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Research Article

Population Genomic Divergence Reveals Uncertainties in Species Identification of Cave-Dwelling Isopods

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Biodiversity conservation is advancing, though much of nature remains unexplored. Subterranean environments hold a significant portion of the world's biodiversity, yet cave biology still largely relies on morphology for species delimitation. The lack of genomics research hinders the resolution of taxonomic classifications and the reconstruction of species evolutionary history. Here, we examined genetic diversity in seven cave-dwelling *Monolistra* isopod species throughout the prealpine arch in Italy and Switzerland. We used genome-wide single nucleotide polymorphisms (SNPs) to reconstruct phylogenetic relationships and infer population structure among 21 cave populations. Phylogenetic tree reconstruction revealed strong divergence among *Monolistra* species, splitting into seven phylogenetic clades. Both species and gene trees confirmed an east–west clade divergence, yet highlighted major conflicts between morphology-based taxonomy and genomic data. Population structure analyses further identified strong within-clade differentiation and genetic isolation between each cave system. Our findings have important taxonomic implications. They suggest that the true level of diversity within the genus *Monolistra* could be much greater than currently understood, stressing the importance of genomics research to improve the general comprehension of the evolution of subterranean dwelling organisms and its implications for conservation.

Keywords: cave biodiversity; ddRADseq; *Monolistra*; population genomics; species delimitation; taxonomy

1. Introduction

Unraveling the forces that shape and sustain biodiversity is key to understanding its intricate web of life, and essential for guiding its conservation [1, 2]. However, many environments remain largely unexplored, for example, abyssal depths [3, 4], subglacial lakes [5], atolls [6], glacier systems [7, 8], and subterranean environments, such as deep subsurface [9] and caves [10]. This is primarily due to their remoteness and inaccessibility, which pose significant logistical challenges for biodiversity sampling. This limitation not only restricts our knowledge of these ecosystems but also leads to an underrepresentation of biodiversity in conservation priorities and policies, as many species remain unidentified or undescribed [11].

Caves harbor a significant yet undiscovered portion of the world's biodiversity [11, 12]. Most of our knowledge about cave biodiversity remains limited to faunistic checklists [12], for which the delimitation of species boundaries still relies on morphological characters. However, these traits can be misleading due to the occurrence of distinct species that appear identical (i.e., cryptic species) [13, 14], local adaptations leading to convergent traits between species or high variability within species [15], and phenotypic plasticity [16]. Genetic data has, therefore, become a tool of choice in taxonomic research, particularly as obtained by high-throughput sequencing techniques in the past decades [17, 18]. Genomic information should, therefore, improve taxonomic identification and influence efforts to conserve cave fauna [19, 20].

In cave organisms, the use of multigene phylogeny and phylogenomics has enhanced accuracy in resolving evolutionary relationships between species [21–24]. The study of genetic variation further allowed a comprehensive understanding of key evolutionary processes, such as local adaptation and niche specialization [25–29], and population demography and connectivity [30–32]. Despite these advances, major challenges persist in fully understanding the evolutionary history of subterranean taxa. First, our current assessment of species diversity may be biased by morphology-based taxonomic approaches (cryptic species; species inflation). Second, the biogeographic barriers that structured epigean biodiversity may not align with subterranean species boundaries. Third, climate factors that shaped species range dynamics may have had very different consequences on cave-adapted organisms [33]. A genomic-based reevaluation of taxonomic classifications is, therefore, critical for further uncovering macroevolutionary patterns of diversification and accurately capturing microevolutionary dynamics in subterranean lineages.

Crustacean isopods of the genus *Monolistra* are a remarkable example of cave-adapted organisms with understudied evolutionary history. *Monolistra* isopods can achieve high densities in groundwater habitats, playing an important role in the food web as detritivores preyed upon by other aquatic cave organisms [34, 35]. *Monolistra* species can be found from the Dinaric Alps in the Balkans to the Western Prealps, where the extreme morphological diversity let to the identification of five subgenera: *Monolistra*, *Microlistra*, *Monolistrella*, *Pseudomonolistra*, and *Typhlosphaeroma* [36]. Early molecular data suggested that the genus colonized cave environments during the Messinian salinity crisis from marine ancestors (~5 million years ago) [37], and that subsequent Pleistocene glaciations may have played a role in species diversification [36, 38]. However, all molecular studies to date on the genetic relationships within *Monolistra* have been using allozymes, or few mitochondrial and nuclear genes, and are limited to species of the eastern Karst Plateau and the Dinaric Alps [38–40], hampering to obtain a clear picture of the overall relationships between the taxa belonging to this genus.

Stretching from the Western Prealps in France to the Eastern Prealps in Slovenia, the prealpine arch hides a vast network of caves [41, 42], harboring several *Monolistra* species [36]. The current taxonomy includes, from east to west: *M. racovitzai* Strouhal, 1928; *M. julia* (Feruglio, 1904); *M. lavalensis* [43]; *M. berica* (Fabiani, 1901); *M. boldorii* Brian, 1931; *M. bergomas* (Arcangeli, 1935); *M. pavani* Arcangeli, 1941. Efforts to determine the geographic distribution of species revealed that most of them are bounded by present-day lakes along an east–west gradient [44]. The distributions of *M. berica* and *M. boldorii* are delineated by the Garda Lake, those of *M. boldorii* and *M. bergomas* by the Iseo Lake, and those of *M. bergomas* and *M. pavani* by the Como Lake (Figure 1). However, the reliance on morphological characters has introduced uncertainties in species delimitation. For instance, *M. bergomas* has been supposed to be a subspecies of *M. boldorii* [47], and *M. julia* has been proposed to be a subspecies of *M. caeca* [48]. Still, lack of data on the genomic divergence across clades complicates the identification of taxa within this genus.

In this study, we assessed the genetic diversity of *Monolistra* species throughout the prealpine arch from the Classic Karst to the Lugano Prealps in Italy and Switzerland (Table 1 and Figure 1). We used genome-wide single nucleotide polymorphisms (SNPs) obtained through double digest restriction-site associated DNA sequencing (ddRADseq) to reconstruct phylogenetic relationships and infer population structure. These analyses allowed us to evaluate genetic divergence across the current taxonomy and to assess the impacts of geographic barriers (e.g., lakes) and geographic isolation (e.g., individual caves) in shaping spatial patterns of genetic differentiation.

2. Material and Methods

2.1. Sampling. The survey of *Monolistra* specimens was based on the described geographic distribution of species (Figure 1 and Table S1). Sampling was performed between 2019 and 2022. We obtained a total of 73 individuals originating from 21 localities, with sample size per described species ranging from $N = 4$ to $N = 29$ (Figure 1 and Table 1). Species were determined based on external morphological traits, including the presence of uropods, the shape and ornamentation of the pleotelson, and the shape of the second pereiopods in males, following the available morphological descriptions ([36, 49]; Table 1). Specimens were stored in absolute ethanol at −20°C.

2.2. DNA Extraction and ddRADseq Library Preparation. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen), with an additional preliminary physical lysis step, and fluorometrically quantified using Qubit Fluorometer (Invitrogen). Given the lack of knowledge on genome size in *Monolistra* and in isopods in general, library construction (selection of restriction enzymes pair; sequencing depth) was designed based on the estimated genome size of *Armadillidium* isopods (1.2–1.7 Gb) [50]. ddRADseq libraries were prepared following Peterson et al. [51] with minor modifications.

The digestion of genomic DNA was performed with 2.4 U of *Pst*I and *Eco*RI endonucleases (New England BioLabs) in 30 μL reaction supplemented with CutSmart Buffer and incubated at 37°C for 90 min, then, at 75°C for 20 min. Fragmented DNA was subsequently ligated with 180 U of T4 DNA ligase (New England BioLabs) and 2.5 pmol of overhang barcoded adapters for both the two cut sites in 50 μL reaction. Ligation was performed at 23°C for 60 min and at 20°C for 60 min, followed by enzyme heat inactivation for 20 min at 65°C. Individual digested-ligated products were pooled on multiplexing batches and purified with 1.5 volumes of AMPureXP beads (Agencourt). For each pool, targeted fragments distribution was collected on BluePippin instrument (Sage Science Inc.). The selected insert length was 400–600 bp. Gel eluted fraction was amplified with indexed primers using Phusion High-Fidelity PCR Master Mix (New England BioLabs) in final volumes of 50 μL. The PCR protocol was: initial denaturation for 3 min at 95°C; 10 cycles of (30 s at 95°C; 30 s at 60°C; 45 s at 72°C); final extension period for 2 min at 72°C. PCR products were purified with 1 volume of AMPureXP beads. Libraries were quantified using Qubit Fluorometer and library size was verified using Bioanalyzer DNA assay (Agilent technologies). Samples DNA

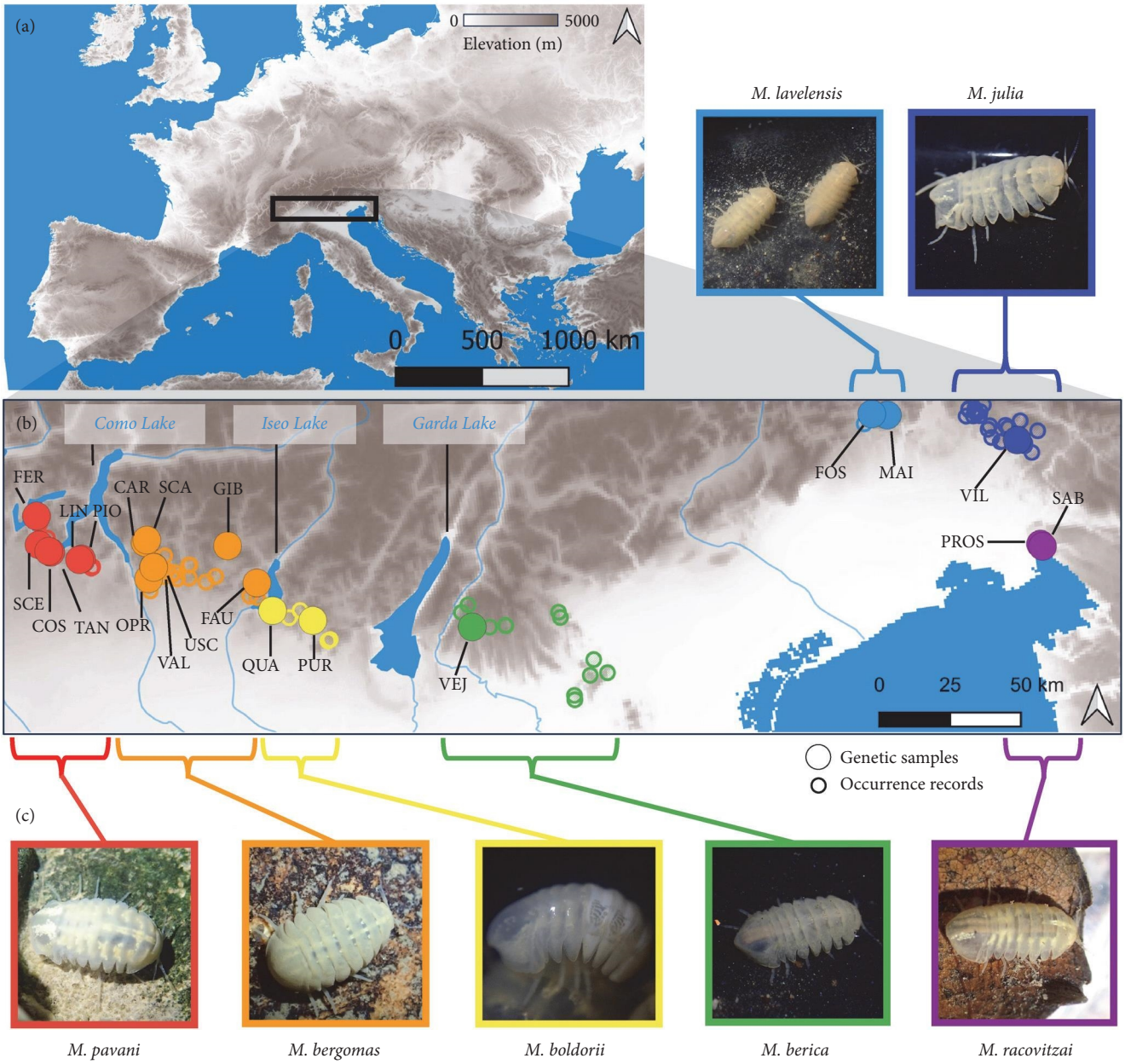

FIGURE 1: Geographic distribution of *Monolistra* species and genetic samples. (a) Map showing the context of the study area. (b) Distribution of the sampling localities for genomics analysis. Dot colors represent the current taxonomy; dot size refers to data type (species occurrences in Table S1). (c) Photos of each *Monolistra* species, Raoul Manenti. All maps are projected in EPSG:3035. The background map was obtained with Natural Earth [45] and Copernicus [46] using QGIS 3.10.8.

sequencing was performed on NovaSeq 6000 (Illumina) with 150 cycles in paired end mode at IGA Technology Services (IGATech).

2.3. SNP Calling and Dataset Filtering. The ddRADseq loci reconstruction and SNP calling was performed using Stacks v2.60 [52]. After demultiplexing, paired-end reads were assembled into de novo loci following the analytical optimization of M and n Stacks parameters as described in Cumer et al. [53]. Optimization of M was performed for each species separately. Bioinformatic treatments allowed for a maximum $M = 5$ and $n = 8$ mismatches to cluster reads within and between samples, respectively. Filtering of SNPs was performed in R [54]. Genotype calls from base coverage <5 or >200 were set to missing data. SNPs showing an excess of shared heterozygosity (>0.5) were discarded. Filter stringencies for the percentage of missing data across individuals and loci were explored following O'Leary et al. [55]. After removing individuals with low-quality data (number of catalog loci <10,000; Table S2), we retained loci with <20% missing data overall individuals. SNPs were further filtered for rare alleles (minor allele count >2) and direct physical linkage (one random SNP per locus).

Table 1: List of *Monolistra* species in the Western Prealps based on the most recent classifications, with their geographic distribution.

Species	Geographic distribution	Sampling locality	Locality code (sample size)	Sampling year	Morphological traits used for species identification
Subgenus *Monolistra*					
Monolistra julia	Julian Prealps	Grotta Nuova di Villanova	VIL (6/2)	2022	Well-developed uropods
Subgenus *Typhlosphaeroma*					
Monolistra racovitzai	Classic Karst, Carniola	Sorgente Sablici 16	SAB (1/0)	2022	Reduced uropods
		Sorgente Pietra Rossa 4a	PROS (3/3)	2022	Regular round pleotelson
Monolistra lavalensis	Carnic Prealps	Caverna Mainarda	MAI (3/1)	2022	Uropods absent
		Grotta Fos di Campone	FOS (1/1)	2022	Pleotelson with a bump Male pereiopods II with a rounded lobe
Monolistra berica	Beric Hills, Vicentine Prealps	Grotta del Ponte di Veja	VEJ (5/5)	2022	Reduced uropods Pleotelson with basal incision and a bump Male pereiopods II with a subtriangular lobe
Monolistra boldorii	Brescia Prealps	Bus Pursì	PUR (5/4)	2022	Reduced uropods
		Bus del Quai	QUA (3/3)	2019	Pleotelson with median incision and a bump with a concavity Male pereiopods II with a rounded lobe
Monolistra bergomas	Bergamo Prealps	—	—	—	Uropods absent Pleotelson with median incision and a bump with a concavity Male pereiopods II with a rounded lobe
	Laghi Bergamaschi	Risorgenza di San Faustino	FAU (3/2)	2022	
	Val Seriana	Grotta Gibelì	GIB (2/2)	2019	
	Valle Imagna	Grotta Uscera	USC (6/4)	2022	
	Valle Imagna	Grotta Valdadda	VAL (2/2)	2020	
	Valle San Martino	Grotta di Opreno	OPR (2/2)	2019	
	Val Taleggio	Grotta dello Scavo	SCA (6/4)	2022	
	Val Taleggio	Bus del Carighun	CAR (8/7)	2020/2022	
Monolistra pavani	Lugano Prealps	—	—	—	Uropods absent Pleotelson with dorsal incision and with a round profile
	Lario	Buco del Piombo	PIO (3/3)	2019/2022	
	Lario	Grotta Lino	LIN (6/4)	2019/2020	
	Valle Intelvi	Grotta El Cos	COS (2/1)	2022	
	Valle Intelvi	Grotta La Tana	TAN (1/1)	2022	
	Valle Intelvi	Grotta di Cava Scerri	SCE (4/4)	2022	
	Valle Intelvi	Grotta Ca del Ferrè	FER (1/1)	2022	

Note: For each species in the present study: list of sampling localities and sampling year. Sample size: Number of sampled individuals/number of genotyped individuals. Species morphology based on Stoch [43] for *Monolistra lavalensis* and Argano [49] for all other species.

2.4. Phylogenetic Tree Reconstruction. Phylogenetic relationships were inferred based on the concatenated sequences of individual samples, using locus consensus sequences including variant and invariant sites (hereinafter species tree). A maximum likelihood (ML) tree was generated using the CAT model. We performed rapid bootstrapping employing Extended Majority Rule (MRE)–based bootstopping and using different data partitions with joint branch length optimization, followed by a thorough ML search implemented in the program RAxML v8.2.12 [56]. Pairwise phylogenetic distances between individuals were obtained from the consensus tree using its branch lengths (all pairs of branch tips), in the ape R package v5.6-2 [57]. We calculated the average phylogenetic distance between monophyletic groups (clades), and the more distant group was used for tree rooting.

The species tree was compared to a multispecies coalescent (MSC) model, where the inferred tree defines a probability distribution on locus tree topologies [58, 59]. The consensus sequence for each phylogenetic group identified in the species tree and each locus was used in RAxML to generate a MSC tree for each locus (hereinafter locus tree), using the same parameters: rapid bootstrapping applying MRE–based bootstopping followed by a thorough ML search. We then used ASTRAL-III [60] to estimate the gene tree from the individual locus trees. Locus trees were visualized using the phangorn R package v.2.12.1 [61]. Finally, we assessed the extent to which locus trees support different tree topologies: (1) the species tree; (2) the gene tree; (3) and (4) two alternative topologies for low-support branches in the gene tree. The normalized quartet score for each topology was calculated using ASTRAL-III.

2.5. Population Structure Analysis. We performed a principal component analysis (PCA) and a discriminant analysis of principal components (DAPCs) on genotypes, using the functions glPca and dapc of the adegenet R package v2.1.1 [62]. For DAPC, the number of genetic clusters K was identified using the K-means procedure in adegenet, identifying the "best" K as the one minimizing the Bayesian information criterion (BIC), with significant ΔBIC between a given K and $K-1$. Then, we inferred global population structure using the ADMIXTURE software v1.3.0 [63]. We tested K possible genetic clusters ranging from 1 to 19 (number of sampled localities; Table 1). For each K, we performed 10 independent runs and determined the "best" K as the one minimizing the tenfold cross-validation error (CVE) using ADMIXTURE [64] and showing spatially informative population structure. We further used ADMXITURE to determine population structure within the main phylogenetic groups by assessing genetic partitioning from $K=2$ to $K=N$ with N being the number of localities sampled in each phylogenetic group.

2.6. Genetic Differentiation. The level of genetic differentiation among populations was estimated using pairwise F_{ST} (Weir and Cockerham) in the hierfstat R package v0.5-11 [65]. Caves with only a single individual sampled were grouped together if they were geographically close and the individuals were genetically similar, as determined by phylogenetic and population structure analyses (localities COS and TAN of *M. pavani*; MAI and FOS of *M. lavalensis*). We performed a hierarchical analysis of molecular variance (AMOVA) using the poppr R package v2.9.6 [66]. We tested the effect of differences between clades based on phylogenetic inference, between populations within clades (sampling localities), and between individuals within populations. Significance was assessed using 10,000 random Monte Carlo permutation tests.

3. Results

3.1. De Novo Sequence Assembly. We obtained 165 million reads for a total of 73 individuals, with 2.3 ± 2.1 (mean $\pm$ SD) million reads per individual (Table S2). We removed 12 individuals with less than 10,000 catalog loci. It resulted in $42{,}230 \pm 15{,}150$ merged stacks per individual on average, with a per-sample depth of $27.0 \pm 15.3X$. Next, removing individuals with a high percentage of missing data (>50%) would further exclude six individuals, but one *M. lavalensis* sample was kept ensuring that each species was represented by at least two samples (sample sizes in Table 1; Table S2). Preliminary phylogenetic reconstruction using a dataset of the seven species (56 individuals) revealed limited resolution to determine the phylogenetic branching of *M. julia* regardless the percentage of missing data (Figures S1 and S2). This is likely due to the low number of informative loci, reflecting both the high divergence between *M. julia* and the six other species and the small sample size in that species. Thus, *M. julia* was excluded from further analyses. The resulting dataset comprising six species and 19 populations (54 individuals) genotyped at 2545 loci was used for phylogenetic reconstruction. Among these loci, 2543 independent SNPs with minor allele count >2 were used for population structure analysis. The percentage of missing data for the 2543 SNPs was $11.95\% \pm 16.21\%$ on average ($\pm$SD), and the individual coverage was $49.40 \pm 33.52X$ on average (Table S2).

3.2. Phylogenetic Relationships. The species tree of concatenated loci was well supported (bootstrap support, BS $\geq$99; Figure 2a). Pairwise phylogenetic distances revealed a higher differentiation of individuals from *M. racovitzai*, which was used for tree rooting ($D=0.028$; Table S3). Mean phylogenetic distances between branch tips revealed seven clades showing within-clade distance $D<0.01$. The seven clades (locality codes) are: Clade (A) *M. racovitzai* (PROS); Clade (B) *M. lavelensis* (FOS; MAI); Clade (C) *M. berica* (VEJ); Clade (D) *M. boldorii* (PUR; QUA) and the population "Risorgenza di San Faustino" (FAU) of *M. bergomas*; Clade (E) populations of Valle Imagna (USC; VAL) and Valle San Martino (OPR) of *M. bergomas*; Clade (F) populations of Val Taleggio (SCA; CAR) and Val Seriana (GIB) of *M. bergomas*; Clade (G) *M. pavani* (PIO; LIN; COS; TAN; SCE; FER).

The gene tree inferred from individual locus trees exhibited partial support across the different nodes (Figure 2b). In total, we detected 24 different topologies. The main represented topologies across locus trees revealed strong differences, and local posterior probabilities (PPs) across branches ranged from 0 to 100, indicating a high level of topological discordance among loci (Figure S3). As a result, both species tree and gene tree had a relatively low evaluation score (0.5; Figure S3). Incongruences between species and gene trees included the branching of Clade D, sister group of Clades E–F–G in

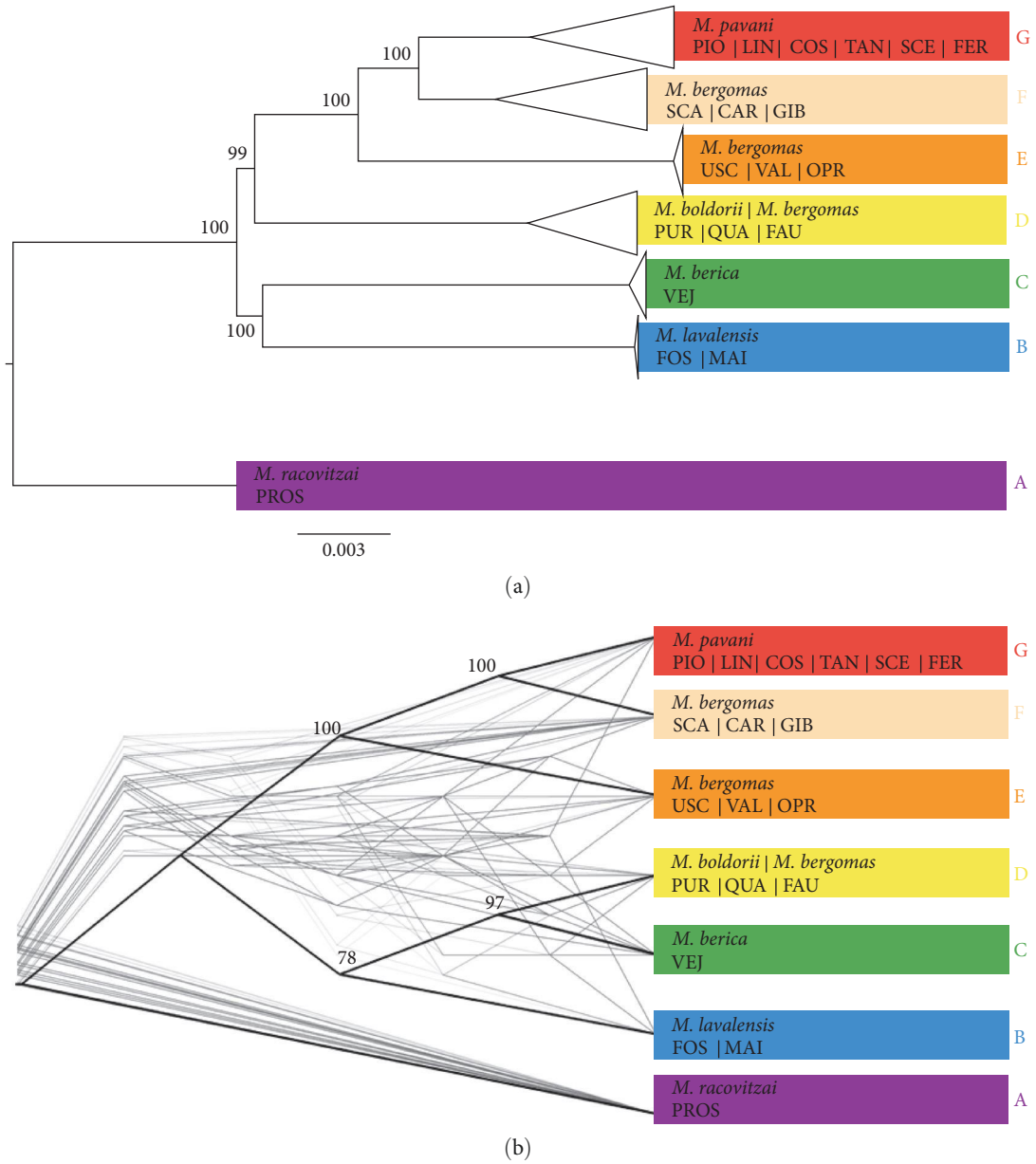

Figure 2: Maximum likelihood phylogenetic relationships among *Monolistra*. (a) Species tree based on concatenated locus sequences. (b) Gene tree based on individual locus trees. *Monolistra racovitzai* was used for tree rooting, according to a higher average phylogenetic distance with all other clades (Table S3). Tip labels report the clade of assignment, the current taxonomy, and the populations ID. Tree support: (a) bootstrap support; (b) local posterior probabilities of the ASTRAL-III consensus gene tree.

the species tree (BS = 99; Figure 2a) whereas sister group of Clade C in the gene tree (PP = 97; Figure 2b).

3.3. Population Structure. The PCA revealed strong genetic differentiation on the first four axes of PCA (71.5% of total variance; Figure 3). The first two axes revealed four different genetic groups (Figure 3a). One of the groups includes the three species *M. racovitzai*, *M. lavalensis*, and *M. berica*, contrary to phylogenetic inference (Clades A, B, and C). The three other groups were consistent with phylogeny and differentiated Clade D (top left), Clade E (bottom), and Clades F and G (top right). The third (14% of variance) and fourth (13% of variance) axes further differentiated between Clades F and G and between Clades A, B, and C, respectively (Figure 3b). The adegenet *K*-means clustering analysis revealed an optimal number of genetic clusters of $K = 10$ (BIC = 198.2; Figure S4). The DAPC based on these 10 groups identified all the seven clades, with further different clusters in E, F and G (Figure 3c).

The global ADMIXTURE analysis revealed an optimal number of $K = 10$ (CVE = 0.06) or $K = 11$ (CVE = 0.05) genetic groups (Figure S5). At $K = 10$ and $K = 11$, all samples were assigned to genetic clusters consistently with phylogeny (Figure 4). The clustering at $K = 4$ was consistent with PCA, separating Clades A–B–C, Clade D, Clade E, and Clades F–G (Figure 3). Clade C (*M. berica*) clustered separately at $K = 6$, while clusters between Clades A (*M. racovitzai*), B

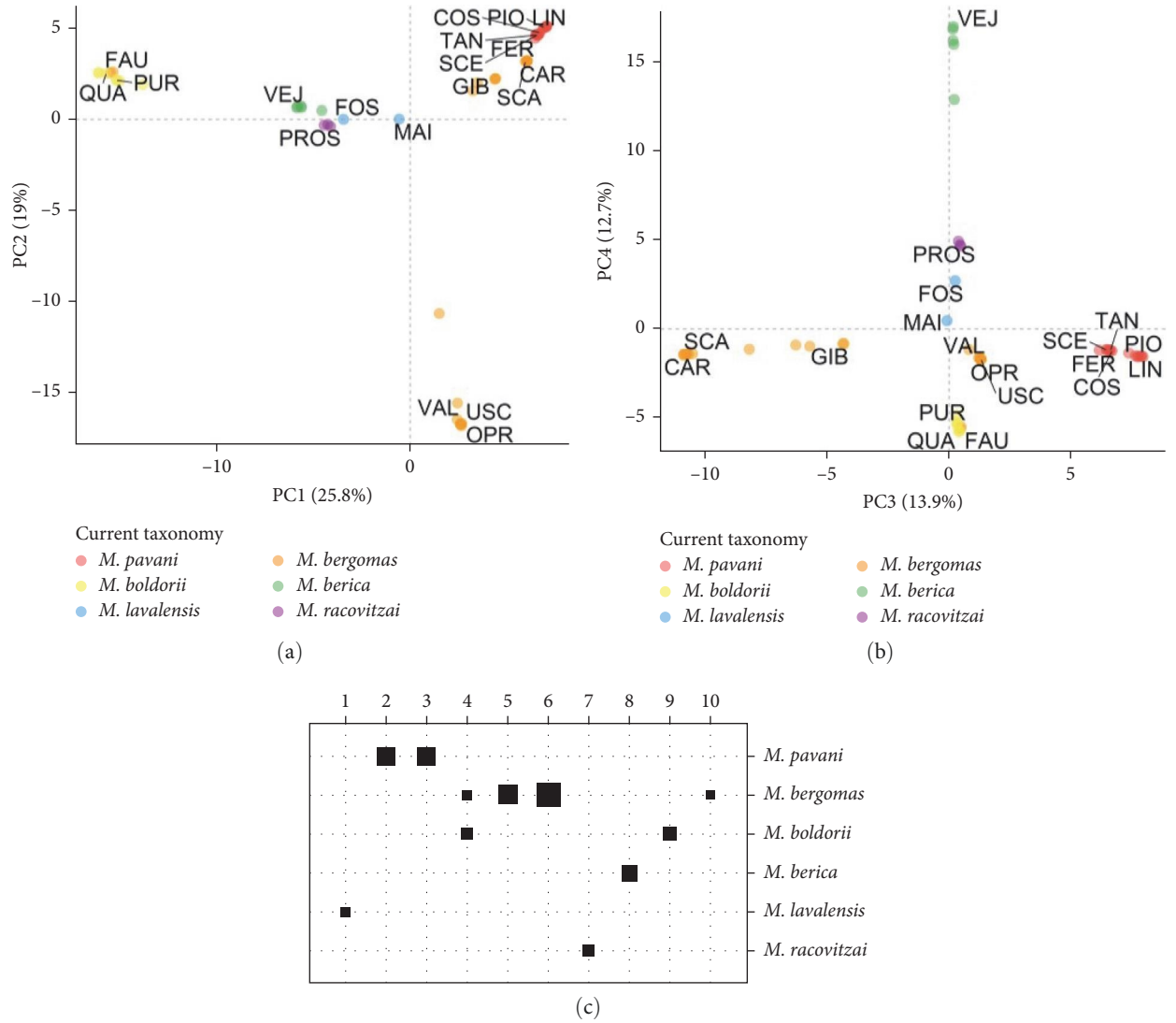

Figure 3: Genetic differentiation among *Monolistra* species. Principal component analysis (PCA) summarizing genetic variation among individuals on the first four PCs of PCA: (a) PC1 vs. PC2; (b) PC3 vs. PC4. Point colors represent the current taxonomy. Labels indicate population ID. (c) Results of the discriminant analysis of principal components (DAPCs), showing assignment probabilities of each individual of the six species (current taxonomy) to each identified genetic cluster (best $K = 10$; Figure S4) based on genetic variation.

(*M. lavalensis*), and C was completely separated at $K = 10$. Clade F (*M. bergomas*) clustered separately from Clade G (*M. pavani*) at $K = 5$. Finally, for Clade G, populations from the Valle Intelvi, West of Como Lake (FER; SCE; TAN; COS), clustered separately from populations in Lario (LIN; PIO) at $K = 7$, according to the DAPC, while further clustering showed admixed populations (Figure S6).

The global ADMIXTURE analysis revealed between-population differentiation for Clades D and F. For Clade D, populations QUA and PUR of *M. boldorii* clustered separately at $K = 8$, and populations FAU (*M. bergomas*) and QUA (*M. boldorii*) at $K = 11$ (Figure S6). Clade F (*M. bergomas*) split into two groups at $K = 9$, with the population GIB in a different cluster than populations CAR and SCA. Both these patterns are in accordance with the DAPC as well. Increasing the K value up to the number of populations (19) did not allow to differentiate them (Figure S6). Nonetheless, running three separate ADMIXTURE analyses on the Clades E, F, and G—respectively on a dataset of 312, 4345, and 6742 independent SNPs—revealed that all populations, corresponding to individual caves, were genetically distinguishable within each clade, except the populations LIN and PIO of *M. pavani* (Figure 4).

3.4. Genetic Differentiation. The level of genetic divergence between the seven phylogenetic clades identified was high and represented a substantial proportion of genetic variation (AMOVA; $V_{\text{clade}} = 78.2\%$ of total variance; p-value < 0.001; Table S5). Pairwise F_{ST} between populations was 0.91 ± 0.18 (mean $\pm$ SD) and ranged between 0.77 and 0.96 among the seven clades (Figure 5; Tables S3 and S4). The genetic differentiation between-populations within clades was low compared to clade divergence (AMOVA; $V_{\text{population}} = 18.0\%$ of total variance; p-value < 0.001; Table S5), with strong variation across clades. In fact, the average pairwise F_{ST} within clades ranged

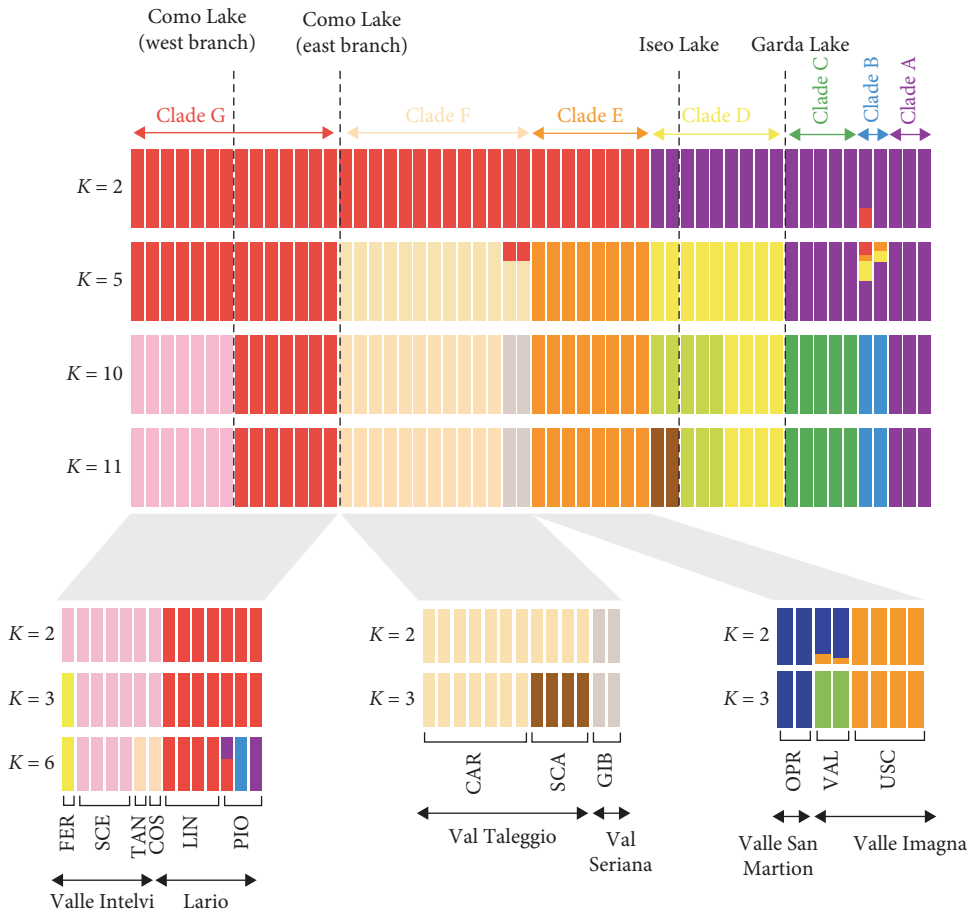

FIGURE 4: Results of ADMIXTURE analysis. ADMIXTURE coefficients among clades (global analysis) and within-clades (separate analyses for Clades E, F, and G) for different numbers of genetic clusters (K). Each bar represents an individual. Each color represents a different genetic group.

between 0.17 and 0.79, respectively, for Clades E and D (Figure 5; Tables S3 and S4). Last, between-samples differentiation within population was not significant (AMOVA; $V_{\text{individual}} = 0.1\%$ of total variance; p-value $= 0.603$), and within sample variability was low (AMOVA; $V_{\text{sample}} = 3.7\%$ of total variance; p-value < 0.001; Table S5).

4. Discussion

Genomic assessment of cave-dwelling *Monolistra* species in the Western Prealps identified seven well-supported phylogenetic clades and suggests that the evolutionary history of these crustaceans is more complex than assumed by current taxonomy. Both between- and within-clade differentiation was high, indicating that the level of underground biodiversity could be greater than currently understood. Our study highlights the value of integrating genomic approaches in species delimitation and for understanding the factors shaping the spatial distribution of genetic variation, providing a valuable contribution for the assessment and monitoring of underexplored biodiversity.

4.1. ddRADseq for Assessing Intra- and Interclade Diversity. Nearly all molecular studies in isopods have been performed using multigene phylogeny [67, 68]. Multigene approaches have been pivotal to obtain quantitative estimates of the evolution of these organisms, but they still cannot provide the genome-wide information that is necessary to resolve complex evolutionary histories, for instance, in fast-evolving groups [69, 70]. Given the lack of knowledge on the genomics of *Monolistra* and of isopods in general, we used a ddRADseq SNP typing to assess evolutionary relationships in this genus. Reduced-representation sequencing datasets have been useful for both resolving species delimitation and discover within-clade diversity [71–76], including in cave organisms [23]. In isopods, RADseq was successfully applied to study intraspecific differentiation [77], but has proven ineffective when divergence between lineages is too large [78]. Our ddRADseq experiment revealed strong genomic divergence between clades (78%) and high support for evolutionary relationships in the species tree. Genetic divergence within clades was generally weak (18%) and ranged from low ($F_{ST} = 0.002$) to very high ($F_{ST} > 0.95$) levels of differentiation among the clades. Nonetheless, most populations were separated in within-clade admixture analysis. Our findings, therefore, show that genome-wide SNPs allow efficient exploration of both between- and within-clade diversity in the highly diverse genus *Monolistra*.

The observed divergence between *M. julia* and all the other six species, as suggested by limited power in phylogenetic tree

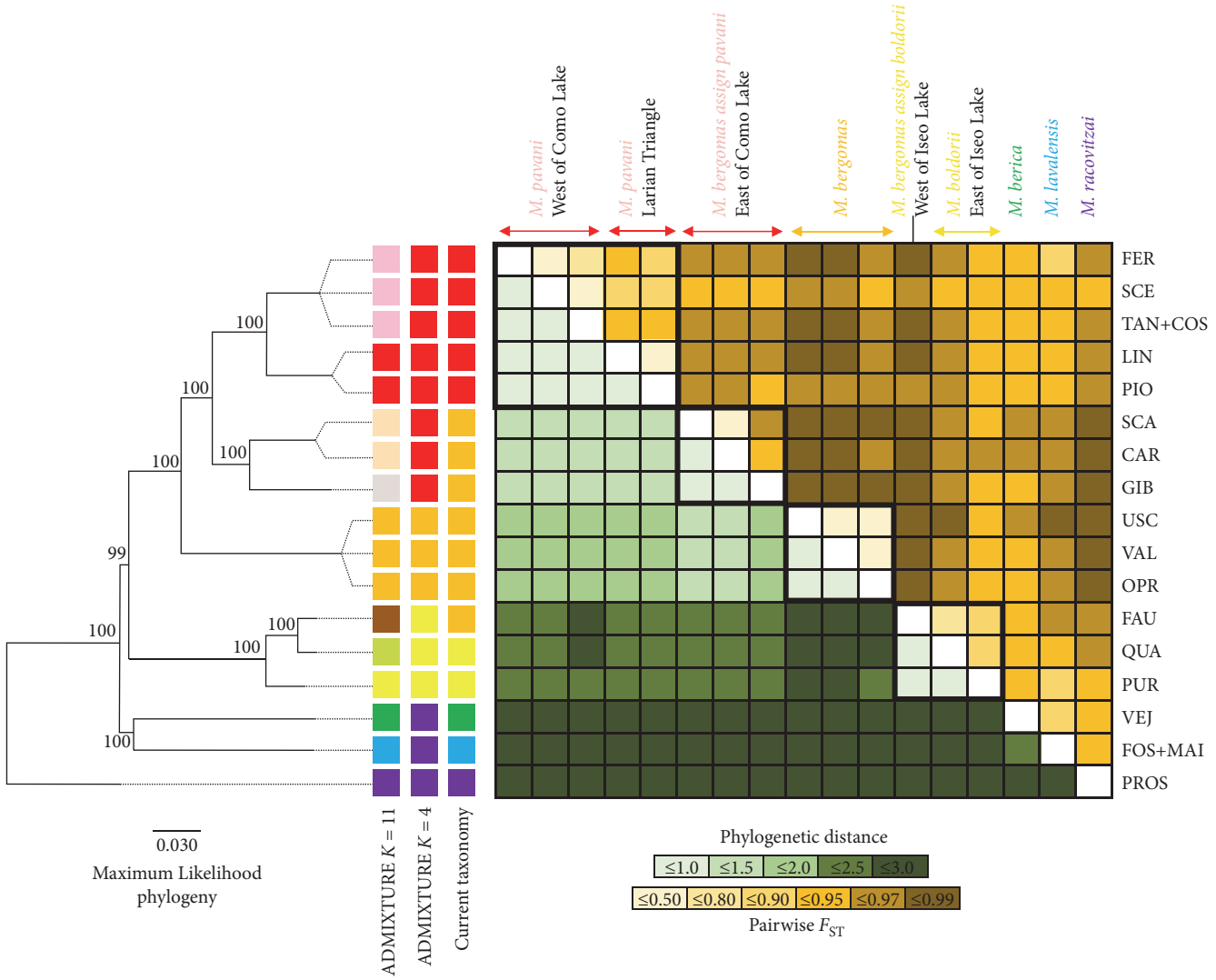

FIGURE 5: Summary result of genomic differentiation in *Monolistra*. From left to right: Maximum Likelihood species tree; ADMIXTURE clustering at $K = 11$ (optimal ADMIXTURE K) and $K = 4$ (main PCA groups); current taxonomy; phylogenetic distances between populations (average between all pairs of individuals; value in 1×10^2); genetic differentiation between populations (pairwise Weir and Cockerham F_{ST}).

inference (Figures S1 and S2) and higher phylogenetic distances (Table S3), supports that *M. julia* belongs to another subgenus (Table 1) [36]. The low genotype call rate of *M. julia* individuals (31%–60%; Table S2) is probably a consequence of the high divergence between *M. julia* and the other analyzed species, resulting in a low number of shared loci and a low phylogenetic support (branch support = 40; Figure S2). Similarly, the call rate was lower for *M. lavalensis* (10%–72%), *M. racovitzai* (0%–76%), and *M. berica* (63%–84%), as compared to *M. bergomas*, *M. boldori*, and *M. pavani* (mean call rate: 92%). These species are only represented by few individuals and a single population in our dataset. As a result, the number of informative loci to resolve deep branches is limited, as compared to those between recently diverged lineages. This is supported by the low consistency between species tree and gene tree, by equivalent support to distinct topologies across locus trees (i.e., collocation of Clade D; Figure S3), and by genetic similarity between the phylogenetically distant species *M. julia*, *M. racovitzai*, *M. lavalensis*, and *M. berica* in population structure analyses. Therefore, our results should be carefully interpreted within an integrative taxonomic framework [18, 79].

4.2. Discrepancy Between Genomic Data and Current Taxonomy. Phylogenetic tree reconstruction and population structure analyses revealed strong mismatches between traditional morphological taxonomy and genetic relationships. Particularly, *M. bergomas*, which geographic distribution has been described to lay between the Iseo Lake to the east and the Como Lake to the west (Figure 1) [44], does not represent a monophyletic group. The seven *M. bergomas* populations sampled split into three different clades (D, E, and F; Figure 2). The "Risorgenza di San Faustino" (FAU) is the easternmost cave where specimens have been determined as *M. bergomas* based

on morphological characters and on the expected distribution of the species. Indeed, the sampled cave is within a system where *M. bergomas* was morphologically identified [44] (Table S1). However, individuals are genetically close (D = 0.003–0.007) to populations of *M. boldorii* (Clade D), and especially with the nearby population QUA (11 km), which is located on the other side of the Iseo Lake (Figure 1; Figure S7). Another population (GIB) just 16 km to the west of FAU carries the morphological features of *M. bergomas*, but belongs to a separate clade (Clade F). Our results therefore suggest to consider the FAU population as *M. boldorii*.

The populations in the core distribution of *M. bergomas* further split into two paraphyletic clades (Clades E and F). These two clades correspond to a north–south differentiation, with the southern populations (Clade E: VAL; OPR; USC) strongly differentiated from all other species, and the northern populations (Clade F: CAR; SCA; GIB) more genetically close to those of *M. pavani* (Clade G) based on both species tree and population structure analyses. Given the strong genetic differentiation, Clade G (i.e., *M. pavani*), Clade E, and Clade F (both currently attributed to *M. bergomas*) likely represent independent evolutionary units. *Monolistra bergomas* was originally described based on specimens from a cave named "Grotta del Salto" [47]. The cadastral number of this cave is not registered in the official cadaster of caves of Lombardy and has never been reported [80], making it impossible to locate and sample in our study. Nonetheless, the type locality of *M. bergomas* is reported to be located within the Imagna Valley [47], where our sampled populations belong to Clade E. Concerning Clade F, it showed a similar and significant genetic divergence with both the Clade G ($D = 0.015$) and Clade E ($D = 0.019$), suggesting that the three clades are still closely related evolutionarily. Clades E and F are morphologically similar, are both located in the Bergamo Prealps and some of the respective sampled caves are only few kms apart (Figure S7). The divergence between these two clades, therefore, calls for detailed morphological analyses, and the use of classical DNA markers to clarify their systematics [79].

Our study reveals previously unrecognized diversity within *Monolistra*, which has gone unnoticed due to the absence of genetic data. We provide the first comprehensive genomic dataset for evolutionary studies of *Monolistra*. In this study, species identities were based on literature-derived morphological descriptions, which limits the direct integration or morphological traits with genomic data. In the future it will be extremely important performing additional studies, combining genomic data with comprehensive quantitative morphological analyses, to capture the different facets of species diversity and to guide taxonomic revisions.

4.3. Spatial Patterns of Genetic Diversification. Palaeoclimatic events promoted the diversification of many cave-specialist species, often resulting in high endemism with many species exclusively found in individual cave systems [81–83]. In *Monolistra*, it appears that evolutionary lineages have a relatively wide geographic distribution, with the same clade being found in multiple caves that can be relatively distant from one another (Figure 1). Pleistocene climate events had a strong impact on the evolutionary history of cave-dwelling organisms [84–87]. The geographical positioning of the prealpine lakes, which were formed during the last glacial maximum, corresponds well to the splits within clades and could provide a paleogeographic framework that explains their present-day distribution. Phylogenetic relationships, population structure, and between-clades genetic differentiation support a longitudinal divergence of clades from east to west [44], with two different common ancestors for eastern (*M. julia*, *M. racovitzai*, *M. lavalensis*, and *M. berica*) and western (*M. bergomas* and *M. pavani*) species, separated by the Garda Lake. This pattern remained consistent when including the genetically distant species *M. julia* into analyses (Figures S1 and S2). An exception to this pattern is *M. boldorii*, whose specimens cluster with the eastern group of species in the gene tree and admixture analysis, whereas with the western group in the species tree. The incongruence between species tree and gene tree, the uncertainty in phylogenetic position of some clades, and the relatively shallow divergence between deep nodes could suggest incomplete sorting of ancestral variation between lineages [59, 88–90], potentially driven by rapid and recent divergence in isolated cave systems [91, 92], or that methodological limitations prevented accurate reconstruction of deep branches (low number of shared loci).

Present-day geographical barriers may not correspond to the historical processes that led to species divergence. For instance, populations genetically identified as *M. boldorii* are found on both sides of Iseo Lake, suggesting that the species may have diverged before this barrier has formed, even if it now restricts gene flow [93–95]. Confirming the observed pattern would require sampling additional populations near Iseo Lake, including three occurrence records that have been reported in the past as as *M. bergomas* (Figure 1; Table S1) [44]. On the contrary, within-clade differentiation seems to be determined by geography. For example, the geographically distant populations GIB and SCA-CAR of Clade E show clear differentiation (28 km; $F_{ST} > 0.95$), whereas the geographically close populations (<22 km) within all three Clades E, F, and G show much lower differentiation ($0.002 < F_{ST} < 0.637$; Figure 5). In *M. pavani*, the genetic differentiation between populations of the Larian Triangle (LIN; PIO) and populations of Valle Intelvi ($F_{ST} > 0.95$), located on opposite sides of the Como Lake, supports that lakes act as effective barriers contributing to genetic divergence (Figure 1). Finally, the only two cave populations genetically undistinguishable (LIN and PIO; same admixture cluster; $F_{ST} < 0.002$) correspond to sample localities located at two different entrances of the same cave system. These results highlight that cave-restricted animals, not expected to disperse, also show classical patterns of geographical isolation [12].

5. Conclusions

Our assessment of evolutionary relationships among *Monolistra* species in the Western Prealps revealed strong genetic distinctions within species. This implies that the true level of diversity within *Monolistra* could be greater than currently understood. Accurate species identification is the fundamental basis to determining evolutionary significant units [71,

96–100]. Further assessing the existence of such diversity is, therefore, crucial for effective monitoring and conservation planning of subterranean biodiversity, which may face extinction before species are even documented or described [11, 101, 102]. Furthermore, each cave system appears to harbor genetically unique populations, indicating highly restricted gene flow and making them uniquely vulnerable to environmental changes and habitat alteration [103]. Such insights underscore the importance of protecting multiple cave habitats to preserve diversity, especially in regions where human activities increasingly threaten subterranean ecosystems. This would help preserve genetic diversity, prevent population declines, and reinforce the link between subterranean and surface ecosystems for a more effective conservation strategy.

Data Availability Statement

Individual sequences have been submitted to the European Nucleotide Archive repository and are accessible through Project Number PRJEB97081, with sample Accession Numbers ERS26771739–ERS26771811 in Table S2. SNP datasets supporting the results of this study are available in the Figshare repository (https://figshare.com/s/6a9d9946333a9ff0747a).

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Stefano Lapadula: formal analysis, investigation, writing – original draft, writing – review and editing. **Raoul Manenti**: conceptualization, investigation, supervision, writing – review and editing. **Benedetta Barzaghi**: conceptualization, investigation, writing – review and editing. **Gentile Francesco Ficetola**: conceptualization, funding acquisition, writing – review and editing. **Stéphanie Sherpa**: conceptualization, formal analysis, supervision, writing – original draft, writing – review and editing.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. (*Supporting Information*)
Figure S1. Preliminary analyses including *Monolistra julia*, using a maximum per-locus missing data threshold of 40%. Figure S2. Preliminary analyses including *M. julia*, using a maximum per-locus missing data threshold of 20%. Figure S3. ASTRAL-III normalized quartet scores. Figure S4. Determination of the optimal number of genetic clusters (K) for adegenet K-means clustering analysis. Figure S5. Determination of the optimal number of genetic groups (K) for ADMIXTURE analysis. Figure S6. Global ADMIXTURE. Figure S7. Distribution of *Monolistra bergomas* and *Monolistra boldorii* (according to current taxonomy). Table S1. Occurrence records of *Monolistra* species. Table S2. Results of the Stacks pipeline. Table S3. Genomic divergence among phylogenetic clades. Table S4. Pairwise F_{ST} (Weir and Cockerham) between localities of the genus *Monolistra*. Table S5. Results of AMOVA.

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