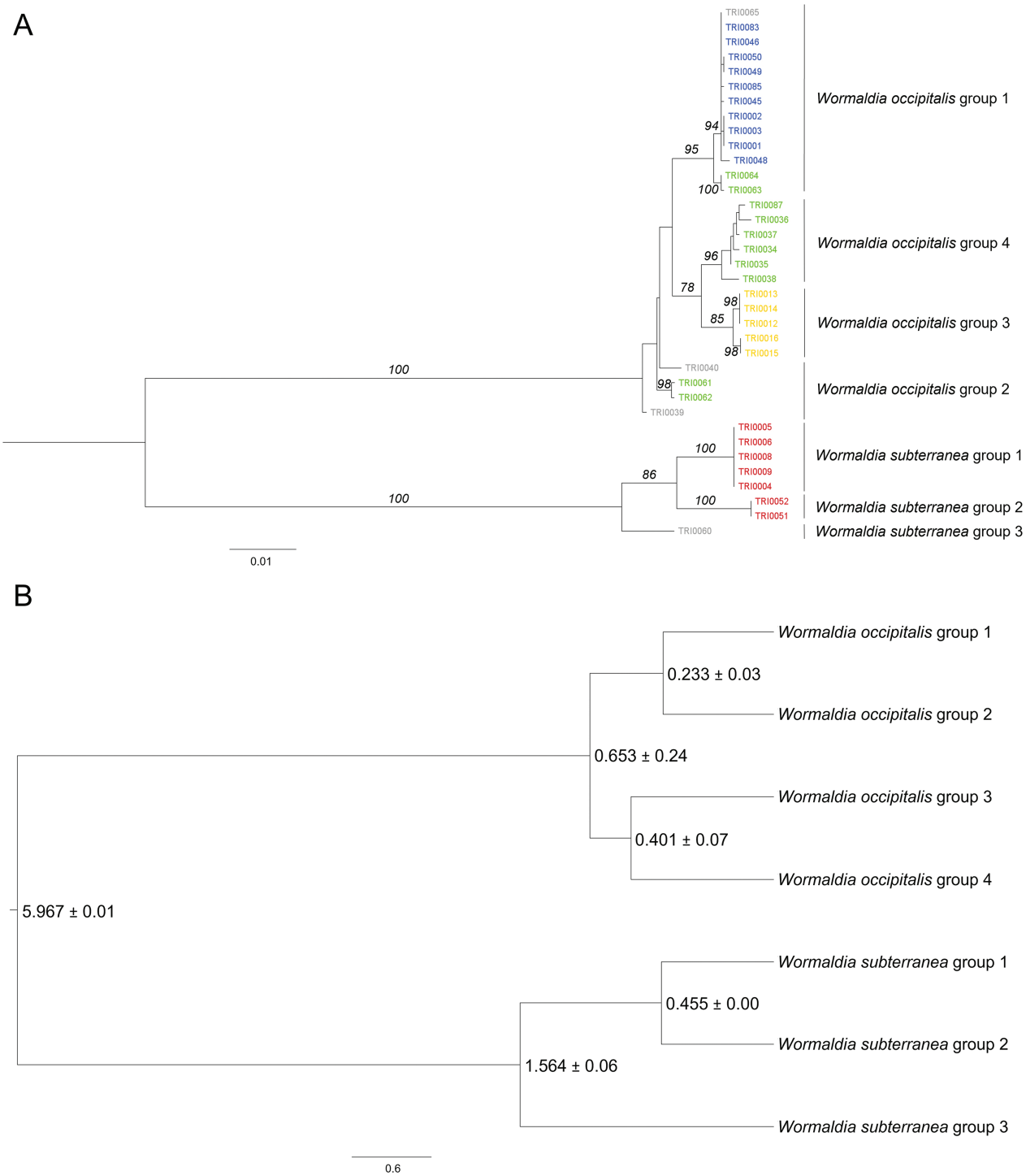

Figure 5. Phylogeny of the *Wormaldia occipitalis* species complex. Species subgroups are marked and labelled with the corresponding name. Individuals are colour-coded by their morphological determination. Blue – *W. occipitalis occipitalis*, green – *W. occipitalis meridionalis*, gold – *W. occipitalis* x *subterranea*, red – *W. subterranea*, grey – *W.* sp. **A** Maximum likelihood based on the combined dataset (COI, wingless, CAD). Numbers on branches present bootstrap values >70 %. **B** Bayesian analysis based on the combined dataset (COI, wingless, CAD). Numbers next to the nodes present the node ages in million years.

termined as *W. o. meridionalis* (i.e., *W. occipitalis* group 4) or *W. o. meridionalis* and *W.* sp. (i.e., *W. occipitalis* group 2). Within the subgroups of *W. occipitalis*, group 2 represented the most basal subgroup. *W. occipitalis* group 3 and 4 formed a monophyletic clade as sister group to *W. occipitalis* group 1. Within *W. subterranea*, group 3 is the most basal group. The nuclear tree also showed different subdivisions in both major groups (File S1 [Fig. S7B]): In contrast to the combined and mitochondrial datasets, two instead of three subgroups were identified within *W. subterranea*, with groups 1 and 2 representing one group. In *W. occipitalis*, groups 1, 3 and 4 were recognised, but samples of group 2 did not show up as a cohesive group. Also group 3 and 4 were not sister groups.

Using all models recommended by BIC for the genes, Bayesian analysis of the molecular data reached conver-

gence after approximately 3,000,000 generations, with an ESS value of 722 for the posterior and 646 for the prior for the combined dataset. The Bayesian analysis showed a similar phylogenetic structure of the *W. occipitalis* species complex, with a clear recovery of *W. occipitalis* and *W. subterranea* as separate groups that diverged about 5.97 ± 0.01 million years ago (Fig. 5B; File S1 [Fig. S6]). As with the Maximum Likelihood, three subgroups were identified in *W. subterranea* and four subgroups in *W. occipitalis*. Here, *W. occipitalis* groups 1 and 2 formed the sister clade to *W. occipitalis* groups 3 and 4. Except for the morphologically determined *W. occipitalis meridionalis* specimens, which are polyphyletic, all other morphological groups are monophyletic (*Wormaldia* sp. is omitted here because it is not specifically determined).

No significant Tajima's *D* values were determined, neither for the entire complex, nor for its groups or subgroups (File S1 [Table S6]). The mismatch distribution analysis illustrated a multimodal distribution with a total of three maxima (File S1 [Fig. S8]).

4. Discussion

4.1. Phylogeny and biogeography

The genetic analyses identified two distinct monophyletic groups, *W. occipitalis* and *W. subterranea*, and four subgroups within *W. occipitalis* as well as three in *W. subterranea* (Figs 3–5). The assumed age of the split between the two major groups was estimated at about six million years (Fig. 5B). Accordingly, these two groups most likely were separated during the Miocene-Pliocene transition (Fig. 6A). During this period, climatic and geological changes occurred in the Alps, which could have promoted vicariance and speciation processes of these two groups (Barron & Keller 1982; Flower & Kennett 1994; Willett et al. 2006). For instance, heaving and depression processes that occurred in the Late Miocene in the Alps may have led to a geographical barrier in the Eastern Alps, likely resulting in range disjunction (Pfiffner 2015). A previous study also assumed diversification of the trichopteran genus *Consorophylax* during this time period due to the geological transformation of the Alps in interaction with climatic changes (Graf & Vitecek 2016).

During the Pliocene, these two groups were not affected by further internal differentiation. From the Pleistocene onwards, we propose several further dispersal processes causing differentiation within both species groups (Fig. 6B): On the one hand, a possible vicariance within *W. occipitalis* could have occurred around 650,000 years ago (Fig. 5B), initially into a south-Alpine (incl. *W. occipitalis* subgroups 3 and 4), and a south-west-Alpine clade (incl. *W. occipitalis* subgroups 1 and 2). This phenomenon is also proposed for other species in this time window (Berends et al. 2021). Each of these two groups in turn diverged into two further subgroups potentially 400–200 kya. Thus, up to and including the Last Glacial Maximum (LGM), four geographically distinct subgroups of *W. occipitalis* existed, distributed across the southern/south-western Alps. On the other hand, according to the hypothesis proposed by the Bayesian tree (Fig. 5B), *W. subterranea* probably originally split into an Alpine and a Balkan group and afterwards spread 650,000–470,000 years ago from the eastern to the western Alps, most likely using a northern route around the Alps. The climatic oscillation during the Pleistocene (Berends et al. 2021) apparently led to a segregation of the eastern Alpine group from the Balkan individuals and to an isolation of the western Alpine population from the eastern Alpine individuals due to glaciation.

Nevertheless, the data obtained from the molecular clock of the Bayesian analyses must be viewed critically. In contrast to the Maximum Likelihood tree, the Bayesian tree exhibits low node support. Secondly, the substitution rates established for insects were used (Papadopoulou et al. 2010). However, since Trichoptera have a lower mobility and a shorter lifespan than other insects (Curry & Baird 2015; Schröder et al. 2021) and may therefore have a different rate of molecular evolution, the applied substitution rates could be less suitable for Trichoptera.

The estimated node ages of the subgroups within both species groups call for multiple peripheral-Alpine extra-Mediterranean refugia (Fig. 6C): The *W. occipitalis* species groups possessed a southern central Alpine (*W. occipitalis* group 3), a north-western Italian (*W. occipitalis* group 4), a south-western Alpine (*W. occipitalis* group 2) and western French refugium (*W. occipitalis* group 1). *W. subterranea* apparently had three refugia in total, in the western Alps (*W. subterranea* group 2), eastern Alps (*W. subterranea* group 1) and, at least one, in the western Balkan region (*W. subterranea* group 3).

This biogeographic hypothesis with a number of extra-Mediterranean refugia is consistent with the phylogeographic pattern of other taxa (Malicky 2006; Pauls et al. 2006; Schmitt & Varga 2012; Schönswetter et al. 2004). Especially for semi-aquatic, stream-inhabiting taxa, such as caddisflies, extra-Mediterranean refugia, e.g. at the margins of the Alps, are more common than for terrestrial species as semi-aquatic taxa could survive glacial periods due to permanently flowing waters whose temperature never dropped below 0 °C (Malicky 1983). Such a 'dinodal' distribution (Malicky 1983) has been demonstrated for further (trichopteran) species (Engelhardt et al. 2011; Garcia-Raventós et al. 2021; Pauls et al. 2006). These refugia were the basis for postglacial dispersal. In our case, the western French *W. occipitalis* group 1 and the eastern-Alpine population *W. subterranea* group 1 both were spreading northwards into Central Europe postglacially (Fig. 6D; File S1 [Fig. S8]). Here, there distribution is overlapping today and even syntopic occurrences are known (Fig. 6E).

Despite not being significant, the high negative value of Taijma's *D* of the *W. occipitalis* group 1 supports a range expansion for this group (File S1 [Table S6]); recent range expansion is also supported by the star-like haplotype structure of the mtDNA with one common central and several rare satellite haplotypes. The lack of

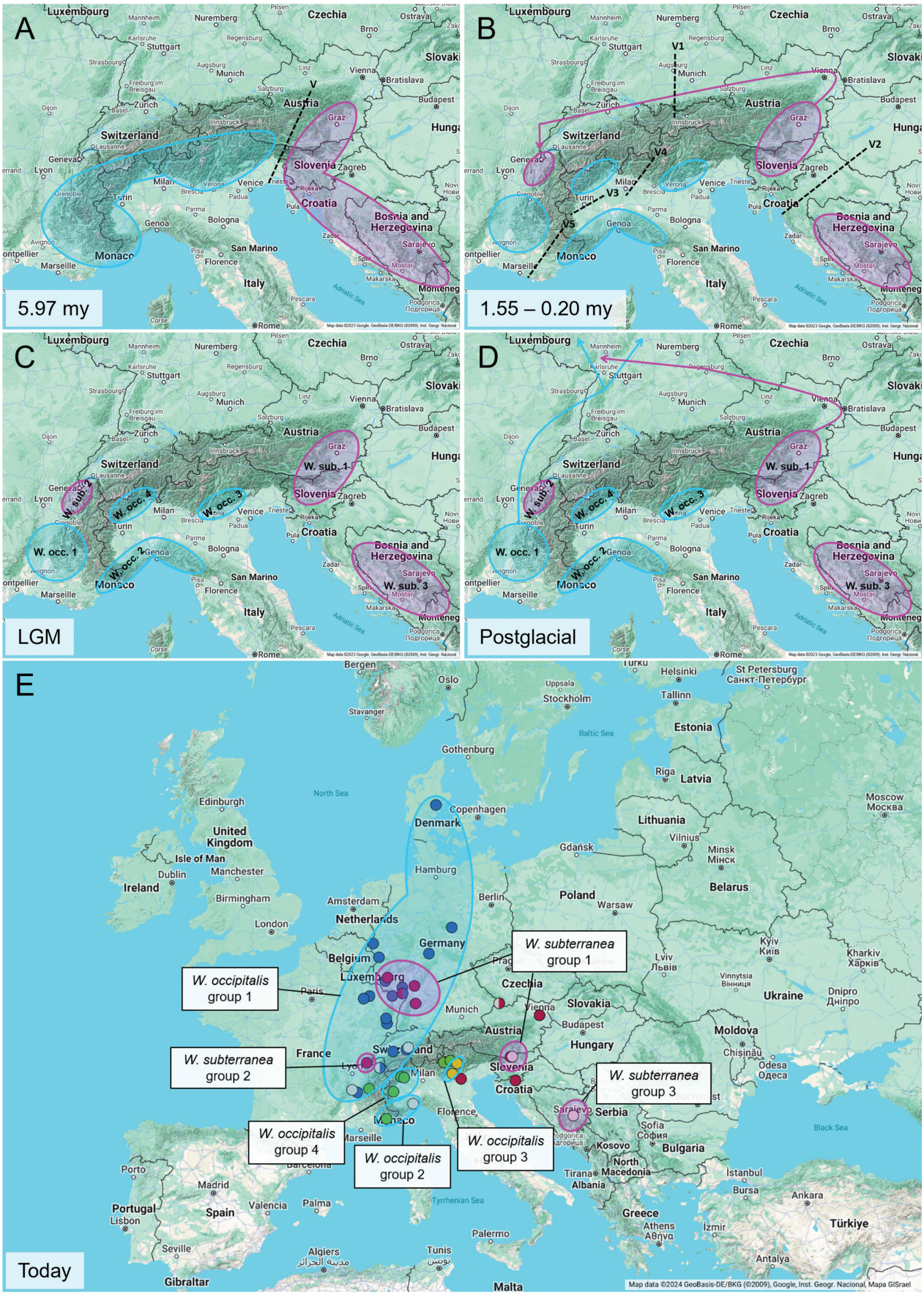

Figure 6. Biogeographic hypothesis of the *Wormaldia occipitalis* species complex. **A**–**D** Biogeographic dynamics of the (sub) groups along time. **E** Recent distribution of the (sub)groups of the *W. occipitalis* species complex. Individuals are colour-coded by their morphological determination. Blue – *W. occipitalis occipitalis*, green – *W. occipitalis meridionalis*, gold – *W. occipitalis* x *subterranea*, red – *W. subterranea*, Grey – *W.* sp. Genetic groups were also colour-coded and additionally labelled. Light blue – *W. occipitalis* ssp., pink – *W. subterranea* ssp.

significance of Taijma's *D* might result from the limited sample size in our analysis and might have become significant if using a higher number of samples. A similar result was obtained for *W. occipitalis* group 3, except that Taijma's *D* is strongly positive, indicating range regression (Schmitt 2020). *W. occipitalis* group 2 and 4 showed neither signs of range expansion nor regression. However, the sample size is not sufficient for a valid test result and should only be interpreted in conjunction with the other results.

4.2. Taxonomy

Our genetic analyses (Figs 3–5; File S1 [Figs S6, S7]) confirm morphological differentiation and accordingly the assumption of several groups and subgroups within the species complex *W. occipitalis* (Neu 2015). The two morphologically distinguishable taxa *W. occipitalis* and *W. subterranea* also showed a clear genetic differentiation with a pairwise difference of almost 6 % in the combined dataset and about 10 % for the mitochondrial COI alone. Among these two taxa, the Alpine specimens do not show a stronger differentiation than the specimens from Central Europe. In general, the detected genetic distance between the two major groups of our analysis is comparable to previous studies in which an interspecific level of differentiation was assumed for members of other caddisfly families (Graf et al. 2015; Pauls et al. 2010). This high degree of differentiation might have resulted from low gene flow due to distribution in a mountain environment and generally low dispersal power of caddisflies (Curry & Baird 2015; Schröder et al. 2021). In addition, there is no detected genetic evidence of hybridisation (Fig. 3) between these two taxa since their presumed separation in the Mio-Pliocene Transition; even in the current area of sympatry, no indication for hybridisation exists, neither genetically nor morphologically. Nevertheless, potential hybridisation events cannot completely be ruled out. This strongly underlines that reproductive isolation may also have fostered the high degree of genetic differentiation between these two groups that definitively should be considered as good species: *W. occipitalis* and *W. subterranea*.

In contrast to morphology, the genetic analyses revealed four instead of two subgroups within *W. occipitalis* and additionally a differentiation into three subgroups within *W. subterranea* (Figs 3–5). The morphologically determined subspecies *W. o. occipitalis* and *W. o. meridionalis* were also identified as groups. However, *W. o. meridionalis* did not represent a monophyletic group but was observed in three genetic subgroups including two monophyletic clades. The morphological group determined as hybrid *W. occipitalis* x *subterranea* was genetically not detected to be of hybrid origin. Instead, the specimens were clearly classified within *W. occipitalis*, forming a monophyletic clade there. The grouping of individuals was mostly consistent between the genetic and morphological determination (Figs 4, 5). Only the specimens TRI0063 and 64 (France, Chamrousse) morphologically grouped within *W. o. meridionalis* but were assigned to the *W. o. occipitalis* cluster by genetic analyses. All specimens morphologically not determined to the species level (*Wormaldia* sp.) clearly clustered within one of the genetic groups; hence no further lineages were recovered. The mitochondrial genetic distance among the subgroups was 1.4–2.8 % within *W. occipitalis* and about 3.0–3.5 % for the three subgroups of *W. subterranea*. Similar intraspecific genetic distances of genetic clusters were previously found within other trichopteran families (Graf et al. 2015; Previšić et al. 2014; Previšić et al. 2009). Higher intraspecific genetic distances have also been detected (Pauls et al. 2006), however, here species status needs to be re-evaluated (Oláh et al. 2015).

Geographic proximity partly correlated with genetic differentiation and phylogenetic relatedness of the species' subgroups: On the one hand, the subgroups of *W. occipitalis* show an exact order in the haplotype network (Fig. 4) according to their geographical distribution from the western to the southern Alps. Hence, they fulfil all criteria of allopatric subspecies. On the other hand, the German specimens within *W. subterranea* group 1 are geographically more distant from the Austrian specimens of the same phylogenetic group than from the genetically more differentiated specimens of *W. subterranea* group 2. Nevertheless, their subspecific status, as well as for *W. subterranea* group 3, is highly likely.

In short, the taxa *W. occipitalis* and *W. subterranea* are distributed in the same area and habitat in western Germany (Fig. 6E). This syntopic distribution is an indication that these two taxa are distinct species, as previous morphological studies already assumed (Neu 2015; Oláh et al. 2019). The high degree of genetic differentiation, lack of genetic evidence of hybridisation and the morphological distinctness support this hypothesis. The subgroups of *W. occipitals* and *W. subterranea* are genetically distinct and have allopatric distributions. However, difficulties in the unambiguous morphological assignment of individuals to a subgroup as well as poor bootstrap values or Bayesian posterior probabilities in phylogenetic analyses and relatively low genetic distances tend to argue for a differentiation at subspecific level.

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Supplementary Material 1

File S1

Authors: Mewis V, Neu PJ, Schmitt T (2024)
Data type: .pdf
Explanation notes: Figure S1. Differentiation characteristics of the male genitalia of the *Wormaldia occipitalis* and *W. subterranea* species group. — **Figure S2.** Aedeagus spination of the *Wormaldia occipitalis* (1) and *W. subterranea* (2) species group. — **Figure S3.** Phenology of the *Wormaldia occipitalis* and *W. subterranea* species group. — **Table S1.** List of samples with geographic information, sex, collection date, morphological determination, and genetic information. AUT – Austria, BIH – Bosnia and Herzegovina, CHE – Switzerland, DEU – Germany, DNK – Denmark, FRA – France, ITA – Italia, SVN – Slovenia. m – male, f – female, NA – no information. NaN – no measurable. 1 – sequence available, 0 – no sequence available. — **Table S2.** Modification of the "DNA Extraction and Purification from Tissue" protocol for DNA extraction. — **Table S3.** Genetic diversity parameter of the two mitochondrial and two nuclear gene fragments as well as the mitochondrial, nuclear and combined dataset of the *Wormaldia occipitalis* species complex. — **Table S4.** Genetic diversity parameter of the combined dataset of the *Wormaldia occipitalis* species complex and species (sub)groups. n – number of haplotypes, h – haplotype diversity, Pi – nucleotide diversity, S – Segregating sites. NaN – no computable. — **Figure S4.** Heatmap presenting the pairwise genetic distances of the mitochondrial dataset (COI) of the *Wormaldia occipitalis* species complex, with dendrogram. The colour codes of the genetic distances are shown in the legend. — **Table S5.** Pairwise genetic distances of the samples of the *Wormaldia occipitalis* species complex dataset. — **Figure S5.** Principal Component Analysis of the *Wormaldia occipitalis* species complex dataset. Individuals are colour-coded by their morphological determination. — **Figure S6.** Phylogeny of all samples of the *Wormaldia occipitalis* species complex using Bayesian analysis based on the combined dataset (COI, wingless, CAD). Numbers on branches represent Bayesian posterior probabilities >0.7. Species subgroups are marked and labelled with their corresponding name. Individuals are colour-coded by their morphological determination. Blue – *W. occipitalis occipitalis*, Green – *W. occipitalis meridionalis*, Gold – *W. occipitalis* x *subterranea*, Red – *W. subterranea*, Grey – *W.* sp. — **Figure S7.** Phylogeny of the *Wormaldia occipitalis* species complex using Maximum likelihood. A – Based on the mitochondrial dataset (COI). B – Based on the nuclear dataset (wingless, CAD). Numbers on branches present bootstrap values >70%. Individuals are colour-coded by their morphological determination. Blue – *W. occipitalis occipitalis*, Green – *W. occipitalis meridionalis*, Gold – *W. occipitalis* x *subterranea*, Red – *W. subterranea*, Grey – *W.* sp. — **Table S6.** Tajima's *D* and corresponding p-value of the *Wormaldia occipitalis* species complex, species groups and species subgroups. NaN – no computable. — **Figure S8.** Mismatch distribution analysis of the mitochondrial dataset of the *Wormaldia occipitalis* species complex.

Link: https://doi.org/10.3897/asp.82.e116205.suppl1