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# Conservation of freshwater biodiversity in Tunisia in a climate change context: combining amphipod distribution data and molecular analyses to improve priorities

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Running headline: Molecular diversity and distribution of amphipods in Tunisia

## **Abstract**

The exceptional diversity of freshwater fauna of the Mediterranean Basin currently faces a crisis in which climate change combined with overexploitation of freshwaters heavily threatens the local fauna. In this context, it is urgent to define conservation priorities on how to best protect freshwater biodiversity. One of the main limits to define such actions remains the lack of knowledge in many countries. In this study, we test the usefulness of molecular data (COI gene) combined with morphological identification to better predict the pattern of biological diversity and threats of climate change on freshwater biodiversity. We focused our study on the freshwater amphipods as model organisms in order to define conservation strategies in Tunisia, one of the most threatened countries. Our results confirmed that amphipods diversity is largely underestimated with 9 species identified by their morphology and 33-39 species assigned depending on delimited with the most parsimonious molecular delimitation method. The distribution of amphipods is mainly restricted to the northern part of Tunisia and seems to be positively correlated with precipitation and negatively correlated with thermal amplitudes and precipitation fluctuations. These environmental factors are sensitive to climate change and confirm that conservation strategies need to be redefined and adjusted in the face of future climate predictions. Moreover, the total diversity and spatial distribution patterns provided by molecular methods seem to be more detailed and accurate than results based on morphology alone and nicely complement traditional species assignment.

**Key words:** freshwater biodiversity, Crustacea, Africa, Maghreb, global warming, flow intermittency

## **Introduction**

The Mediterranean Basin ranks among the twenty-five most important biodiversity hotspots worldwide (Myers et al. 2000). Due to its high degree of endemism, it is even considered as a "hyper-hot" candidate for conservation support. The freshwaters of the circum-Mediterranean countries are indeed known for their exceptional biodiversity, housing ca. 35% of Palearctic freshwater species and more than 6% of the world's freshwater species with a high rate of endemism (Tierno de Figueroa et al. 2013). However, Mediterranean freshwater biodiversity is greatly threatened by anthropogenic pressure. The ongoing destruction of habitats, invasions of alien species, and finally, global climate change are among the most important factors causing biodiversity loss in the Anthropocene (Caro et al. 2012; Pereira et al. 2010; Sala et al. 2000), at an estimated speed estimated to be ca. 100 times greater than during geological epochs (Pimm and Raven 2000).

In the Mediterranean region, the North African countries are particularly subjected to anthropogenic impacts, and Tunisia is a prime example. The country currently faces a crisis in which climate change combined with overexploitation of freshwaters heavily threatens the local, highly understudied biodiversity. The water stock in Tunisia is relatively low compared to other countries, particularly on the northern shore of the Mediterranean Sea (Peel et al. 2007). With ca. 201 m<sup>3</sup>/year/inhabitant of exploitable water resources (Romagny and Cudennec 2006), Tunisia is one of the countries with the scarcest water supplies, which should translate to between 500 m<sup>3</sup>/year/inhabitant and 1000 m<sup>3</sup>/year/inhabitant according to international standards (Margat 2008). The country extends latitudinally through four different bioclimatic zones. In the northwestern part of the country, total annual rainfall ranges from 10 mm/y to 1500 mm/y, to even less than 50 mm/y in the southwesternmost part of the country (NIM 2016). The construction of hydraulic facilities has grown considerably since the 1980's to compensate for this scarce rainfall, and several surface water mobilisation structures have been built on

watercourses in catchment basins, mainly in the northern part of the country. So far, 40 reservoirs, 237 hillside dams, and 902 hillside lakes have been built, mobilising more than 90% of surface waters (Ben Rejeb Jenhani et al. 2019).

It can be argued that climate change and its direct or indirect consequences will become one of the greatest threats to biodiversity and one of the main causes of changes in the biogeographic ranges of species (Both et al. 2006; Genner et al. 2004; Le Roux and McGeoch 2008; Parmesan 2006; Walther et al. 2002). In this context, it is urgent to define conservation priorities on how to best protect freshwater biodiversity. However, temporal changes in the distribution of aquatic invertebrates in most regions of the world have generally received less attention than changes in the distribution of vertebrates (e.g., fish, amphibians) (Dudgeon 2000). Moreover, data about species extinctions or inventory lists are still lacking for most of river and stream biodiversity (Palmer et al. 2000).

In this study, we propose to use amphipods as model organisms to define conservation strategies in Tunisia. This widespread crustacean group exhibits extraordinarily high diversity in freshwaters, with around 2000 known freshwater species on a global scale (Väinölä et al. 2008), inhabiting all kinds of freshwater ecosystems (running waters, lakes, groundwaters). Amphipods are among the most important freshwater macroinvertebrates in terms of biomass. They greatly facilitate energy transfer, both in continental Europe and in Mediterranean freshwater ecosystems (Forrow and Maltby 2000; Macneil et al. 1997), and are considered as a key species of freshwater trophic food webs (Piscart et al. 2009). They are also considered as a very good model for ecological (Piscart et al. 2011; Dehedin et al. 2013a; Foucreau et al. 2014; Pellan et al. 2016), evolutionary (Copilaş-Ciocianu et al. 2017, 2020) and ecotoxicological studies (Dehedin et al. 2013b; Maazouzi et al. 2016; Henry et al. 2017; Hupało et al. 2018b).

Several systematic and ecological studies have focused on freshwater amphipods found in the Mediterranean region (Karaman and Pinkster 1977, 1987; Karaman and Ruffo 1977; Piscart et

al. 2013; Rewicz et al. 2014; Ayati et al. 2018; 2019), and identified more than 100 distinct amphipod species reported from Mediterranean islands alone (Hupało et al. 2021). Nevertheless, the freshwater amphipods of the Mediterranean basin have remained less studied than their marine counterparts. Also, an extraordinarily high level of cryptic and pseudocryptic diversity has recently been discovered among freshwater amphipods in Europe, including the northern Mediterranean region (Grabowski et al. 2017a, 2017b; Hou et al. 2011, 2014; Mamos et al. 2014, 2016; Weiss et al. 2014; Katouzian et al. 2016; Wattier et al. 2020). One can also assume similar levels of as yet undiscovered diversity in Northern Africa, especially given the scarcity of molecular research led in the region. In Tunisia, previous studies have revealed the presence of one species of the genus *Rhipidogammarus* Stock, 1971, another one belonging to the genus *Gammarus* Fabricius, 1775 and seven other species belonging to the genus *Echinogammarus* Stebbing, 1899 (Ayati et al. 2019 and references therein). Yet, based on previous findings from Mediterranean freshwaters, one may expect the real level of diversity to be much higher, and the number of currently reported species may well be underestimated.

In this context, the present study aims to (i) complement knowledge on the diversity and distribution of freshwater amphipods in Tunisia, (ii) quantify the level of hidden diversity within the conventionally recognised morphospecies, (iii) determine the climate-related threats on amphipods throughout their spatial distribution along the north-south climatic gradient, and (iv) estimate the share of hidden diversity in the conservation plan for freshwater biodiversity in Tunisia.

## **Materials and methods**

### *Study area*

Tunisia is the smallest country in Northern Africa, with 165,000 km<sup>2</sup> and a vast coastline along the Mediterranean Sea (1,300 km) (Figure 1). The climate strongly varies between the north and the south of the country according to elevation and the distance from the sea. As a result, Tunisia is characterised by a latitudinal gradient of climatic conditions ranging from a hot-summer subtropical Mediterranean climate in the mountainous north to a hot desert climate in the south (Figure 1a).

The water potential is very small (FAO 2014). Rainfall is also unequally distributed in space (from 31.6 mm/y in the southwest near Tozeur to 709.8 mm/y in the northwest near Jendouba) and very irregular over time. The resulting hydrological network comprises mainly intermittent streams and lakes, with a few permanent rivers throughout the year (Figure 1b).

### *Sampling*

Material was collected from 212 sites, including 27 sites visited in March 2010 (for details, see Rewicz et al. 2014) and 185 sites surveyed between November 2015 and April 2017, among which 76 (17 sites from Rewicz et al. 2014 and 59 sites from our study) harboured amphipods (Figure 1b; Table S1). Amphipods were sampled in the different habitats with a hand net (500- $\mu$ m mesh size) to collect at least 30 individuals at most sites. A minimum of 10 individuals was collected from scarcely populated sites, without standardization of the sampling time but usually less than 2h of sampling effort. Indeed, when two amphipods species co-occurred in the same site, the proportion of each species is relatively balanced (Ayati et al. 2019) and rarely exceed 90% of the number of individuals for the dominant species (except in the case of biological invasions, Piscart et al. 2011). In the laboratory, the amphipods were identified down to the morphospecies level based on the available taxonomic keys (Ayati et al. 2018; Fadil and Dakki 2003; Grintsov 2009; Özbek and Ustaoglu 2007; Pinkster 1993, Piscart et al. 2013).

### *DNA extraction, PCR amplification and sequencing*

DNA was extracted using the standard phenol/chloroform method (Hillis et al. 1996), following the protocol previously described by Hupało et al. (2018a). Extracted DNA was stored at 4°C until amplification and finally long-term stored at -20°C. A fragment of the cytochrome *c* oxidase subunit I gene (COI) was amplified using primers LCO1490-JJ and HCO2198-JJ (Astrin and Stüben, 2008), under the following PCR conditions: 60 s at 94°C, 5x (30 s at 94°C, 90 s at 45°C, 60 s at 72°C), 35x (30 s at 94°C, 90 s at 51°C, 60 s at 72°C), 5 min at 72°C, as proposed by Hou et al. (2007). All PCR products (5 µl) were cleaned up using exonuclease I (ThermoFisher Scientific) and alkaline phosphatase FastAP (ThermoFisher Scientific) according to the manufacturer's guidelines. Direct sequencing was performed using the same forward primer as the one used for amplification, and the BigDye terminator technology in Macrogen Europe sequencing company (Netherlands).

### *Sequence data authentication, editing, alignment and deposition*

All sequences were confirmed as belonging to *Echinogammarus* and *Rhipidogammarus* following BLAST searches in GenBank (Altschul et al. 1990). Then, they were assembled and aligned with the default Geneious algorithm in the Geneious 10.0.9 software package (Kearse et al. 2012).

All the sequences were deposited in GenBank (ON258344 - ON258599; Table S2). Additionally, all the DNA sequences were deposited in a separate DS-TUNAMP dataset in the public repository of the Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert 2007), where all the relevant metadata and sequence trace files will be publicly available upon publication (<http://dx.doi.org/10.5883/DS-TUNAMP>).



### *MOTU delimitation and phylogeny reconstruction*

The Molecular Operational Taxonomic Units (MOTUs) were delimited using the COI sequence dataset, according to the distance-based Barcode Index Number (BIN) System implemented in BOLD (Ratnasingham and Hebert 2007). The results of BIN delimitation were cross-validated with another distance-based method, namely the Assemble Species by Automatic Partitioning (ASAP) methodology (Puillandre et al. 2021) using K2P distance and run online (<https://bioinfo.mnhn.fr/abi/public/asap/>), as well as with a phylogenetic tree-based delimitation method, namely Multi-rate Poisson tree processes (mPTP; Kapli et al. 2017), which was also run online (<https://mptp.h-its.org/>).

For final visualisation, the Neighbour-Joining tree of all COI sequences was created, using Tamura-Nei model of evolution with 1,000 bootstrap replicates, in MEGA7 software (Kumar et al. 2016).

### *DNA barcode analysis*

Mean and maximum genetic distances, as well as the distance to the nearest species, were calculated based on the Kimura 2-parameter model (K2P; Kimura 1980), using the analytical tools of BOLD workbench (Barcode Gap Analysis, Distance Summary).

We assessed genetic diversity as the number of haplotypes (k), haplotypic diversity (h), and nucleotide diversity ( $\pi$ ) (Nei 1987) with DnaSP v5 software (Librado and Rozas 2009). Values were calculated for each species and particular MOTU.

### *Geographical patterns of species diversity*

We illustrated the spatial distribution of amphipod diversity in Tunisia regarding morphospecies and the MOTUs. The two types of diversity were interpolated using the ordinary kriging method incorporated in the spatial analyst toolbox of ArcMap 10.8.1 (ESRI®) by taking into account the five closest neighbouring sites with a maximum spatial distance fixed at 100 km. The ordinary kriging for which the assumption of stationarity (that the mean and variance of the values is constant across the spatial field) was chosen because the number of species/MOTUs is relatively low (1-5) and relatively stable (>90% of sites with amphipod have only one species/MOTU). The distance of 100 km was chosen as a compromise between the spatial distribution of amphipod species which is rather limited (Ayati et al. 2019) and the ability of amphipods to disperse spatially.

### *Statistical analyses*

In order to study the relationship between amphipod assemblages and climatic variables, we used the mean climatic variables between 1970 and 2000 collected from WorldClim database Version 2 (<http://www.worldclim.org>): minimum (Tmin), maximum (Tmax) and mean temperature (Tmean); minimum (Pmin), maximum (Pmax) and mean precipitation (Pmean). We also computed additional climatic variables corresponding to the thermal amplitude (Delta Tmean) – the difference between Tmax and Tmin –, and the mean annual coefficients of variation of temperature (CVTmean) and precipitation (CVPmean). The climatic variables were collected for all 212 sampling sites.

Generalised linear models (GLMs) assuming binomial distributions were used to test if the presence/absence of species was related to climatic variables. Tmin, Tmax and CVTmean were omitted from the models because they were strongly correlated with other variables. The models were selected using a backward process based on the Akaike Information Criterion (AIC). Two

models were considered significantly different when  $\Delta AIC > 2$  (Burnham and Anderson, 2002). The simplest model was preferred after omitting non-significant fixed effects step by step, based on the t-statistic.

Moreover, sites with amphipods were classified using a K-mean clustering algorithm using climatic variables and a Principal Component Analysis (PCA) was used to assess the link between the clusters obtained and climatic variables. A distance-based Redundancy Analysis (db-RDA) was performed on Bray-Curtis similarities (Legendre & Anderson, 1999) with a forward variables selection by permutation procedure (Blanchet, Legendre & Borcard, 2008) was used to detect linear relationships between climatic variables as predictors and amphipod assemblages as the response variable. The significant impact of each climatic variable on amphipod assemblages were tested by distance based linear modelling applied with a backward procedure and Bayesian Information Criterion (BIC) criterion using the DistLM function in Primer 6 software (PRIMER-E™).

In order to characterise the clusters by testing whether they could be characterised by one or more indicator MOTUs or morphospecies, we used the indicator value (IndVal) using the *IndVal* function of the 'indicspecies' package (Cáceres and Legendre 2009). More precisely, we focused on the specificity of the species as an indicator of the site (i.e., the value of component A) which is the probability for the surveyed site to belong to the target site group given the fact that the species has been found. All subsequent analyses were carried out using R 3.3 software and Rstudio version 2022.7.1 (RStudio Team 2022).

## **Results**

### *Presence of amphipods in freshwaters of Tunisia*

We did not find a strong difference between the geographical distributions of sites harbouring or not harbouring amphipods, even if the proportion of sites without amphipods seemed slightly higher in southern and eastern Tunisia (Figure 1b). The best model explaining the presence of amphipods (McFadden Pseudo- $R^2 = 0.90$ ,  $p < 0.001$ ) included mean precipitation, CVP<sub>mean</sub> and Delta T. In this model, the mean precipitation was significantly and positively correlated with the presence of amphipods ( $p < 0.001$ ), whereas CVP<sub>mean</sub> and Delta T<sub>mean</sub> were negatively correlated ( $p < 0.001$  and  $p = 0.002$ , respectively).

#### *Diversity of freshwater amphipods based on morphological assignment*

Amphipods were collected only in 76 sampling sites out of 212 (59 in the present study and 17 in 2010) (Figure 1b, Table S1). Nine gammarid species were identified using their morphological characters: six species already known in Tunisia (*Rhipidogammarus rhipidiophorus*, *Echinogammarus afer*, *Echinogammarus olivii*, *Echinogammarus pungens*, *Echinogammarus simoni*, *Echinogammarus tacapensis*), a new species for the country (*Echinogammarus haraktis*), and two species (*Echinogammarus carthaginiensis* and *Echinogammarus tunetanus*) recently described by Ayati et al. (2018) based on the material also used in the current study. Moreover, one species collected in 2010 (*Echinogammarus sp.*) remained undetermined morphologically and could represent a new species for science.

#### *Diversity of freshwater amphipods based on MOTU delimitation*

We generated 256 new COI sequences, covering all identified morphospecies, and used two additional sequences from Tunisia available from GenBank (KF478550, KF478551; Hou et al. 2014). Three MOTU delimitation methods provided similar results (Figure 2a). The BOLD clustering method revealed 39 BINs, varying from one BIN within *E. tacapensis* and *R.*

*rhipidiophorus* to 15 within *E. simoni* (Table S3). Best ASAP clustering also revealed 39 MOTUs (score: 7.5,  $p = 1.44^{e-01}$ ), which were fully congruent with those pointed out by BINs (Figure 2a). Delimitation using mPTP revealed only 33 MOTUs, and differences were observed in five cases compared to the other two methods. Within *E. olivii* two BINs (AEN3245; ADE1906) were merged into one MOTU. *Echinogammarus tunetanus* MOTU (ADH0211) was split into two MOTUs, and two BINs (ADH1060; ADK9117) merged into one MOTU. Within *E. simoni* two MOTUs (ADK9114, ADK9208) were merged into one MOTU, with another five BINs (ADK9118, ADE2063, AEM8341, ADH1058, and ADE1881) also merged into one MOTU (Figure 2a). Based on the delimitation results, we decided to focus on entities (MOTU) delimited with BINs, also supported by ASAP delimitation in the downstream analyses. All MOTUs were generally characterised by a relatively high haplotype and nucleotide diversity (Table S3). The mean K2P distance within morphospecies varied from 0.12% and 0.17% minimum in the case of *E. tacapensis* and *R. rhipidiophorus* to 8.96% and 13.87% maximum in the case of *E. sp.* and *E. simoni*, respectively. The maximum K2P distance within morphospecies had lowest values (varied from 0.65% and 0.75%) for *E. tacapensis* and *R. rhipidiophorus* (only single MOTU species). On the contrary maximum values (17.75% and 24.1%) were detected for *E. haraktis* and *E. simoni*, respectively (Table S3). The nearest distances between different *Echinogammarus* morphospecies varied from 12.51% minimum K2P between *E. afer* and *E. tacapensis* to 21.42% maximum between *E. pungens* and *E. haraktis*. The highest value for the nearest K2P distance parameter (25.85%) was observed between *R. rhipidiophorus* and *E. simoni* (Table S3).

### *Species distribution*

The distribution pattern of morphospecies and MOTU followed a very similar trend, with a higher diversity in the northwestern and western parts of the country near Tozeur (Figure 2b, Figure 3). The overall spatial variations seem to be more intense for morphological diversity than for MOTU diversity. Contrary to morphospecies with distribution areas reaching several hundreds of km, MOTUs were generally found in a few localities (one to four different localities), and only four MOTUs (ADE2848, ADD7985, ADE2420, ADE1881) were detected in more than two localities, but still within the same region.

#### *Climate-species associations*

The PCA of climatic variables showed that the first PCA axis explained 63.9% of variation and was determined by temperature (Figure 4a). The second PCA axis only explained 19.4% of variation. Forty-eight percent of this variation was related to mean precipitation. Based on the similarity of climatic variables, the clustering procedure identified four clusters composed of 7, 29, 10, and 30 sites, respectively, with various BINs harboured (Table 1). The four clusters of the K-mean clustering analysis were clearly distributed into three groups in terms of climatic variables (Figure 4b). The sites of cluster 1 were characterised by high temperature and a low coefficient of variation, those of clusters 2 and 4 by high and low precipitation and high variation in temperature and those of cluster 4 differing from cluster 2 by lower values. The sites of cluster 3 were characterised by high precipitation and high seasonal variations in terms of deltaT and CVPmean.

The first three axes of the dbRDA showed that the climatic variables explained only 10% of the MOTU distribution and increased to 16.7% when considering morphospecies distribution. Among these variables, only Tmin, Tmean, Tmax, and deltaT ( $P = 0.006$ ) were significantly correlated with the MOTUs ( $P$ -values  $< 0.006$ ) or morphospecies assemblages ( $P$ -values  $< 0.009$ ).

In term of MOTU composition, faunal composition of cluster 1 were characterised by only two BINs: *E. haraktis* (ADD7985) and *E. afer* (ADE2848) with a specificity of 25% ( $P = 0.016$ ) and 23% ( $P = 0.016$ ), respectively. The same indicator species were found using morphospecies only with a specificity of 23% for *E. haraktis* ( $P = 0.036$ ) and 35% for *E. afer* ( $P = 0.001$ ), respectively (Table 1). This cluster pooled together the warmest sites located in the south of Tunisia (Figure 5). Clusters 2 did not harbour significant indicator species ( $P$ -values  $>0.084$ ), neither with MOTUs nor with morphospecies. For cluster 4, only *E. carthaginiensis* is considered as indicator when using morphospecies with a specificity of 19% ( $P = 0.047$ ) but no indicator MOTUs were found ( $P$ -values  $> 0.1$ ). Cluster 4 pooled together sites located near the Mediterranean coasts of Tunisia (Figure 4) with three BINs: *R. rhiphidiophorus* (ADH0194), *E. pungens* (ADH11973), and *E. carthaginiensis* (ADK9348) well distributed in the cluster. Finally, sites of the third cluster were characterised by only one BIN corresponding to *E. pungens* (ADE0367) with 20% specificity ( $P = 0.017$ ). The sites of cluster 3, as for cluster 2, were distributed in northwestern Tunisia. (Figure 5).

## Discussion

### *Diversity of freshwater amphipods in Tunisia*

Ten morphospecies were identified in our study, including two brackishwater species (*R. rhiphidiophorus*, *E. olivii*), seven freshwater species including four already well known species in Tunisia (*E. afer*, *E. pungens*, *E. simoni*, and *E. tacapensis*), a new species for Tunisia (*E. haraktis*), and two new species (*E. carthaginiensis* and *E. tunetanus*) recently described by Ayati et al. (2018), including one morphologically undetermined *Echinogammarus* sp. However, our samplings failed to find the endemic *Echinogammarus dactylus*, known only from its type locality (not accessible during the sampling campaigns). The diversity of epigean

amphipods in Tunisia was lower than in west Mediterranean countries in Europe, such as Spain (25 species; Pinkster 1993), Italy (25 species; Ruffo and Stoch 2006), Greece (22 species; Karaman and Pinkster 1977a, b, 1987), and Turkey (49 species; Ipek and Ozbeck 2022). Our study notably increased the number of freshwater gammarid morphospecies recorded in Tunisia from 7 to 11. As a result, Tunisia, with 0.06 species per km<sup>2</sup> is now at a similar level of freshwater amphipod species diversity as other countries of the northern part of the Mediterranean coast (ranging from 0.05 species per km<sup>2</sup> for Spain to 0.17 species per km<sup>2</sup> for Greece) regarding the number of species to country surface ratio.

Molecular species delimitation revealed the presence of 33 (mPTP) to 39 (BIN, ASAP) MOTUs, that can be considered as rough and imperfect but handy proxies for species. If these results are confirmed, the number of known gammarid species in Tunisia will be tripled or even quadrupled. High hidden diversity has been already evidenced in numerous conventionally recognised and widely distributed morphospecies of gammarids in Europe (e.g., Mamos et al. 2016, Copilas-Ciocianu et al. 2017, Grabowski et al. 2017a, Wattier et al. 2020) and such phenomenon seems to be a rule in this group. In several cases, using the molecular markers and integrative taxonomy approach it was even possible to describe such cryptic or pseudocryptic species (e.g. Grabowski et al. 2017b, Hupało et al. 2018a, Rudolph et al. 2018, Mamos et al. 2021). However, given that our results are based on a single mitochondrial polymorphism marker, no definitive taxonomic conclusion can be drawn based on this sole evidence (Mamos et al. 2021, Hupało et al. 2022). On the other hand, MOTUs within the confamiliar genus *Gammarus* divergent by more than 4% K2P genetic distance do not usually form precopulas in the wild; this suggests the presence of prezygotic isolation at this level of divergence (Lagrué et al. 2014). Following this assumption and taking into account that the mean K2P distance exceeds 4% in *E. pungens*, *E. haraktis*, *E. tunetanus*, *E. cartaginiensis*, *Echinogammarus* sp., and *E. simoni*, one can suppose that these morphospecies may harbour as yet undiscovered



species. Optimal testing of this hypothesis will at least require further multimarker studies employing several independently evolving nuclear genes, but also preferably an integrative approach including morphological studies at the ultrastructural level (see Hupało et al. 2018a; Morhun et al. 2022 for a comparison). Nevertheless, in malacostracan crustaceans, polymorphism of mitochondrial markers, including COI, is known to satisfactorily reflect divergence and diversification events in groups of closely related species at both the intraspecific and intrageneric levels (e.g. Rudolph et al. 2018, Jabłońska et al. 2021, see Coates et al. 2018 for review). Assuming that modern nature conservation largely relies on the preservation of natural evolutionary processes, revealing the genetic structure of biota is fundamental for identifying conservation units and priorities. As such, it is within the focus of protection and conservation programs (Hughes et al. 2008; Van Dyke 2008). Our results showing the high number of operational taxonomic units within the conventionally recognised amphipod species indicate that inland waters throughout Tunisia harbour a very high and unique diversity of amphipods. This points out that a proper conservation strategy aimed at preventing further degradation of critically endangered freshwater resources in Tunisia should use molecular data. Thus, the genetic structure of amphipods can serve as a handy proxy for identifying freshwater diversity, endemism hotspots, and hydrological systems deserving conservation.

### *Species distribution*

The distribution of amphipods in Tunisia is relatively fragmented, with less than half of the visited sites harbouring amphipods, most of them located in the north of the country. Our results indicate that the presence of amphipods in Tunisia seems to be positively correlated with precipitation and negatively correlated with thermal amplitudes and precipitation fluctuations. They suggest that the scarcity of epigeal waters, especially in Mediterranean climate

conditions, is likely the most important factor explaining the presence of amphipods. The vulnerability of crustaceans, mainly amphipods, to climate change has been observed globally (Imberger et al. 2016; Ledger et al. 2013; Stubbington et al. 2009, 2017). Sensitivity to drought is indeed greater among species with permanently aquatic life cycles, such as crustaceans (Aspin et al. 2019). To survive a drought event, many crustaceans have developed specific biological (e.g., small body size, diapause) or behavioural traits (e.g., burrowing, desiccation-resistant stages) (Aspin et al. 2019; Bêche et al. 2006; Bogan et al. 2017; Boulton 2003). Surface amphipods can hide in the river hyporheic zone to face the water flow recession (Stubbington et al. 2011). The knowledge on the different abilities of various amphipod species to cope with drought is scarce, some data shows that *Gammarus* species outperform *Echinogammarus* in the intermittent stream sections in temperate Europe (Meyer et al. 2004). However, this behaviour is heavily limited by (i) the sediment porosity (Dole-Olivier 2011; Vadher et al. 2015, 2017) and (ii) the permanence and the quality of hyporheic water (Boulton 2003). If severe droughts cause the water level to decrease in the hyporheic zone (also called 'subsurface drought'), amphipods have limited chances of survival (Vander Vorste et al. 2016).

The presence of amphipods is also affected by the stability of the environment across seasons/years. The negative effect of the thermal amplitude (i.e., seasonal variation) and changes in precipitation may indeed promote extreme seasonal events that add to the effect of drought (Brooks 2009 and references therein). A previous study in California found that the overall precipitation pattern for a given year (e.g., whether it is a dry or average to wet rainfall year) either exacerbated or mediated the impacts of seasonal drought on aquatic communities (Bêche et al. 2006). Like all ectotherms, amphipods are affected by temperature, which drives their survival and distribution limits (Chown 2001; Foucreau et al. 2014). However, our study revealed that climatic variables (mainly temperature) contribute to explain until 16.7% of the species distribution. We highlighted four groups of sites according to climatic variables and

geographic distribution, each group harbouring different amphipod assemblages. The first group of sites (i.e., cluster 1) included sites with the highest temperature in southern Tunisia. These sites harboured five morphospecies corresponding to five MOTUs, among which *E. afer* and *E. tacapensis* were the indicative species preferentially living in the sites of this group. These species are likely adapted to survive higher water temperatures (Cottin et al. 2015; Foucreau et al. 2014) and are only known in Algeria, Tunisia, and Libya (Pinkster 1993). The second group of sites (clusters 2 and 4) harboured 11 morphospecies and 34 MOTUs, including 11 of the 15 MOTUs associated with the morphospecies *E. simoni*. The species of these groups are widely distributed in Atlas Mountain in Tunisia and are located in the sites with the lowest precipitation and a high seasonal variation in temperature. Sites of the cluster 4 are restricted to a narrow stretch along Mediterranean coasts of Tunisia with *E. olivii* and *R. rhipidiophorus* present only in this cluster. This distribution is congruent with the ecology of these species that are known either in brackish water (*E. olivii*; Pinkster 1993) or from springs along the seashore (*R. rhipidiophorus*; Ayati et al. 2019). Finally, the last group of sites (cluster 3) harboured four morphospecies corresponding to eight MOTUs, among which one MOTU (ADE0367) of the morphospecies *E. pungens* is the only one indicative species. The geographical distribution of the cluster 3 largely overlaps those of the cluster 2 in Northern Tunisia but in sites with the highest precipitation rate and a strong seasonal variation. Among the 4 clusters, the cluster 1 and 3 could be strongly affected by climate change and reduced precipitation. In a global change context, rising temperature (by up to +5°C) and the reduction of precipitation (down to -27%) during the next decades in Tunisia (CMIP6 2022) may differently impair the distribution of species, even if local adaptations of the populations living under contrasting thermal conditions might slightly influence the consequences of rising temperature on their distribution (Cottin et al. 2012). We predict that the amphipod assemblages of both groups will be affected by increasing temperature. The sensitive species of clusters 1 and 3 will probably decline, which

also holds true for the drought-tolerant species of cluster 3 because they are already located in the southernmost limit of their geographic distribution (Pinkster, 1993) and their ability to evolve in the upcoming decades may be limited. Consequently, 8 of the 39 MOTUs of amphipods in Tunisia are directly threatened by climate change. Moreover, climate change may also affect the other MOTUs, which did not exhibit specific distributional patterns associated with precipitation and temperature. Some of these 31 BINs might be affected by increased intermittency of streams, which remains the main factor of the presence of amphipods in Tunisia.

#### *Impact on conservation strategies*

Areas of high conservation interest are traditionally defined as biodiversity hotspots, which are generally identified as areas with high species richness and endemism that are or could be subjected to severe anthropogenic impact (Myers et al. 2000). These two diversity attributes are commonly used in conservation biology because they reflect the complexity and uniqueness of ecosystems (Caldecott et al. 1996). However, information on species richness and local endemic species is missing for aquatic invertebrates, which represent more than 80% of the freshwater animal biodiversity (Balian et al. 2008). Our results show that more than 75% of amphipod species could remain undiscovered if only conventional morphological traits were used for species assignment (Mamos et al. 2016; Wattier et al. 2020). Therefore, using molecular data and a highly integrative taxonomic approach appears to be crucial to improve our knowledge of biodiversity for highly diverse and relevant taxonomic groups such as freshwater amphipods (Mamos et al. 2021). The distribution of biodiversity hotspots based on molecular species delimitation methods (MOTUs) generally converged with the findings solely based on morphological data, pointing to north-western Tunisia as the diversity hotspot. It is interesting, given that the morphology-based identification is often biased by the low morphological polymorphism in amphipods irrespective of the molecular divergence between

species (see Mamos et al. 2014), which results in the already mentioned high level of cryptic diversity within this group. It must be noted that diversity surveys are also highly dependent on the sampling effort. Proper sampling strategy, adequate not only to the survey goal, but also to the studied taxonomic group, spatial scale, and surveyed terrain, must be taken into account. In the Mediterranean region, with the generally scarce and highly fragmented hydrological network, maximising the number of sampled unconnected watersheds instead of dense sampling over one watershed seems to be a proper strategy. It boosts the possibility of finding isolated and presumably divergent populations/species instead of sampling more uniform populations tentatively in places prone to mutual gene exchange.

Our study also highlights that, contrary to species identified only based on their morphology, most MOTUs have very limited distribution and may represent endemic lineages. Consequently, molecular tools may also improve our knowledge of local endemism and improve the detection of relevant conservation units (e.g., Jabłońska et al. 2020). Molecular tools could specifically help, especially in developing countries, to establish biodiversity strategies and action plans, monitor local biodiversity, and highlight and maintain adequate national systems of conservation areas.

### *Conclusions*

Our study shows that both molecular and morphological data are needed to fully identify the diversity and distribution of freshwater amphipods in Tunisia. Total diversity provided by molecular methods seems to be much more detailed and accurate than results based on morphology alone. The spatial distribution pattern provided by molecular methods at the country scale is very similar to the one obtained using morphology. Water availability appears to mainly explain the presence of amphipods in freshwater ecosystems in Tunisia, and the distribution of many species is strongly mediated by their thermal tolerance. These two

environmental factors are sensitive to climate change and confirm that current conservation strategies need to be redefined and adjusted in the face of future climate predictions.

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## Statements & Declarations

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### *Competing Interests.*

All authors declare that there is no conflict of interest.

*Author Contributions.* All authors participated in data collection and curation, molecular analyses and writing. KA, TR, CP performed the morphological identification of Amphipods. SD, MG and CP supervised the work.

### *Data Availability.*

“The molecular dataset generated during the current study and their metadata are available in the DS-TUNAMP dataset in the public repository of the Barcode of Life Data Systems repository, <http://dx.doi.org/10.5883/DS-TUNAMP> and on GenBank database (ON258344 - ON258599), <https://www.ncbi.nlm.nih.gov/genbank>. Site and species location are given in supplementary table S1. All other datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.”

**Figure captions:**

**Figure 1** (a) Maps of Tunisia showing the climatic conditions and (b) the locations of the 212 sites where amphipods were sought for.

**Figure 2** (a) Neighbour-joining tree obtained from COI data. Numbers at the nodes, bootstrap values  $\geq 0.80$ . Scale bar, number of substitutions per site. Bars next to the tree, different delimitation methods used in this study, with the coloured ones corresponding to BIN delimitation with respective BINs annotated. (b) Distribution of each detected BIN and species, with colours and symbols corresponding to the ones annotated on the tree. The white symbols refer to respective species' occurrences confirmed with morphological data only.

**Figure 3** Distribution of morphological diversity (a) and molecular diversity (b). Warmer colour (red), high interpolated diversity between neighbouring localities; colder colour (purple), areas of lower interpolated diversity.

**Figure 4** Results of the principal component analysis (PCA) on climatic variables. (a) Correlation circle with selected climatic variables (Tmin, Tmax, Tmean, Pmean, Delta T, CVTmean, CVPmean). (b) Distribution of the four clusters of sites obtained by K-means analysis on the first two axes. The centre of each cluster (white circle) is positioned at the weighted average of the sites. Solid lines link the corresponding sites to the corresponding cluster.

**Figure 5** Distribution of sites according to the 4 clusters resulting from the K-means clustering of climatic variables.

**Table S1** List of sampling sites with or without amphipod and the name of morphospecies identified and their associated BINs.

**Table S2** List of all individuals analysed genetically including the name of morphospecies identified, BOLD process ID, individual sample ID and their associated GenBank accession numbers and BINs.

**Table S3** Summary of statistics from molecular analyses.

**Table 1** Species composition of the four clusters of the SOM analysis. The probabilities of occurrence of each species in the cluster are given in brackets. The numbers in brackets give the Indval values (probabilities of occurrence in the cluster). They are only provided if values were significant.

Clusters	Composition in BINs	Composition in morphospecies
<b>Cluster 1</b>	ADD7705, ADD7985 (25%), ADE2421, ADE2847, ADE2848 (23%),	<i>E. afer</i> (35%), <i>E. haraktis</i> (23%) <i>E. olivii</i> , <i>E. simoni</i> , <i>E. tacapensis</i>
<b>Cluster 2</b>	ADD7705, ADD7985, ADE1881, ADE2063, ADE2420, ADE2421, ADH0211, ADH1057, ADH1059, ADH1072, ADH1073, ADH1173, ADF5926, ADH1058, ADH1074, ADK9117, AEN0934, ADK9114, ADK9208, ADK9347, AEN8341	<i>E. haraktis</i> , <i>E. pungens</i> , <i>E. simoni</i> , <i>Echinogammarus</i> sp., <i>E. tacapensis</i> , <i>E. tunetanus</i> ,
<b>Cluster 3</b>	ADE0367 (20%), ADE0629, ADE0708, ADE2419, ADE2420, ADE2063, ADE1881, ADE2860	<i>E. carthaginiensis</i> , <i>E. pungens</i> , <i>E. simoni</i> , <i>Echinogammarus</i> sp.
<b>Cluster 4</b>	ADE1881, ADE1906, ADE2848, ADE2847, ADF5926, ADF6964, ADF8387, ADH0194, ADH1060, ADH1172, ADH1173, ADK9117, ADK9118, ADK9348, AEN1155, AEN3245, AEN8114, AEN8341	<i>E. afer</i> , <i>E. carthaginiensis</i> (19%), <i>E. olivii</i> , <i>E. pungens</i> , <i>E. tunetanus</i> , <i>Echinogammarus</i> sp., <i>E. simoni</i> , <i>R. rhipidiophorus</i>

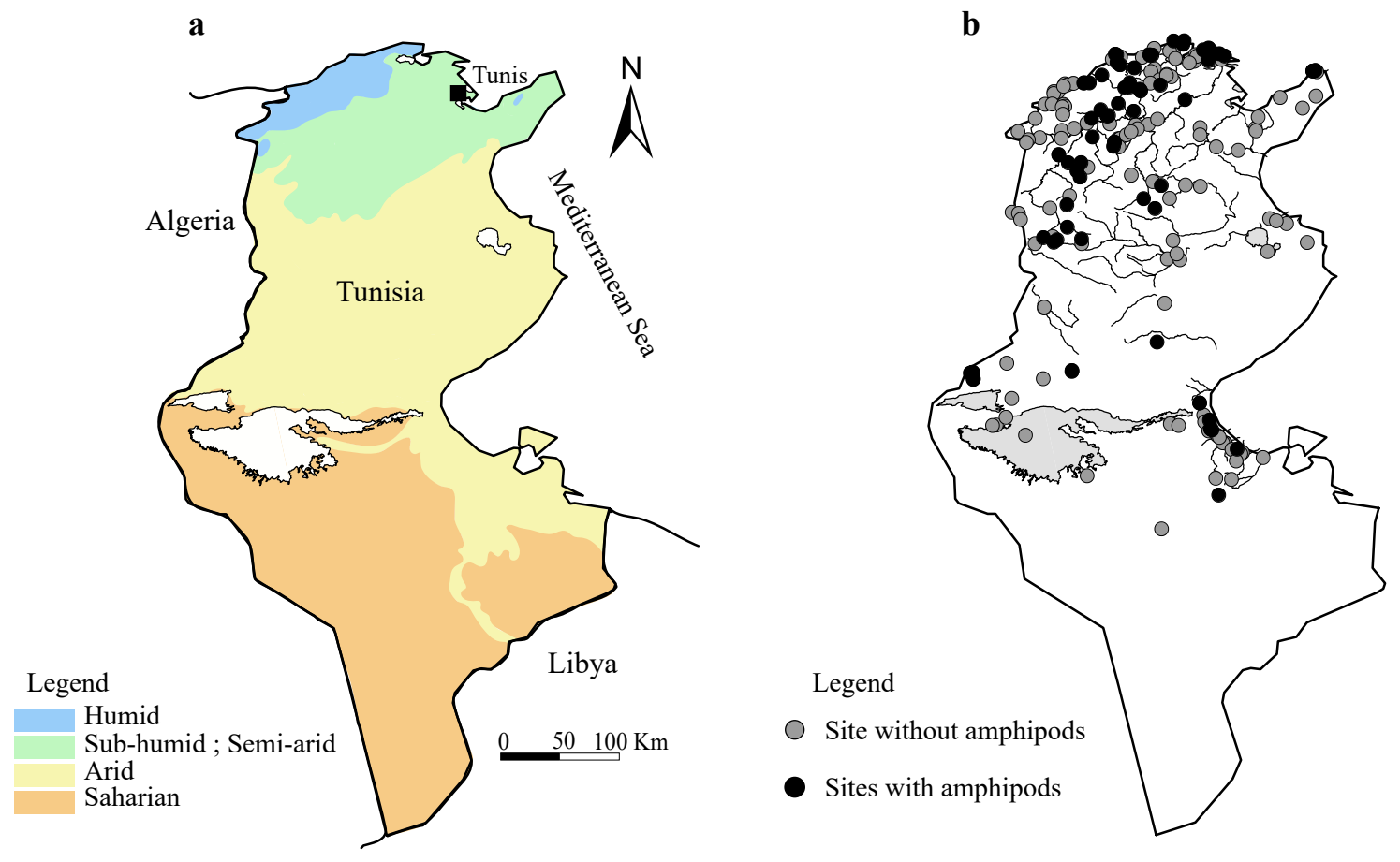


FIGURE 1

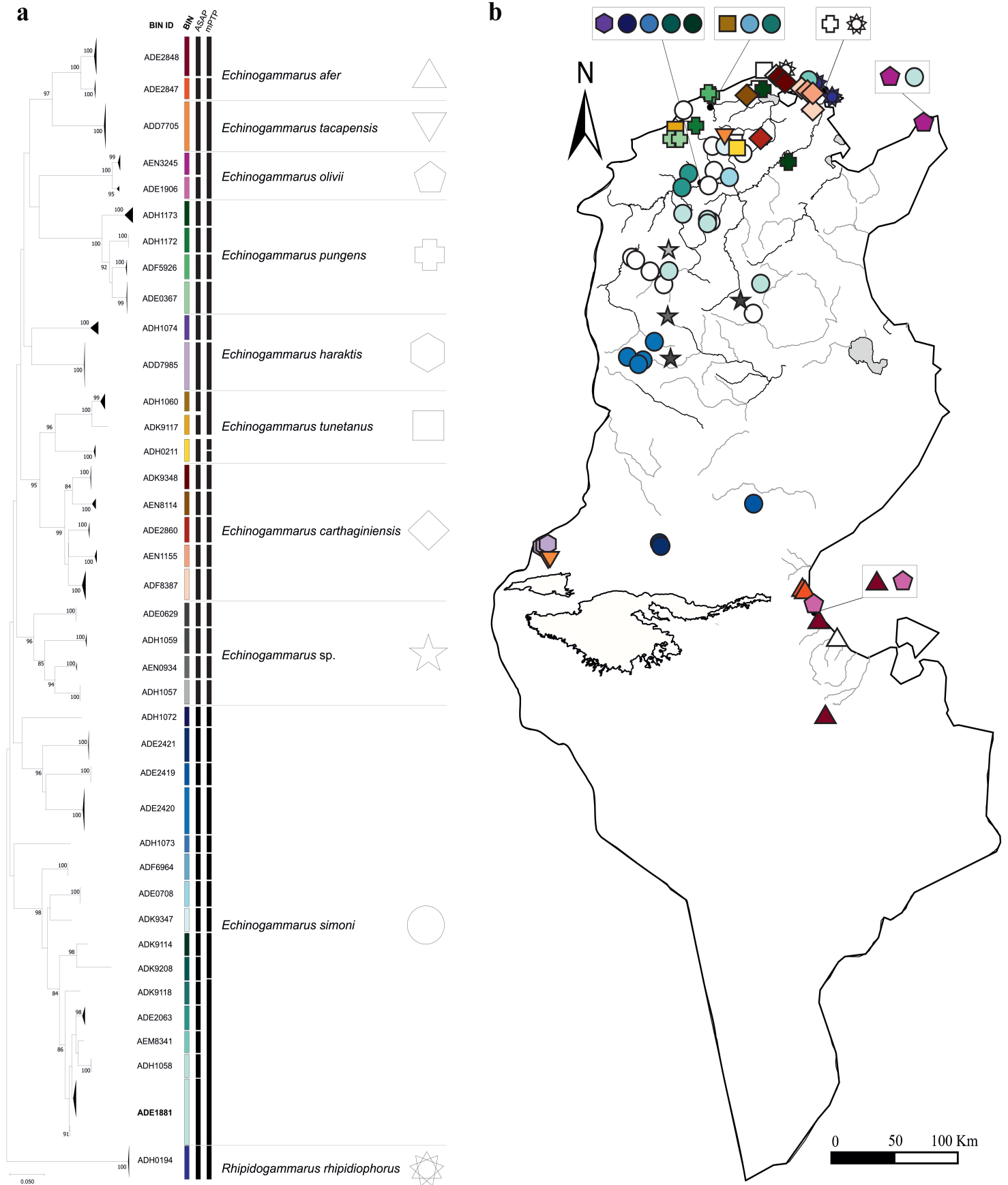
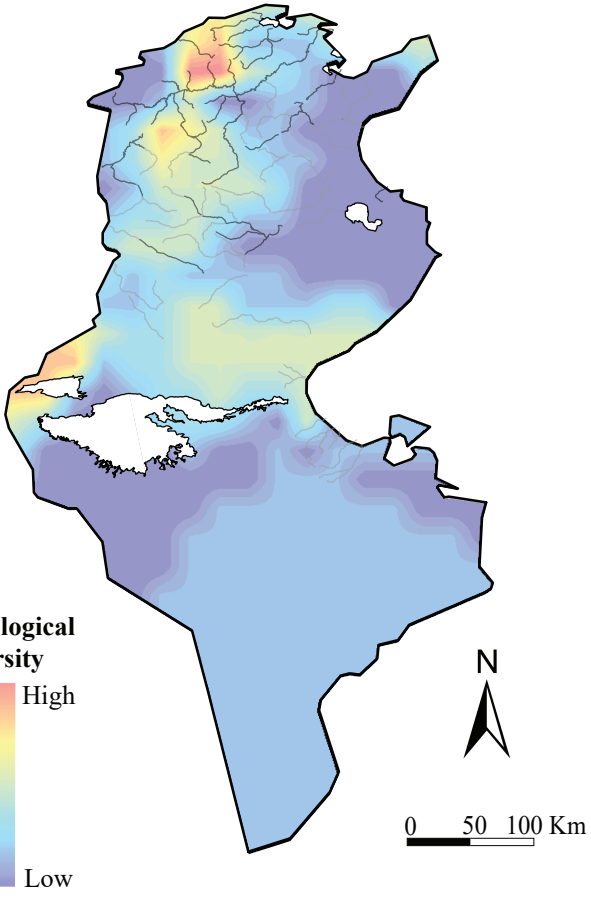


FIGURE 2

**a**



**b**

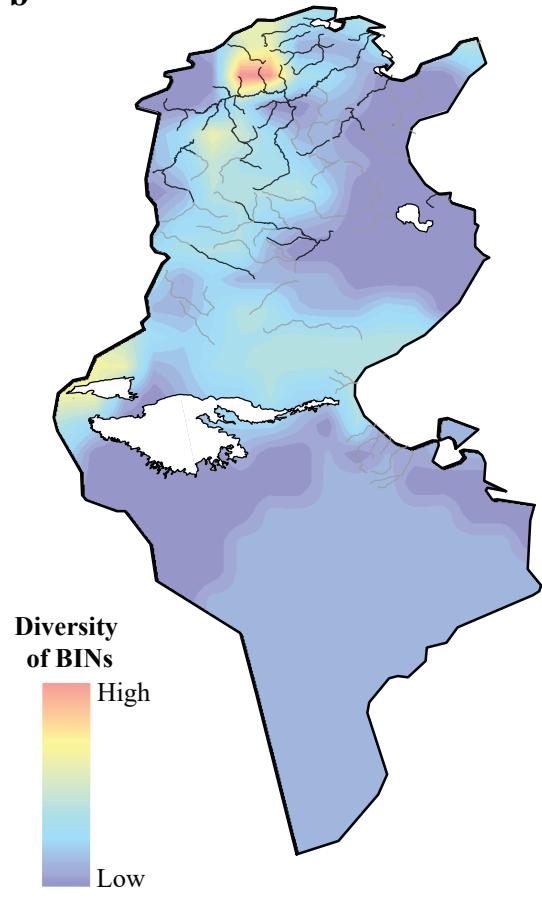


FIGURE 3

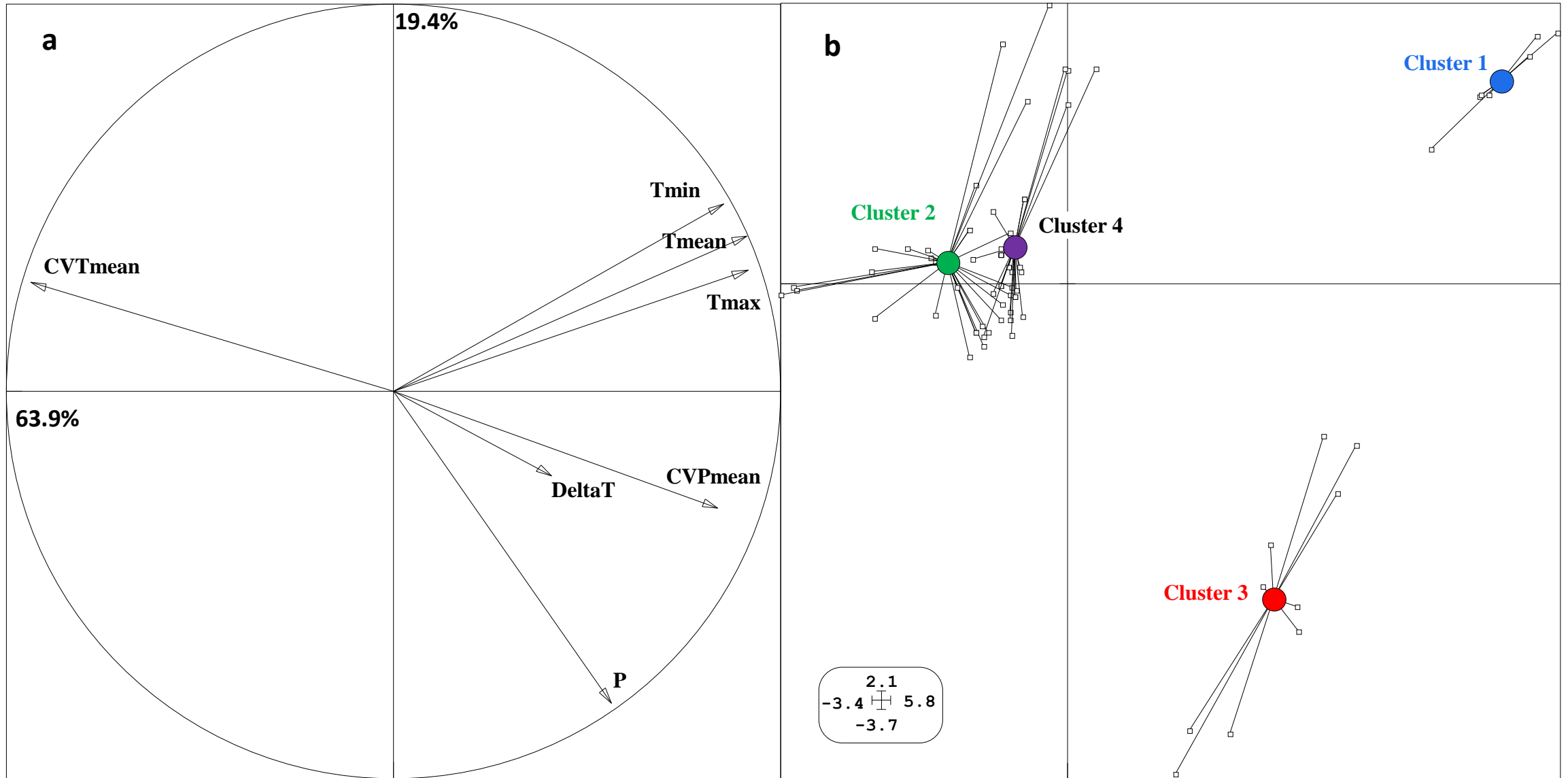


FIGURE 4



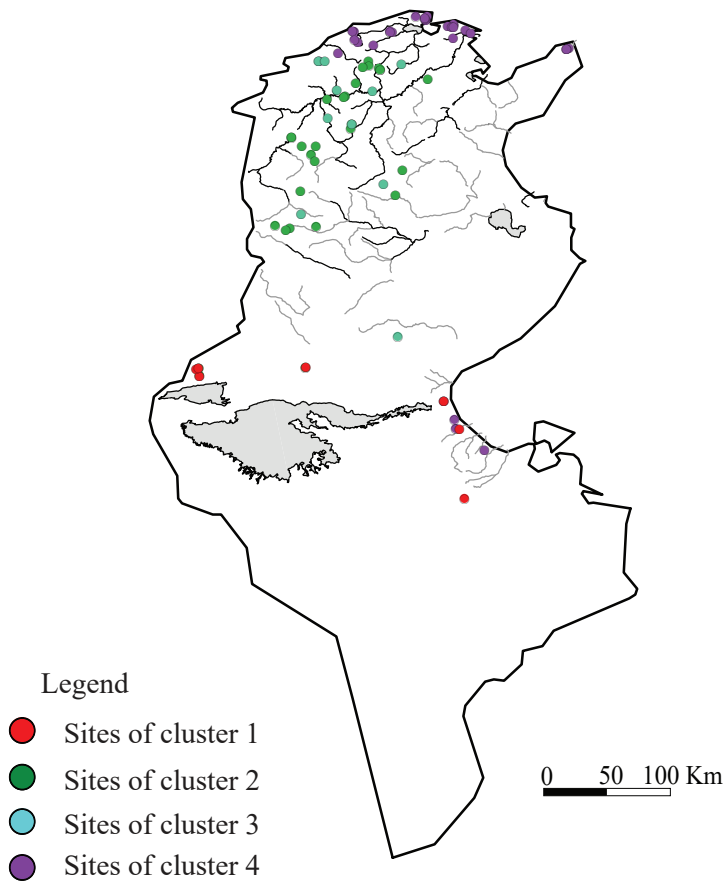


FIGURE 5