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Phylogeography and Species Delimitation of *Asaccus montanus* (Gekkota, Phyllodactylidae)
New insights into cryptic diversity and conservation in the Hajar Mountains

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Student Contributions

The student started working in the project on March 3rd, 2025.

Experimental design: the study was conceived by members of the *Systematics, biogeography and evolution of reptiles and amphibians* research group, including Salvador Carranza and Bernat Burriel-Carranza.

Sampling: specimen collection was conducted by Salvador Carranza, Bernat Burriel-Carranza and other collaborators.

Laboratory work: DNA extractions, quantification (Qubit and gel electrophoresis), PCR amplification and sample preparation for Sanger sequencing and ddRADseq were performed by the student with occasional help from other lab members. The ddRADseq protocol was conducted by the Genomics Unit staff.

Data analysis: the student conducted all the analysis included in the present study with guidance and supervision from the directors and the other lab members. This included the production, curation and management of several genetic and genomic datasets, and the use of several programs, computers and remote servers to run the different analyses.

Graphical work and mapping: all the figures present in the study were created by the student using R, Affinity Designer and QGIS.

Writing: the student made the writing of the manuscript with supervision and corrections from the directors.

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Phylogeography and Species Delimitation of *Asaccus montanus* (Gekkota, Phyllodactylidae). New insights into cryptic diversity and conservation in the Hajar Mountains

Abstract

Our knowledge of global biodiversity has many gaps regarding the total number of species and their geographic distribution. The lack of investigation in specific environments, together with the huge impact of human activity on natural ecosystems, only helps to worsen this situation. Over the last decades, several studies have unveiled mountain ranges in arid regions as reservoirs of hidden diversity. The Hajar Mountains of southeast Arabia constitute a clear example, with high levels of squamate endemism. Previous work in Jebel Akhdar identified two deeply divergent lineages within the Mountain leaf-toed gecko (*Asaccus montanus*), suggesting a possible case of cryptic diversity. In this study, we further explore the phylogeography and species boundaries of *A. montanus* by incorporating new genomic data. Using a combination of population structure analyses, phylogenetics, and coalescent-based species delimitation methods, we evaluated the extent of genetic divergence and the possible presence of independently evolving lineages within the species. Our results reveal strong population structure across the distribution range of *A. montanus*, although current data remains insufficient to determine whether the observed lineages represent distinct species. We therefore highlight the need for a more extensive sampling and the integration of complementary approaches to address this question. Overall, our study sheds light on the evolutionary history of *A. montanus*, underscores the challenges of delimiting species in recently diverged lineages, and highlights the role of arid mountain systems as relevant diversity hotspots, with remarkable implications for conservation strategies.

Keywords: biodiversity crisis | arid regions | Hajar Mountains | endemism | *Asaccus montanus* | phylogeography | species delimitation

1. Introduction

Since the Industrial Revolution in the 18th and 19th centuries, human activity has profoundly impacted life on Earth and is responsible for an unprecedented biodiversity crisis (Keck et al., 2025). Factors that cause a change in an ecosystem are referred to as drivers, and can be either indirect or direct. Indirect drivers include demographic growth, changes in economic activity, sociopolitical factors influencing decision-making, cultural and religious characteristics, and technological development. These factors interact in complex ways in different locations influencing the level of production and consumption of ecosystem services, thus determining pressures on ecosistems shaped by direct drivers. The main factors corresponding to this latter category are habitat modification or destruction (including land use change and physical modification or water withdrawal from rivers), resource overexploitation, pollution, climate change, and the introduction of invasive alien species, which currently remain constant or even grow in intensity in most ecosistems (Millennium Ecosystem Assessment Board, 2005). Consequently, a marked increase in species extinction rates has brought them to levels comparable to previous mass extinction events (Barnosky et al., 2011; Ceballos et al., 2015). This situation results in many species becoming extinct before being even discovered or adequately described.

Beyond the biodiversity crisis itself, research has been unequally distributed across ecosystems. While scientific effort has focused on hotspots traditionally considered more relevant, such as tropical forests, others areas have been systematically understudied. This tendency becomes clearer within the harshest dryland regions (i.e., arid and hyper-arid), which represent 17.2% of the total terrestrial surface on Earth (Durant et al., 2012; Safriel et al., 2005). Altogether, the accelerated extinction rates and lack of investigation in specific areas aggravate the existing knowledge gap regarding the total number of species and their geographic distribution, defined as the Linnean and Wallacean shortfalls, respectively (Hortal et al., 2015).

The Arabian Peninsula is a suitable example for this kind of environment, with 99% of its area defined by an arid or hyper-arid climate (Kotwicki & Al Sulaimani, 2009). In the last decades, several studies have unveiled a great amount of animal diversity, challenging the conventional perception of the region as biodiversity-poor (Carranza & Arnold, 2012; Machado et al., 2019; Melnikov et al., 2015; Sindaco et al., 2018; Tamar et al., 2023; Vasconcelos & Carranza, 2014). Most of this variety corresponds to reptiles, a group particularly prominent in arid regions that constitutes the main component of its vertebrate fauna (Šmíd et al., 2021).

Together with the Arabian Gulf coast and the central region of Saudi Arabia, three major mountain ranges account for most of the Arabian Peninsula's biodiversity: the Asir Mountains of western Saudi Arabia and Yemen, the Dhofar Mountains of south-eastern Yemen and southern Oman, and the Hajar Mountains of northern Oman and eastern United Arab Emirates (UAE). These formations represent the most relevant diversity hotspots when considering endemic species, especially in the case of squamate reptiles (Ficetola et al., 2018; Šmíd et al., 2021). In particular, the latter mountain range stands out as a geographically well-delimited area with an exceptional degree of endemicity and higher evolutionary history than the others, with local communities characterised by increased phylogenetic diversity and smaller distribution ranges. Overall, these features have led them to become a crucial centre of squamate diversity both in Arabia and the rest of the world (Burriel-Carranza et al., 2022, 2025b; Carranza et al., 2018, 2021; Šmíd et al., 2021).

The Hajar Mountains form a curved range of 650 km parallel to the Gulf of Oman coastline, from Ras al Hadd to the Musandam Peninsula. In the west and south, the system is isolated from the rest of the Arabian Peninsula by a large extension of sand and gravel drylands that include part of Rub' al-Khali, the largest desert in the region. The mountains can be delimited into three major blocks: the Western, Central, and Eastern Hajars. Within Jebel Akhdar, the main massif in the central region, Jebel Shams constitutes the highest peak with an elevation of 3,009 m above sea level (asl), although other areas along the mountain range also exceed 2,000 m asl (Burriel-Carranza et al., 2022). Despite being considered a mountain desert (Edgell, 2006), the topographic complexity and high elevations of the terrain make the Hajars one of the most climatically variable regions in south-eastern Arabia (Burriel-Carranza et al., 2019; Carranza et al., 2018, 2021). The combination of these characteristics with the desertic conditions of the region compared to other more temperate mountain systems seems to have prompted the diversification of squamates rather than any other vertebrate group. Up to 32 reptile species are found within the Hajar Mountains, with 23 of them being endemic (Burriel-Carranza et al., 2022, 2025a).

The formation and evolution of this community appears to have been mainly driven by climatic oscillations and hyper-arid periods that have affected the region since the formation of the current mountain topography (Burriel-Carranza et al., 2025a). As a result, non-adaptive allopatric speciation due to the isolation of populations has apparently been responsible for this great amount of diversification. Together with the selection of similar adaptive traits among species driven by overlapping niches in an ecologically homogeneous habitat, this process has triggered the emergence of a high degree of cryptic diversity with very low morphological variation (Burriel-Carranza et al., 2025a; Simó-Riudalbas et al., 2017). In a recent review on cryptic species conservation, Hending (2025) defines the concept as "species that have been, or currently are, classified as single species due to their near-identical morphological appearance, and if already described, are still very hard or impossible to tell apart by traditional identification methods based on visual appearance". This matches the more broadly used definition given by Bickford et al. (2007), although there is no clear consensus within the scientific community. Recent studies in the Hajars region have unveiled several cases corresponding to this type of diversity within the genera *Pristurus* (Burriel-Carranza et al., 2025a; Garcia-Porta et al., 2017), *Asaccus* (Carranza et al., 2016; Simó-Riudalbas et al., 2018; Tamar et al., 2019), *Ptyodactylus* (Simó-Riudalbas et al., 2017), *Hemidactylus* (Carranza & Arnold, 2012) and *Omanosaura* (Mendes et al., 2018).

The genus *Asaccus* Dixon & Anderson, 1973, commonly known as Middle Eastern leaf-toed geckos, is represented in the Arabian Peninsula by seven species endemic to the Hajar Mountains (Burriel-Carranza et al., 2019, 2022; Carranza et al., 2018, 2021). The taxonomic review conducted by Arnold & Gardner (1994) already described four of these species, but recent works based on more in-depth genetic and morphological analyses unveiled the existence of three more taxa (Carranza et al., 2016; Simó-Riudalbas et al., 2018). Also in this framework, cryptic diversity has been recently hypothesized within the Mountain leaf-toed gecko *Asaccus montanus* Gardner, 1994, endemic to the Central Hajars and restricted to rocky areas >1,700 m asl (Burriel-Carranza et al., 2022). *A. montanus* represents the oldest split within its genus, diverging from all other Arabian and Iranian congeneric species around 30 million years ago (mya; Carranza et al., 2016; Kamali et al., 2024; Papenfuss et al., 2010; Simó-Riudalbas et al., 2018). In a recent work, Tamar et al. (2019) identified a divergence event within the species around 2.7 mya, with two main lineages corresponding to geographically separated western and eastern populations on either

side of Jebel Shams. Their results from molecular genetics and species delimitation analyses revealed two well-differentiated lineages and suggested the presence of a cryptic species within *A. montanus*, highlighting the need for broader sampling combined with genetic, genomic, and morphological analyses before concluding any taxonomical hypothesis.

The concept of species plays a central role in numerous fields of biological science, as they are commonly used as fundamental units for assessing biodiversity and setting conservation priorities (Myers et al., 2000; Whittaker et al., 2005). However, the question of what exactly defines a species has been a subject of debate in the scientific community for a long time. One of the oldest and most widely known definitions is the biological species concept, based on pre- or postzygotic reproductive isolation to distinguish between species, although it is not the only one. At the beginning of this century, De Queiroz (2007) reviewed most of the existing conceptualizations of species and proposed a unified definition: the General Lineage Concept (GLC). Rather than relying on a single requirement for splitting two taxa as different species, the GLC defines speciation as a continuum, integrating the requirements for all the previously proposed species concepts at different stages of the process. In this way, species are understood as independently evolving metapopulation lineages, with attributes like reproductive isolation, morphological divergence or reciprocal monophyly (i.e., strict monophyletic groups for each species) becoming empirical lines of evidence for species delimitation (De Queiroz, 2007, 2025; Fišer et al., 2018).

In modern systematics, species delimitation methods (SDMs) play a fundamental role. In particular, those based on the Multispecies Coalescent (MSC) model are of special relevance when assessing species that have recently diverged (Knowles & Carstens, 2007; Leaché et al., 2014; Rannala & Yang, 2003; Wiens, 2007). Unlike other SDMs, the MSC model addresses the problem of incomplete lineage sorting (ILS), which arises from discordance between the evolutionary history of genes and that of species. By considering genetic processes that can contribute to the persistence of genetic lineages across multiple species (e.g., mutation and genetic drift), the model provides a strong framework to integrate incongruence between gene trees and the species tree (Fujita et al., 2012; Jiao et al., 2021). Most of these SDMs rely on the a priori assignment of samples to putative taxa, followed by an evaluation of their species status (Leaché et al., 2014; Sukumaran et al., 2021; Yang & Rannala, 2010). Such groupings into biologically coherent units can be based on diverse criteria, including morphological, ecological, or behavioural attributes, population structure analyses, and phylogenetic reconstructions using specific genes or genome-wide data (e.g., Burriel-Carranza et al., 2023, 2025a). MSC-based SDMs then enable to test whether the observed differences among groups support the existence of independently evolving metapopulation lineages, or whether they reflect variation within a single species (Hillis, 2019).

Our study aims to clarify the taxonomic status of the two lineages identified within *A. montanus*. To do this, 28 new specimens were collected near Jebel Shams in the Jebel Akhdar mountain range, covering the unsampled area in previous studies. Molecular and genomic data were generated to assess the population structure and evolutionary history of the species, as well as to conduct species delimitation with an integrative approach using several methodologies.

2. Materials and Methods

2.1 Sampling and DNA extraction

This study included 62 individuals of the species *Asaccus montanus* collected from 15 localities across its known distribution range in the Central Hajar Mountains (Fig. 1; Table 1). Of these, 28 individuals were sampled in November 2024 and stored in 8 ml plastic tubes with 90% ethanol at -20ºC. Subsequently, DNA was extracted from tail tips using the *DNEasy Blood & Tissue Kit* (QIAGEN, Hilden, Germany). The remaining 34 specimens were obtained from previous works (Burriel-Carranza et al., 2025b; Tamar et al., 2019). Additionally, 51 individuals encompassing nine species from the *Asaccus* genus were included in some analyses (Table 1).

2.2 Mitochondrial data

A fragment of the *12S* mitochondrial gene was amplified for the new *A. montanus* specimens using two sets of primers: 12S RUP (Garcia-Porta et al., 2017) and 12S Gekko (Metallinou et al., 2015). Amplification conditions used were 94ºC (5′), [94ºC (2′′), 54ºC (5′′), 72ºC (8′′)] x 35, 72ºC (2′) and 94ºC (3′), [94ºC (2′′), 52ºC (5′′), 72ºC (8′′)] x 35, 72ºC (2′), respectively. PCR products were purified and Sanger sequenced by Macrogen Spain (Madrid, Spain) to obtain ~400 bp *12S rDNA* gene fragments. Chromatographs were checked, edited, and assembled using *Geneious 2025.1.3* (Kearse et al., 2012; Dotmatics, Boston, MA, USA). Sequences for the remaining *A. montanus* and the other *Asaccus* species were retrieved from GenBank, and alignments were performed using *MUSCLE v5.1* (Edgar, 2004) implemented in *Geneious 2025.1.3* (Kearse et al., 2012). Accession numbers and specimen codes for all individuals with available mitochondrial data are provided in Table 1.

2.3 Genomic data

For the new 28 *A. montanus* specimens, genomic libraries were produced following Peterson et al. (2012) protocol for double-digest restriction site-associated DNA sequencing (ddRADseq). The procedure consisted of double-digesting 500 ng of the previously extracted DNA with two restriction enzymes, one common (Msp1) and one rare (Sbf1). Barcoded Illumina adaptors were ligated to the resulting fragments and subsequently size-selected to retain only those between 415 and 515 bp. Finally, the product was sequenced on an Illumina NextSeq 500 platform for 75 bp single-end reads.

Raw ddRADseq data produced for this project and the reads recovered from Burriel-Carranza et al. (2025) were processed through a bioinformatics pipeline using *Ipyrad v.0.9.94* (Eaton & Overcast, 2020). We discarded sites with Phred score <33, reads containing more than five low-quality bases (Q < 20), consensus sequences with low coverage (<6), excessive undetermined or heterozygous sites (>5%), or more than two haplotypes. The clustering threshold, a parameter that establishes the level of sequence similarity at which two sequences are identified as being homologous and therefore are clustered together, was set to 89% and applied both within individuals to assemble reads into consensus sequences and across individuals to cluster them into loci. Thereafter, we applied *vcftools* (Danecek et al., 2011) and *Plink2* (Chang et al., 2015) to generate an iterative filtering to remove low-quality samples and genotypes (see Burriel-Carranza et al., 2023 for a similar approach). Values of missing data per individual allowance started at 98% and were progressively reduced until 88%, decreasing by 2% in each iteration.

With the remaining genomic data, we built five different datasets based on the further analyses to be conducted. Individuals included in each dataset are specified in Table 1. Dataset 1 was built with the exact same parameter configuration as before, and the rest differed only in the minimum number of samples per locus. We retained only loci present in at least 60% of the samples for datasets 2 and 3, 80% for dataset 4, and 75% for dataset 5. Additionally, we removed non-biallelic SNPs and selected one SNP per locus for datasets 1 and 5, and applied a hard threshold of missing genotype call rate, a minor allele frequency filter (maf <0.05), and removed monomorphic sites for dataset 1. Dataset types can be summarised into concatenated loci (*c_loci*), which kept variant and invariant sites, and putatively unlinked SNPs (*uSNPs*), which only retained the SNP with the highest read depth at each locus. For further specifications regarding genomic datasets, see Table 2.

2.4 Population structure

Dataset 1 was used to analyse the population structure and ancestry of *A. montanus* with *ADMIXTURE v.1.3.0* (Alexander et al., 2009; Alexander & Lange, 2011), a model-based software that estimates both population allele frequencies and individual ancestry proportions from multilocus SNP data, allowing the assignment of specified ancestry contributions from K populations to each specimen. We evaluated a range of possible ancestral populations from K=1 to K=12 using the script *admixture-wrapper* (https://github.com/dportik/admixture-wrapper), running 10 replicates per K, computing 15-fold cross-validation (CV) for each, and selecting the number of populations with the lowest average CV value. Afterwards, using the same dataset, we conducted a Principal Component Analysis (PCA) with *Plink v1.90b6.21* (Chang et al., 2015) to further assess population structure and identify genetic clustering. Results from this section were visualized with *R v.4.4.2* (R Core Team, 2024), and the ancestral genetic proportions for each individual were represented geographically at their sampling sites using *QGIS v.3.40.4-Bratislava* (QGIS Development Team, 2025).

2.5 Phylogenetic analyses

Phylogenetic trees were built using both Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. For ML analyses, we constructed two phylogenies differing in the specimens included in each. The first tree comprised *A. montanus* individuals together with the other *Asaccus* detailed in Table 1 and was built using the concatenated loci *dataset 3*. The second one was constructed using *dataset 2* and included only *A. montanus* specimens. For both analyses, we used *RAxML-ng v.1.0.2* (Kozlov et al., 2019) with a GTR+G model, a total of 100 starting trees (50 random and 50 parsimony), and 1,000 bootstrap replicates to estimate branch support.

For BI analyses, we built two reconstructions with mitochondrial and genomic data using *mt dataset 1* and *dataset 4*, respectively. Both trees were inferred with *BEAST2 v.2.7.4* (Bouckaert et al., 2019), although time-calibrated using different data. For our mitochondrial phylogeny, we calibrated the deepest node with the mean age of the node separating *A. montanus* from the rest of *Asaccus* species in the time-calibrated species tree of Tamar et al. (2019), and applied a normal distribution covering the 95% HPD intervals (mean = 27.81 mya, 95% HPD = 20.05 – 36.52 mya). For our genomic phylogeny, the mean age and 95% HPD intervals were extracted from the complete *Asaccus* phylogeny in Burriel-Carranza et al. (2025) to calibrate the split between the western and eastern clades within *A. montanus* (mean = 4.02 mya, 95% HPD = 1.62 – 7.27 mya).

The two Bayesian analyses were performed applying a GTR model with four gamma categories, estimating base frequencies, and using a strict clock LogNormal with a Calibrated Yule process tree prior. We conducted three independent runs of 10^8 generations, sampling every 10,000 generations. Convergence and stationarity were checked with *Tracer v.1.7.2* (Rambaut et al., 2018), posterior distributions were merged with *LogCombiner v.2.7.7* (Bouckaert et al., 2019) applying a 10% burnin, and the resulting trees were summarized with *TreeAnnotator v.2.7.7* (Bouckaert et al., 2019). Additionally, as the mitochondrial dataset included other *Asaccus* species (see Table 1), we used the package *treeio* (Wang et al., 2020) implemented in *R v.4.4.2* (R Core Team, 2024) to remove them and facilitate the comparison between genomic and mitochondrial phylogenies.

Furthermore, we inferred a species tree under the MSC model using *SNAPP v.1.6.1* (Bryant et al., 2012) implemented in *BEAST2 v.2.7.4* (Bouckaert et al., 2019). The MSC model provides a robust framework for estimating divergence times and effective population sizes and for delimiting species by explicitly accounting for discordances caused by ILS (Jiao et al., 2021). Since species tree inference and species delimitation analyses are often computationally demanding, downsampling datasets to a few specimens per lineage is a common practice (e.g., Burriel-Carranza et al., 2023; Tonzo et al., 2019). For this purpose, *dataset 5* was built including the 3 non-admixed individuals with less missing data from each lineage identified in the population structure and phylogenetic analyses (see Table 1 for specimen codes).

The input file for building the species tree was prepared with *dataset 5 – uSNPs* using the script *snapp_prep.rb* (https://github.com/mmatschiner/snapp_prep). We dated the deepest node with a normal distribution from a mean age of 2.31 and a σ of 1.15, extracted from Burriel-Carranza et al. (2025). Priors were left in their default configuration as follows: mutation rates (u & v) were fixed to 1 and a uniform distribution with 0-1,000 boundaries was set for the population mutation rate θ, which was constrained to be identical on all branches. This latter prior is assumed by the script to decrease the computational load of the analysis (Stange et al., 2018). Then, we made three independent runs of 5,000,000 generations, sampling every 2,500, and generated the final summarised tree as in the BI analyses. *DensiTree v.2.7.7* (Bouckaert, 2010) was used to visualize the posterior trees along with the consensus.

We also estimated the species tree with *BPP v.4.3.8* (Yang, 2015) analysis A01 (Flouri et al., 2018). BPP is a Bayesian Markov chain Monte Carlo (MCMC) program for implementing species tree estimation and species delimitation under the MSC model using multilocus genomic data. MCMC algorithms generate samples from the posterior distribution without explicitly computing complex mathematical calculations such as the normalizing constant, allowing to approximate parameter estimates and their uncertainty under the specified model (Nascimento et al., 2017). Analysis A01 reconstructs the species phylogeny given predefined assignments of individuals to species, allowing the estimation of posterior probabilities (pp) for each split (Rannala & Yang, 2017; Yang & Rannala, 2014). The sequence alignment files were generated from *dataset 5 – c_loci* following the scripts in https://github.com/airbugs/Dynastes_delimitation. We estimated θ and τ priors (a = 3, b = 0.045 and a = 3, b = 0.092, respectively) and retained only those loci present in at least one individual of each putative taxa in the species tree. Then we conducted three independent runs of 1,500,000 generations, sampling every 5 after a burnin of 20,000 and combined them to obtain the final output.

2.6 Species delimitation

We conducted guided species delimitation with *BPP v.4.3.8* (Yang, 2015) analysis A10 (Rannala & Yang, 2013; Yang & Rannala, 2010), which compares alternative delimitation models based on the inferred species tree topology. Input files were prepared from *dataset 5 – c_loci* following the same procedure as for analysis A01, and three independent runs with identical configuration were executed and combined. The species delimitation model with the highest pp was selected.

Additionally, we calculated the genealogical divergence index (*gdi*) proposed by Jackson et al. (2017) to test the robustness of the putative species. Assuming complete isolation (i.e., no gene flow after speciation), *gdi* is defined as:

$$1 - e^{-2\tau_{AB}/\theta_A},$$

where τ_{AB} represents the divergence time between species *A* and *B*, and θ_A represents the population size of species *A*. To properly test species status between two taxa, the index must be calculated reciprocally (*A vs. B* and *B vs. A*). According to Jackson et al. (2017), low *gdi* values (*gdi* < 0.2) indicate that *A* and *B* are the same species, while high values (*gdi* > 0.7) suggest a distinct species status of the two taxa. Values in between, known as "the grey zone", are considered ambiguous and lacking the necessary support to consider the two taxa as distinct species. We assessed species status using *dataset 5 – c_loci* at two levels: (i) considering the four putative taxa identified in the species tree (West1, West2, East1, and East2), and (ii) lumping them into two broader lineages (West and East). *BPP v.4.3.8* (Yang, 2015) analysis A00 output was used to compute *gdi* as proposed by Leaché et al. (2019), since this analysis allows the estimation of parameters under the MSC (including species divergence times and population sizes) on a fixed species phylogeny (Burgess & Yang, 2008; Rannala & Yang, 2003; Yang, 2002). We executed three runs with the same configuration as for the previous BPP analyses, and the output was used to calculate and visualize *gdi* indexes with *R v.4.4.2* (R Core Team, 2024).

3. Results

3.1 ddRADseq data processing

From sequencing, we obtained a total of 2.53 x 10^8 raw reads and retained >99% after applying quality filters, with an average of 2.38 x 10^6 reads per individual. Subsequently, by conducting post-processing filtering, we identified 10 individuals (all *A. montanus*) with low coverage levels and excluded them from the genomic datasets (see Table 1). Determined by the number of individuals included and the filters applied for each, the number of loci in *c_loci* datasets ranged from 985 to 9,973, and the two *uSNP* datasets included 5,582 and 8,433 SNPs (see Table 2 for further information on each dataset and Materials and Methods section 2.3 for details in dataset preparation).

3.2 Population structure

After running ADMIXTURE, the cross-validation suggested K = 2 as the most likely number of ancestral populations (average CV = 0.4131). In this arrangement, ancestry proportions geographically separate *A. montanus* individuals into West and East within their distribution range in Jebel Akhdar, with almost no signals of admixture between them. Higher K values further split these lineages into West, East1 and East2 for K = 3, and West1, West2, East1 and East2 for K = 4 (Fig. 2a&b). Within the western lineage, CN5838 (locality 3) showed admixed ancestry with a higher proportion from West2, and was therefore assigned to this group. In the eastern lineage, specimens CN10755 and CN19330 (locality 10) showed nearly all ancestry proportion from East1. CN8279 (locality 11) also exhibited some admixture with East1, although the predominant ancestry was from East2.

In the PCA, PC1 (65.5% of the total variance) strongly segregated *A. montanus* into West and East clusters. PC2 (3.62% of the total variance) clearly split the western lineage into West1 and West2, whereas PC3 (2.57% of the total variance) slightly differentiated group East1 from East2 while keeping the western lineage clustered together (Fig. 2c&d). Curiously, PC3 isolated the two admixed individuals from locality 10 (CN10755 and CN19330) from the rest of the eastern specimens.

3.3 Phylogenetic reconstructions

3.3.1 Mitochondrial tree

To explore the evolutionary relationships and the arrangement of groups identified in the population structure analyses, we constructed several phylogenies. The BI tree built with the *12S rDNA* mitochondrial gene was concordant with the results in Tamar et al. (2019). *A. montanus* was inferred as a monophyletic group sister to another one comprising the remaining *Asaccus* species (Fig. S1). Focusing on the studied species, two main lineages were recovered, separating western from eastern specimens (pp = 1) and one additional subdivision within the eastern clade (pp = 1 & 0.98; Fig. 3b).

3.3.2 Genomic phylogenies and topological discordances

Both ML trees, one including *A. montanus* and other *Asaccus* species and another restricted to *A. montanus*, recovered West and East lineages as two monophyletic groups (bootstrap = 100) without further subdivision (Fig. S2&S3). The BI phylogeny also revealed a clear geographical structure, but this time with four distinct lineages, corresponding to two separate monophyletic

clades within both the western and eastern groups (pp = 1 for each). These divisions mostly match the groups inferred from population structure analyses, with localities 1-2 falling into the West1 clade, 3-7 into West2, 8-9 into East1, and 10-13 into East2. Curiously, specimen CN5838, which was assigned to West2 based on its predominant ancestry, fell into the clade corresponding to West1. By contrast, specimens CN10755 and CN19330, which showed predominant ancestry from East1, fell in the phylogeny within the East2 clade (Fig. 3a).

The mitochondrial and genomic phylogenies were discordant regarding the western group, as the latter clearly split the lineage into two monophyletic lineages. However, both analyses recovered the same structure for the eastern lineage, with two strongly supported monophyletic groups within it (Fig. 3).

3.4 Species tree and species delimitation

Species trees inferred using SNAPP and BPP analysis A01 resulted in the same topology as the BI genomic phylogeny, with 4 strongly supported putative species: West1, West2, East1, and East2 (pp = 1 for all nodes). According to the time-calibrated tree obtained from SNAPP, the first split occurred 3.96 mya (3.00 – 4.82 mya HPD95%) between western and eastern populations, followed by a much more recent split within each clade: 0.25 mya (0.18 – 0.32 mya HPD95%) between West1 and West2, and 0.21 mya (0.16 – 0.27 mya HPD95%) between East1 and East2 (Fig. 4a).

The two approaches used to test the status of the identified lineages as new putative species yielded different results. From BPP analysis A10, the most confident species delimitation model (pp = 1) supported the species status for all four putative taxa (Fig. 4b). However, the *gdi* did not support species status for any of them. First, all sister lineages were compared between them reciprocally (West1 *vs*. West2 & West2 *vs*. West1; East1 *vs*. East2 & East2 *vs*. East1), and the results supported the same species status, with *gdi* values below 0.2. Then, we lumped the subdivisions within each major clade and compared them (West *vs*. East & East *vs*. West), but results still did not completely support a distinct species status as *gdi* values fell within the ambiguous zone at 0.563 and 0.592, respectively (Fig. 4c).

4. Discussion

Our results reveal *Asaccus montanus* to be a geographically structured species, divided into western and eastern populations within the Jebel Akhdar massif in the Central Hajars. While both lineages are supported by all the analyses conducted, they show intraspecific discordances depending on the method used. Maximum likelihood (ML) phylogenies reflect the split between the western and eastern lineages as two well-supported monophyletic groups, but Bayesian inference (BI) trees offer more refined resolution. The phylogenetic reconstruction based on the *12S rDNA* mitochondrial gene presents an additional subdivision within the eastern lineage as two well-supported monophyletic lineages. Moreover, the phylogenomic tree adds complexity to its topology, with the split of the western lineage aslo into two monophyletic lineages (Fig. 3&S3). In turn, population structure analyses show almost no signals of admixture in the most likely scenario of two main lineages (K = 2). However, some admixed individuals (i.e., specimens CN5838, CN10755, CN19330, and CN8279) are identified when considering further subdivisions within these lineages (K = 4; Fig. 2).

The mitochondrial tree topology obtained in this study is concordant with the one recovered by Tamar et al. (2019), and the incorporation of genome-wide data adds further insight into the species phylogeographic structure. As it was hypothesised, the split between the western and eastern lineages could have occurred contemporaneously to the final uplift of some regions in the Eastern and Central Hajars during the late Miocene to Pliocene (~4-6 mya; Hansman et al., 2017). While the divergence time inferred from our time-calibrated BI genomic phylogeny matches their previous estimation (2.3 and 2.7 mya, respectively), our species tree places the split at 3.96 mya (3.00 – 4.82 mya HPD95%; Fig. 4a), falling within the time range of the final uplift events in the Hajar Mountains. Wadi Nakhur, a deep gorge running west of Jebel Shams, was suggested to be the geographical barrier responsible for the cladogenesis of the two main lineages. However, our study shows individuals sampled east of Jebel Shams that belong to the western lineage.

The phylogeographic patterns recovered in our analyses naturally raise the question of whether the identified lineages represent only population structure or instead correspond to independently evolving species. To address this, we built a species tree and implemented various SDMs. All coalescent-based approaches (BPP analyses A01 and A10) supported species status for the four putative taxa (West1, West2, East1 and East2; Fig. 4a&b), whereas none of them was recovered as a distinct species when computing the heuristic genealogical divergence index (*gdi*) (Fig. 4c). Considering the very recent divergence times estimated for the splits within the western and eastern lineages (0.25 and 0.21 mya, respectively), as well as the evidence of admixture in some specimens (Fig. 2a&b), we evaluated *gdi* both at the fine scale (i.e., the four putative taxa) and at the broad scale (West and East). While values between sister clades (West1-West2 and East1-East2) clearly support single-species status, those between the western and eastern lineages fall within the so-called grey zone of species delimitation, close to the species-level threshold (0.563 for West *vs.* East and 0.592 for East *vs.* West).

According to the GLC, the continuous process of speciation requires the existence of old and young species (De Queiroz, 2007). When two metapopulation lineages have been evolving independently for a long time, their species status is often easy to define as it is supported by most lines of evidence (e.g., reproductive isolation, morphological divergence, and reciprocal monophyly). However, when this diversification has started recently, the discrimination between

population-level processes and species-level divergences might become more challenging due to the limited criteria available for assessing lineage separation (De Queiroz, 2007). In some cases, this latter situation can be considered a specific type of cryptic diversity. The classical definition refers to the selection and maintenance of similar morphological traits by both diverging lineages, which makes the two resulting species visually indistinguishable but easy to differentiate using other techniques such as molecular or genomic data (e.g., Badiane et al., 2014). However, in other situations, recent divergence between two species might be the reason why they have not yet acquired morphological differentiation. These are known as sibling species, and as young independently evolving lineages, it is often difficult to determine whether the divergences reflect population- or species-level separation since some gene flow may still persist (Chambers et al., 2023; Fišer et al., 2018; Hending, 2025).

In addition to this challenge, SDMs based on the MSC model present some limitations. This approach tests the coalescence of different reconstructed gene histories with a species hypothesis, assuming that all incongruences among genes are caused by ILS. However, other biological processes can also generate such conflicts (Hillis, 2019). For example, species exhibiting gradual genetic variance across a geographical range may pose a challenge for assessing lineage divergence with MSC-based methods. In such cases, the choice of sampling localities is critical, as the same set of gene trees can be expected in individuals from genetically divergent populations within a single taxon or from well-differentiated species (Chambers & Hillis, 2020). Another weakness of the MSC model is its reliance on several assumptions, one of which is the absence of gene flow across populations. This requirement often forces the selection of only a limited number of specimens meeting this criterion, which may, in turn, cause clusters of geographically proximate samples to be misidentified as distinct species (Barley et al., 2018; Hillis, 2019).

With the advent of Next-Genration Sequencing (NGS) technologies, the unprecedented resolution now achievable does not always align with the SDMs that were originally conceptualized from much smaller datasets. In the specific case of MSC-based approaches, this increased resolution often leads to population splits being mistakenly interpreted as species-level divergences (Jackson et al., 2017; Leaché et al., 2019; Sukumaran & Knowles, 2017). Aware of these limitations, in this study, we have incorporated complementary methodologies to reduce the risk of oversplitting *A. montanus* into erroneously delimited species. Specifically, the calculation of the *gdi* provides a heuristic approach designed to address this problem by applying stricter thresholds to separate lineages, and can be directly implemented on outputs derived from MSC-based methods (Jackson et al., 2017; Leaché et al., 2019). Nevertheless, as our analyses did not prove a definitive conclusion on whether the western and eastern groups represent independently evolving lineages or merely reflect patterns of genetic structure within a single species, further investigation remains necessary.

One of the points from SDMs on which most authors agree is the need to consider the geographical distribution of the candidate species, designing a comprehensive and adequate sampling strategy that covers the potential contact zones between both (Chambers & Hillis, 2020; Dufresnes et al., 2023; Hillis, 2019). Our study has revealed that neither Wadi Nakhur nor Jebel Shams act as a geological barrier separating the two lineages, thus further sampling focused on localising the transition area should be carried out in future investigations. Following this, a way of analysing and representing patterns of genetic variation across a geographical range is the buildup of sigmoid clines. Using admixture parameters or mitochondrial allele frequencies from

specimens sampled along a transect, this analysis would allow us to evaluate if the change in genetic structure is abrupt or gradual (e.g., Dufresnes et al., 2020a, 2020b). Still focusing on genetic divergence, another way of complementing SDMs is the implementation of reference-based taxonomy. This approach allows the comparison of the levels of differentiation between the analysed putative taxa and those observed between other already described species, based on genetic distance calculations (Leaché et al., 2021). Tamar et al. (2019) already computed the uncorrected *p*-distances between *A. montanus* western and eastern lineages and compared them to those between other *Asaccus species*. For instance, the distance between the two clades (8.15%) was similar to that between *Asaccus caudivolvulus* and *Asaccus gardneri* (~7%; Carranza et al., 2016), but relatively low compared to the distances among other species of the genus. Furthermore, with the genomic data now available, other parameters such as F_{ST} and d_{xy} could be analysed (see Burriel-Carranza et al., 2023; Leaché et al., 2021 for methodological examples).

Additionally, non-genetic data sources should also be considered to increase the robustness of species delimitation (Hillis, 2019; Sukumaran & Knowles, 2017). Among these, morphology is one of the most relevant traits, although it can be particularly challenging to assess in cases of cryptic diversity. In such situations, morphometric or meristic variables can be established based on distances between body parts or on the number of specific structures present in individuals, which can subsequently be used in univariate and multivariate analyses (see Simó-Riudalbas et al., 2017). The same strategy can be followed with osteological information, which can be obtained through micro-computed tomography (μ-CT) scanning (see Burriel-Carranza et al., 2025a). Finally, ecological parameters can be evaluated to test whether candidate lineages exploit distinct environmental or microhabitat conditions. For instance, the degree of ecological differentiation can be quantified through the assessment of environmental niche overlaps using a PCA calibrated on the environmental space of the study area (PCA-env) and an Ecological Niche Factor Analysis (ENFA). At a finer scale, microhabitat preferences can also be assessed by recording substrate type and vertical position above the ground, two variables considered relevant in geckos that can then be statistically compared between lineages (see Simó-Riudalbas et al., 2017).

The concern about the incongruences of some SDMs and the insistence on the need for integrative studies arise from the growing trend of describing species without solid evidence, a phenomenon that has been named taxonomic inflation (Hillis, 2019; Isaac et al., 2004). This practice poses a problem for the development of conservation and protection strategies, both for the species themselves and for the habitats they live in, since jurisdictions in most parts of the world rely exclusively on already described taxa (Dufresnes et al., 2023). Consequently, considering the limited resources commonly dedicated to environmental management, the exitence of inadequately described species may lead to an unnecesary waste of resources. However, all lineages play an essential role in the generation, sharing and recombination of adaptive alleles, which in turn are fundamental for species diversification (López-Goldar & Agrawal, 2021; Martin et al., 2013). To adress this situation, several proposals exist that aim to facilitate the incroporation of relevant phylogeographic lineages in conservation planning without contributing to an uncontrolled and unfounded description of species.

Specifically, Dufresnes et al. (2023) suggest classifying divergent lineages that lack sufficient evidence for species status under the rank of subspecies, and they provide a workflow to guide this process. In our case, phylogeographic studies based on mitochondrial and genomic data show a clear differentiation between the western and eastern populations, and species

delimitation analyses yield results that, although inconclusive, approach the threshold for species-level recognition according to a heuristic criterion. This may represent robust genomic evidence for evolutionary divergence between lineages, but reproductive isolation preventing gene flow, another pivotal aspect in the workflow, can not be determined with the data currently available. In this situation, Dufresnes et al. (2023) recommend relying on lineage age to determine their assignment as species or subspecies. Just like with genetic distances, the estimated divergence time between western and eastern *A. montanus* (mean = 3.96 mya; 95% HPD = 3.00 – 4.82 mya) is comparable to that between *A. caudivolvulus* and *A. gardneri* (mean = 4.0, 95% HPD = 2.1 – 6.7 mya; Carranza et al., 2016), but more recent than that inferred for other *Asaccus* species (e.g, *Asaccus gallagheri* and *Asaccus platyrhynchus*; mean = 7.7 mya, 95% HPD = 4.4 – 12.4 mya; Carranza et al., 2016). Therefore, further investigations are needed to fill the current knowledge gap and help establishing a definitive taxonomic designation of *Asaccus montanus*.

Tables and Figures

Table 1. List of all individuals included in this study, with information regarding location, clade assignment, GenBank accession numbers, number of ddRADseq raw and filtered reads, as well as indication of their inclusion in mitochondrial and genomic datasets. Sequences marked with "XXX" were newly generated in this study and will be uploaded to GenBank.

	Species	Specimen Code	Locality	Latitude	Longitude	Clade	12 S Acession nº	Raw reads	Filtered reads	*mt dataset 1*	*dataset 1*	*dataset 2*	*dataset 3*	*dataset 4*	Raw data
1	*Asaccus montanus*	CN7084	1	23,1558	57,0327	W1	MH752874	2523384	2520518	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
2	*Asaccus montanus*	CN7217	1	23,1558	57,0326	W1	MH752872	3087388	3083152	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
3	*Asaccus montanus*	CN4030	2	23,2952	57,1317	W1	MH752861	2322486	2319309	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
4	*Asaccus montanus*	CN4045	2	23,2952	57,1317	W1	MH752864	3340718	3336857	YES	YES	YES	YES	YES	Burriel-Carranza et al., 2025
5	*Asaccus montanus*	CN6982	2	23,2958	57,1317	W1	MH752865	3273010	3269160	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
6	*Asaccus montanus*	CN6987	2	23,2966	57,1311	W1	MH752871	3734389	3729782	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
7	*Asaccus montanus*	CN7220	2	23,2952	57,1317	W1	MH752866	3599026	3595707	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
8	*Asaccus montanus*	CN7224	2	23,2952	57,1317	W1	MH752867	3780222	3773366	YES	YES	YES	YES	YES	Burriel-Carranza et al., 2025
9	*Asaccus montanus*	CN7263	2	23,2952	57,1317	W1	MH752868	4063101	4058906	YES	YES	YES	YES	YES	Burriel-Carranza et al., 2025
10	*Asaccus montanus*	CN7267	2	23,2964	57,1311	W1	MH752862	1034163	1033013	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
11	*Asaccus montanus*	CN7270	2	23,2952	57,1317	W1	MH752863	2556777	2551605	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
12	*Asaccus montanus*	CN7275	2	23,2952	57,1317	W1	MH752869	2645053	2640887	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025

	Species	Specimen Code	Locality	Latitude	Longitude	Clade	12 S Acession nº	Raw reads	Filtered reads	*mt dataset 1*	*dataset 1*	*dataset 2*	*dataset 3*	*dataset 4*	Raw data
13	*Asaccus montanus*	CN7278	2	23,2958	57,1317	W1	MH752870	2177515	2174616	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
14	*Asaccus montanus*	CN5838	3	23,1931	57,1994	W2	MH752873	3072278	3067729	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
15	*Asaccus montanus*	BC00756	4	23,2286	57,2014	W2	XXX	547154	546460	YES	YES	YES	YES	NO	Present study
16	*Asaccus montanus*	BC01003	4	23,2286	57,2014	W2	-	621292	620538	NO	YES	YES	YES	NO	Present study
17	*Asaccus montanus*	BC01039	4	23,2286	57,2014	W2	XXX	41156	41104	YES	NO	NO	NO	NO	Present study
18	*Asaccus montanus*	BC00890	5	23,2422	57,2134	W2	XXX	3400815	3396595	YES	YES	YES	YES	YES	Present study
19	*Asaccus montanus*	BC01037	5	23,2426	57,2131	W2	XXX	1151294	1149932	YES	YES	YES	YES	NO	Present study
20	*Asaccus montanus*	BC01038	5	23,2426	57,2141	W2	XXX	4044254	4040398	YES	YES	YES	YES	YES	Present study
21	*Asaccus montanus*	BC00875	6	23,2729	57,2169	W2	XXX	240728	240446	YES	YES	YES	YES	NO	Present study
22	*Asaccus montanus*	BC01031	6	23,2717	57,2166	W2	XXX	312962	312711	YES	NO	NO	NO	NO	Present study
23	*Asaccus montanus*	BC01004	6	23,2709	57,2167	W2	XXX	210026	209804	YES	NO	NO	NO	NO	Present study
24	*Asaccus montanus*	BC00935	7	23,1746	57,3130	W2	XXX	2796551	2793173	YES	YES	YES	YES	NO	Present study
25	*Asaccus montanus*	BC00958	7	23,1754	57,3141	W2	XXX	3236510	3233283	YES	YES	YES	YES	YES	Present study
26	*Asaccus montanus*	BC00937	7	23,1745	57,3134	W2	XXX	191626	191111	YES	NO	NO	NO	NO	Present study
27	*Asaccus montanus*	BC00422	7	23,1755	57,3111	W2	XXX	41736	41699	YES	NO	NO	NO	NO	Present study
28	*Asaccus montanus*	CN7742	8	23,1670	57,4125	E1	MH752854	2855088	2852693	YES	YES	YES	YES	YES	Burriel-Carranza et al., 2025
29	*Asaccus montanus*	CN8261	8	23,1670	57,4125	E1	MH752857	1646372	1644363	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
30	*Asaccus montanus*	CN8265	8	23,1670	57,4125	E1	MH752860	585369	584786	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
31	*Asaccus montanus*	CN8272	8	23,1670	57,4125	E1	MH752858	1994708	1992451	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025

	Species	Specimen Code	Locality	Latitude	Longitude	Clade	12 S Acession nº	Raw reads	Filtered reads	*mt dataset 1*	*dataset 1*	*dataset 2*	*dataset 3*	*dataset 4*	Raw data
32	*Asaccus montanus*	CN8277	8	23,1670	57,4125	E1	MH752855	225455	225244	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
33	*Asaccus montanus*	CN8994	8	23,1670	57,4125	E1	MH752856	4015342	4010463	YES	YES	YES	YES	YES	Burriel-Carranza et al., 2025
34	*Asaccus montanus*	CN9003	9	23,1566	57,4239	E1	MH752859	3820505	3813345	YES	YES	YES	YES	YES	Burriel-Carranza et al., 2025
35	*Asaccus montanus*	CN10737	10	23,1481	57,5626	E2	XXX	4033009	4028474	YES	YES	YES	YES	NO	Present study
36	*Asaccus montanus*	CN10738	10	23,1479	57,5603	E2	XXX	2812589	2808952	YES	YES	YES	YES	NO	Present study
37	*Asaccus montanus*	CN10740	10	23,1481	57,5627	E2	XXX	2523779	2521717	YES	YES	YES	YES	NO	Present study
38	*Asaccus montanus*	CN10745b	10	23,1484	57,5589	E2	XXX	3469529	3466240	YES	YES	YES	YES	NO	Present study
39	*Asaccus montanus*	CN10751	10	23,1481	57,5625	E2	XXX	3427630	3424664	YES	YES	YES	YES	NO	Present study
40	*Asaccus montanus*	CN10755	10	23,1481	57,5626	E2	XXX	769974	769252	YES	YES	YES	YES	NO	Present study
41	*Asaccus montanus*	CN10757	10	23,1478	57,5620	E2	XXX	4383791	4377897	YES	YES	YES	YES	YES	Present study
42	*Asaccus montanus*	CN19330	10	23,1479	57,5621	E2	XXX	1365236	1363933	YES	YES	YES	YES	NO	Present study
43	*Asaccus montanus*	CN19335	10	23,1486	57,5594	E2	XXX	1263475	1262455	YES	YES	YES	YES	NO	Present study
44	*Asaccus montanus*	CN19346	10	23,1484	57,5589	E2	XXX	3198354	3194996	YES	YES	YES	YES	NO	Present study
45	*Asaccus montanus*	CN19353	10	23,1481	57,5596	E2	XXX	4466521	4461902	YES	YES	YES	YES	YES	Present study
46	*Asaccus montanus*	CN19367	10	23,1480	57,5596	E2	XXX	2161717	2159553	YES	YES	YES	YES	NO	Present study
47	*Asaccus montanus*	CN19392	10	23,1486	57,5595	E2	XXX	647808	647209	YES	YES	YES	YES	NO	Present study
48	*Asaccus montanus*	CN19407	10	23,1487	57,5592	E2	XXX	7182373	7172375	YES	YES	YES	YES	NO	Present study
49	*Asaccus montanus*	CN19646	10	23,1485	57,5595	E2	XXX	607056	606401	YES	NO	NO	NO	NO	Present study
50	*Asaccus montanus*	CN7727	11	23,1339	57,5985	E2	MH752850	3568560	3565050	YES	YES	YES	YES	YES	Burriel-Carranza et al., 2025
51	*Asaccus montanus*	CN8267	11	23,1339	57,5985	E2	MH752851	1983008	1980432	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025

	Species	Specimen Code	Locality	Latitude	Longitude	Clade	12 S Acession nº	Raw reads	Filtered reads	*mt dataset 1*	*dataset 1*	*dataset 2*	*dataset 3*	*dataset 4*	Raw data
52	*Asaccus montanus*	CN8268	11	23,1339	57,5985	E2	MH752852	698416	697635	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
53	*Asaccus montanus*	CN8279	11	23,1339	57,5985	E2	MH752853	2936320	2931461	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
54	*Asaccus montanus*	AO43	12	23,0781	57,6061	E2	KX550530	160832	160575	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
55	*Asaccus montanus*	AO42	12	23,0781	57,6061	E2	MH752841	134966	134766	YES	NO	NO	NO	NO	Burriel-Carranza et al., 2025
56	*Asaccus montanus*	AO44	12	23,0781	57,6061	E2	MH752843	-	-	YES	NO	NO	NO	NO	-
57	*Asaccus montanus*	CN196	13	23,0807	57,6698	E2	MH752848	1173845	1172511	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
58	*Asaccus montanus*	CN5819	13	23,0807	57,6698	E2	MH752849	1775094	1773032	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
59	*Asaccus montanus*	CN7101	13	23,0807	57,6698	E2	MH752845	1086150	1085153	YES	YES	YES	YES	NO	Burriel-Carranza et al., 2025
60	*Asaccus montanus*	AO45	14	23,0794	57,6383	E2	MH752844	66328	66194	YES	NO	NO	NO	NO	Burriel-Carranza et al., 2025
61	*Asaccus montanus*	S1555	15	23,0711	57,6042	E2	MH752846	85613	85473	YES	NO	NO	NO	NO	Burriel-Carranza et al., 2025
62	*Asaccus montanus*	S8057	15	23,0713	57,6042	E2	MH752847	33244	33179	YES	NO	NO	NO	NO	Burriel-Carranza et al., 2025
63	*Asaccus arnoldi*	CN4013	-	22,1070	59,3570	-	-	1010988	1009671	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
64	*Asaccus arnoldi*	CN10782	-	22,6157	59,0936	-	-	4282431	4276110	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
65	*Asaccus arnoldi*	S7576	-	22,6161	59,0937	-	-	1798955	1795092	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
66	*Asaccus arnoldi*	CN3190	-	23,0853	58,8718	-	-	3241643	3237577	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
67	*Asaccus arnoldi*	CN4310	-	22,1071	59,3570	-	KX550526	-	-	YES	NO	NO	NO	NO	-

	Species	Specimen Code	Locality	Latitude	Longitude	Clade	12 S Acession nº	Raw reads	Filtered reads	*mt dataset 1*	*dataset 1*	*dataset 2*	*dataset 3*	*dataset 4*	Raw data
68	*Asaccus arnoldi*	S7252	-	22,6161	59,0937	-	MG019492	-	-	YES	NO	NO	NO	NO	-
69	*Asaccus caudivolvulus*	CN10292	-	25,4783	56,3622	-	-	1678889	1676100	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
70	*Asaccus caudivolvulus*	CN10293	-	25,4783	56,3622	-	-	2086414	2082419	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
71	*Asaccus caudivolvulus*	CN10295	-	25,4783	56,3622	-	-	2179733	2175951	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
72	*Asaccus caudivolvulus*	CN10308	-	25,4783	56,3622	-	-	1755821	1752288	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
73	*Asaccus caudivolvulus*	S7445	-	25,4783	56,3622	-	KX550491	-	-	YES	NO	NO	NO	NO	-
74	*Asaccus caudivolvulus*	S7866	-	25,4783	56,3622	-	KX550492	-	-	YES	NO	NO	NO	NO	-
75	*Asaccus caudivolvulus*	S8088	-	25,4783	56,3622	-	KX550493	-	-	YES	NO	NO	NO	NO	-
76	*Asaccus elisae*	MVZ234315	-	32,8487	48,2642	-	KX550527	5089985	5083360	YES	NO	YES	NO	NO	Burriel-Carranza et al., 2025
77	*Asaccus gallagheri*	CN3739	-	24,5131	56,4634	-	-	10555723	10544667	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
78	*Asaccus gallagheri*	CN5817	-	24,5131	56,4634	-	-	2350157	2347123	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
79	*Asaccus gallagheri*	CN7823	-	25,1823	56,2290	-	-	4572571	4568357	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
80	*Asaccus gallagheri*	CN204	-	25,4592	56,1837	-	-	2780208	2776549	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
81	*Asaccus gallagheri*	CN126	-	25,7826	56,2137	-	-	2438214	2435458	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
82	*Asaccus gallagheri*	CN8387	-	25,9785	56,2045	-	-	2415247	2412601	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
83	*Asaccus gallagheri*	UAE20	-	26,0992	56,3294	-	-	1976954	1972657	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025

	Species	Specimen Code	Locality	Latitude	Longitude	Clade	12 S Acession nº	Raw reads	Filtered reads	*mt dataset 1*	*dataset 1*	*dataset 2*	*dataset 3*	*dataset 4*	Raw data
84	*Asaccus gallagheri*	AO75	-	23,1733	57,4319	-	MG019478	708248	707215	YES	NO	YES	NO	NO	Burriel-Carranza et al., 2025
85	*Asaccus gallagheri*	CN14	-	23,0690	57,4732	-	MG019476	-	-	YES	NO	NO	NO	NO	-
86	*Asaccus gallagheri*	CN2800	-	-	-	-	MG019450	-	-	YES	NO	NO	NO	NO	-
87	*Asaccus gardneri*	CN9008	-	25,4621	56,1815	-	-	4976839	4971656	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
88	*Asaccus gardneri*	CN8673	-	25,6505	56,2665	-	-	2751266	2747712	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
89	*Asaccus gardneri*	CN8370	-	25,6722	56,2122	-	-	2675149	2671740	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
90	*Asaccus gardneri*	CN3955	-	25,7464	56,2783	-	-	149894	149725	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
91	*Asaccus gardneri*	CN8700	-	25,9758	56,1504	-	-	800145	799019	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
92	*Asaccus gardneri*	CN763	-	26,0385	56,2156	-	-	763069	762022	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
93	*Asaccus gardneri*	UAE2	-	26,0392	56,2150	-	-	3425248	3416404	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
94	*Asaccus gardneri*	CN3681	-	26,0474	56,2316	-	-	3405005	3402061	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
95	*Asaccus gardneri*	CN3907	-	26,2070	56,2434	-	-	685949	685092	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
96	*Asaccus gardneri*	CN2702	-	26,2121	56,2356	-	-	2766269	2762888	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
97	*Asaccus gardneri*	CN3686	-	26,2275	56,2135	-	-	2654911	2651447	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
98	*Asaccus gardneri*	CN3905	-	26,1493	56,1619	-	KX550485	-	-	YES	NO	NO	NO	NO	-
99	*Asaccus gardneri*	CN5771	-	-	-	-	KX550451	-	-	YES	NO	NO	NO	NO	-

	Species	Specimen Code	Locality	Latitude	Longitude	Clade	12 S Acession nº	Raw reads	Filtered reads	*mt dataset 1*	*dataset 1*	*dataset 2*	*dataset 3*	*dataset 4*	Raw data
100	*Asaccus griseonotus*	MVZ234325	-	33,2595	47,8042	-	-	4932156	4926085	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
101	*Asaccus griseonotus*	MVZ234326	-	33,2595	47,8042	-	KX550528	9933401	9921921	YES	NO	YES	NO	NO	Burriel-Carranza et al., 2025
102	*Asaccus margaritae*	CAS250892	-	25,2651	56,3068	-	-	3144566	3141047	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
103	*Asaccus margaritae*	CN840	-	25,0077	56,2152	-	-	3319990	3313690	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
104	*Asaccus margaritae*	CN8195	-	25,9648	56,2034	-	-	1088045	1086396	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
105	*Asaccus margaritae*	CN7126	-	25,9648	56,2034	-	-	1845139	1842423	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
106	*Asaccus margaritae*	CN3966	-	25,0077	56,2152	-	KX550517	-	-	YES	NO	NO	NO	NO	-
107	*Asaccus margaritae*	CN3967	-	-	-	-	KX550523	-	-	YES	NO	NO	NO	NO	-
108	*Asaccus nasrullahi*	MVZ234330	-	32,8487	48,2642	-	KX550529	3218182	3215640	YES	NO	YES	NO	NO	Burriel-Carranza et al., 2025
109	*Asaccus nasrullahi*	FTHM002705	-	33,8167	47,8167	-	-	1151899	1149953	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
110	*Asaccus platyrhynchus*	CN3651	-	23,0558	57,0211	-	-	2985126	2981737	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
111	*Asaccus platyrhynchus*	S1959	-	23,0620	57,1304	-	-	2667493	2664497	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
112	*Asaccus platyrhynchus*	CN994	-	23,4466	57,8819	-	-	9126938	9117028	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
113	*Asaccus platyrhynchus*	CN717	-	23,4468	57,8810	-	-	1022124	1021310	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
114	*Asaccus platyrhynchus*	S7750	-	23,1981	57,3905	-	-	3430161	3424413	NO	NO	YES	NO	NO	Burriel-Carranza et al., 2025
115	*Asaccus platyrhynchus*	S1751	-	-	-	-	KX550525	-	-	YES	NO	NO	NO	NO	-

Table 2. Dataset specifications. c_loci: concatenated loci; uSNPs: Unlinked Single Nucleotide Polymorphisms; gen: missing genotype call rate allowed; bp: base pairs.

Dataset name	Dataset type	Analysis	Postprocessing filtering (%)	Individuals (nº)	dataset length (bp)	loci (nº)	SNPs (nº)	Missingness (%)
dataset 1	uSNPs	Admixture & PCA	40 gen	51	5,582	5,582	5,582	24.22
dataset 2	c_loci	RAxML-ng	40 gen	51	477,488	7,326	25,012	16.12
dataset 3	c_loci	RAxML-ng	40 gen	92	64,409	985	8,032	30.26
dataset 4	c_loci	BEAST	20 gen	51	195,434	2,999	10,393	10.97
dataset 5 - c_loci	c_loci	BPP: species tree inference, species delimitation and *gdi*	25 gen	12	646,550	9,973	24,314	15.64
dataset 5 - uSNPs	uSNPs	SNAPP: species tree inference	25 gen	12	8,433	8,433	8,433	16.14

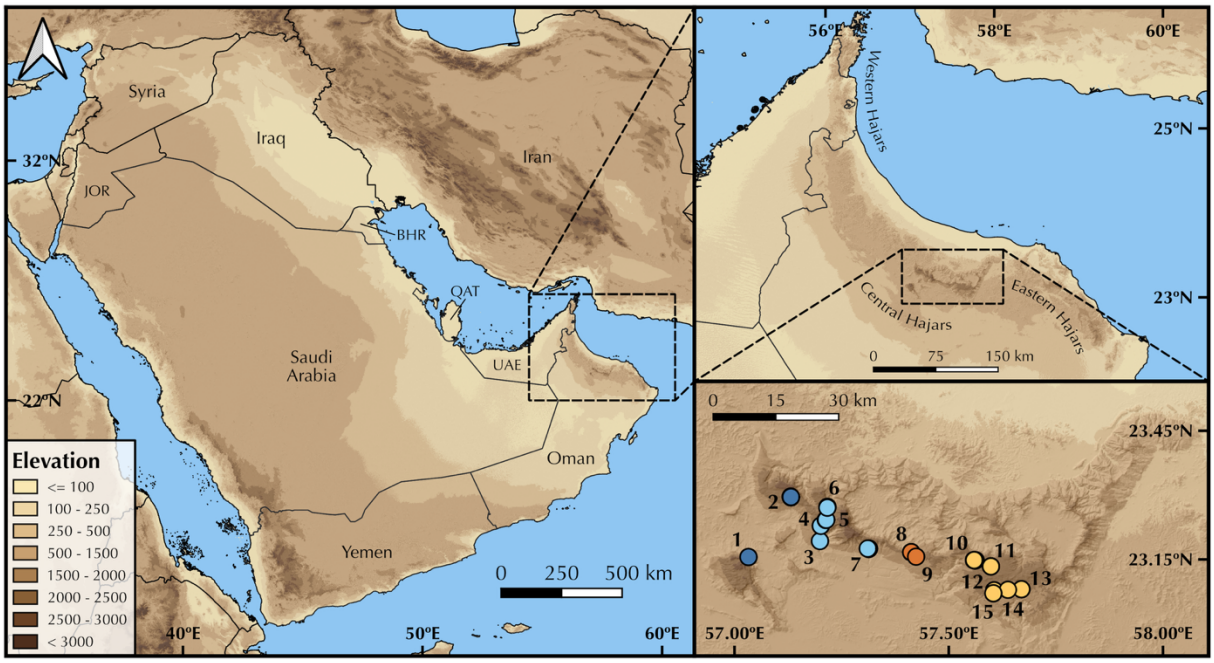

Figure 1. Topographic map of the study area showing the 15 localities from where *Asaccus montanus* specimens were sampled. Colours represent the four lineages identified in our analyses: West1 (dark blue), West2 (light blue), East1 (dark orange), and East2 (light orange). See Table 1 for the correspondence between the 15 localities and the 62 sampled individuals.

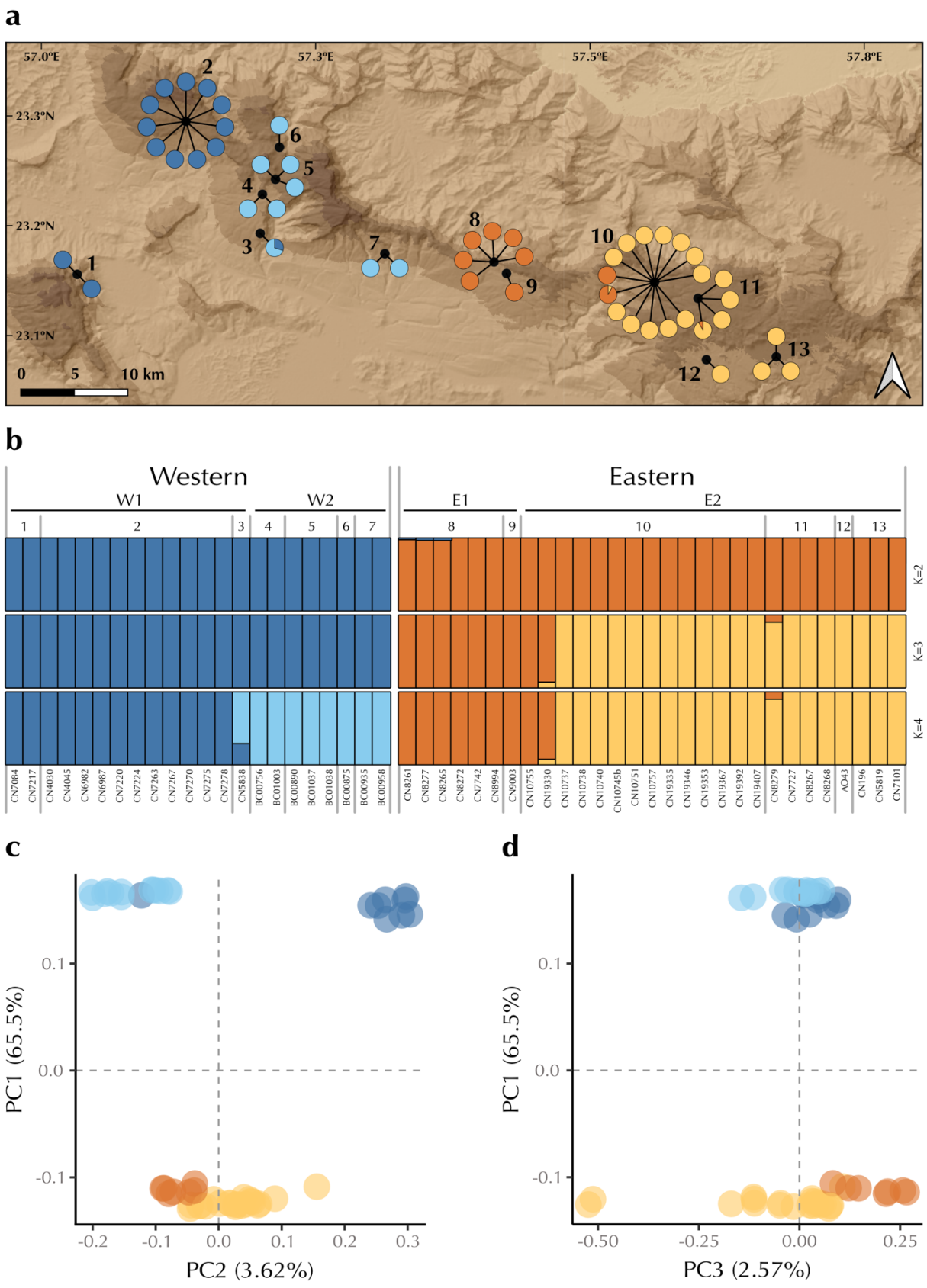

Figure 2. Population structure of *Asaccus montanus*. **a**: Geographic distribution of the analysed specimens, with pies showing the ancestry proportions for K = 4 from ADMIXTURE analysis. **b**: Bar plot showing the inferred ancestry proportions per individual for *K* = 2-4 from *ADMIXTURE* analysis. **c-d**: Principal Component Analysis (PCA), with points representing specimens coloured according to the inferred groups from ADMIXTURE analysis: West1 (dark blue), West2 (light blue), East1 (dark orange), and East2 (light orange).

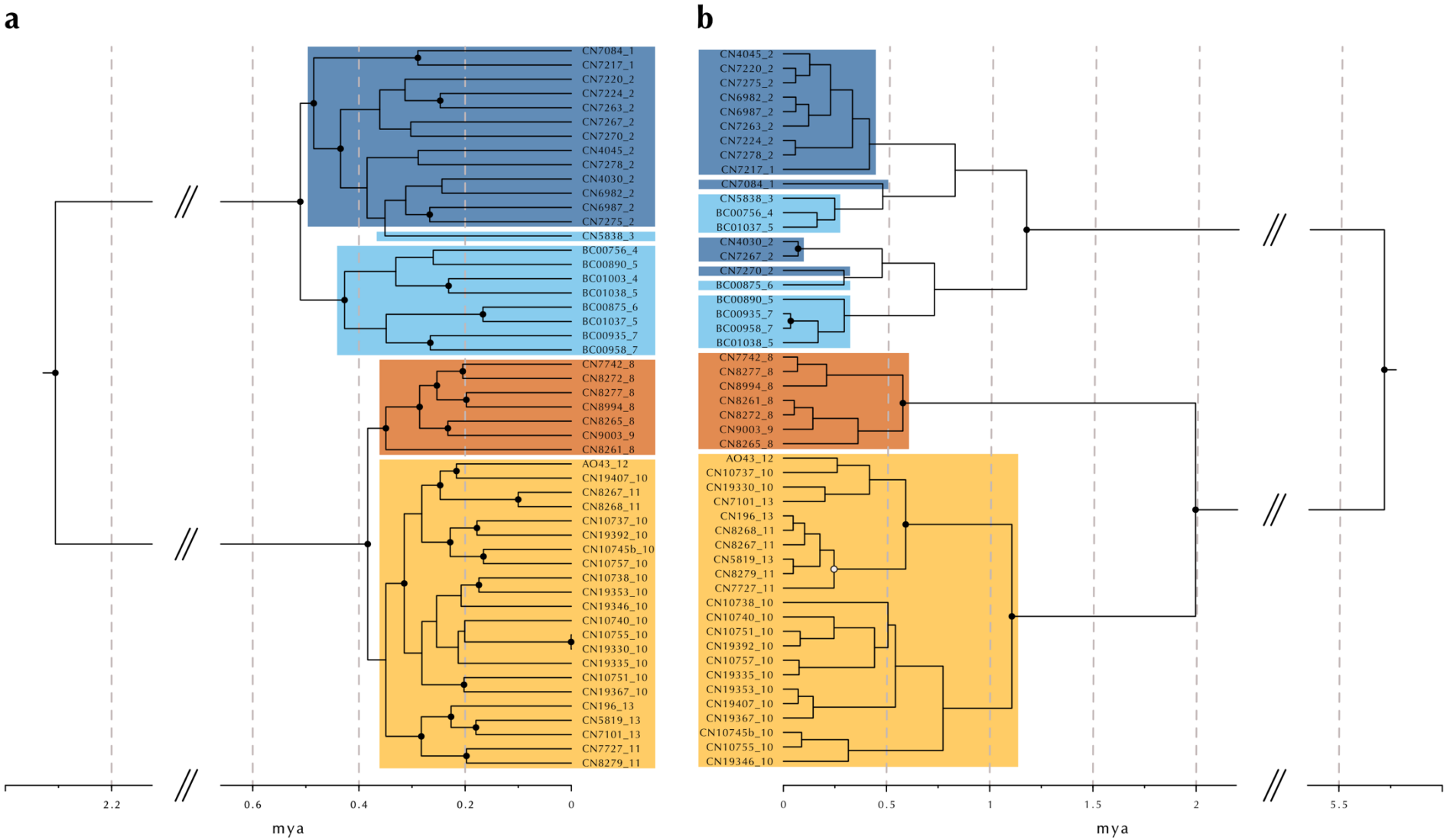

Figure 3. Bayesian inference time-calibrated trees. **a**: Phylogenomic tree inferred with a concatenated dataset of 2,999 nuclear loci from 51 *Asaccus montanus* specimens. **b**: Phylogenetic tree based on the *12S rDNA* mitochondrial gene from 50 *A. montanus* specimens. Posterior probability (pp) values above 0.95 and pp > 0.85 are shown with black and white dots at each node, respectively; Numbers after the last dash correspond to locality numbers in Fig. 1&2a and Table 1; Colours correspond to West1 (dark blue), West2 (light blue), East1 (dark orange), and East2 (light orange) lineages inferred with population structure analysis.

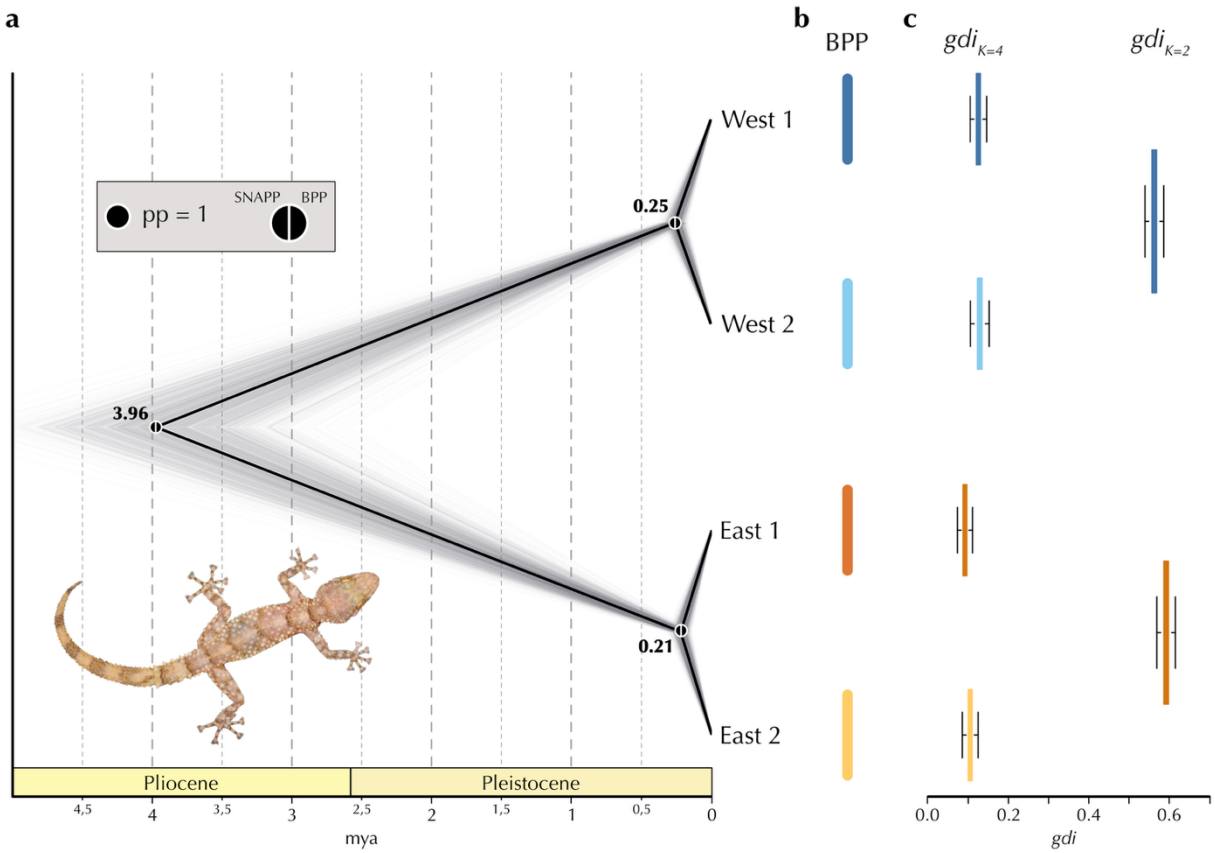

Figure 4. Species tree and species delimitation. **a**: Time-calibrated Multispecies Coalescent (MSC) species tree inferred from 8,433 uSNPs in 12 *Asaccus montanus* individuals. Mean divergence times are shown at each node, with a cloudgram (grey lines) representing temporal uncertainty across the posterior distribution of trees. Circles at nodes indicate posterior probabilities (pp) from species tree inference with SNAPP and BPP analysis A01. **b**: Species-level assignments inferred with BPP analysis A10. **c**: Posterior distribution of the genealogical divergence index (*gdi*) for the two pairs of sister taxa (West1-West2 and East1-East2) and the two main lineages (West-East).

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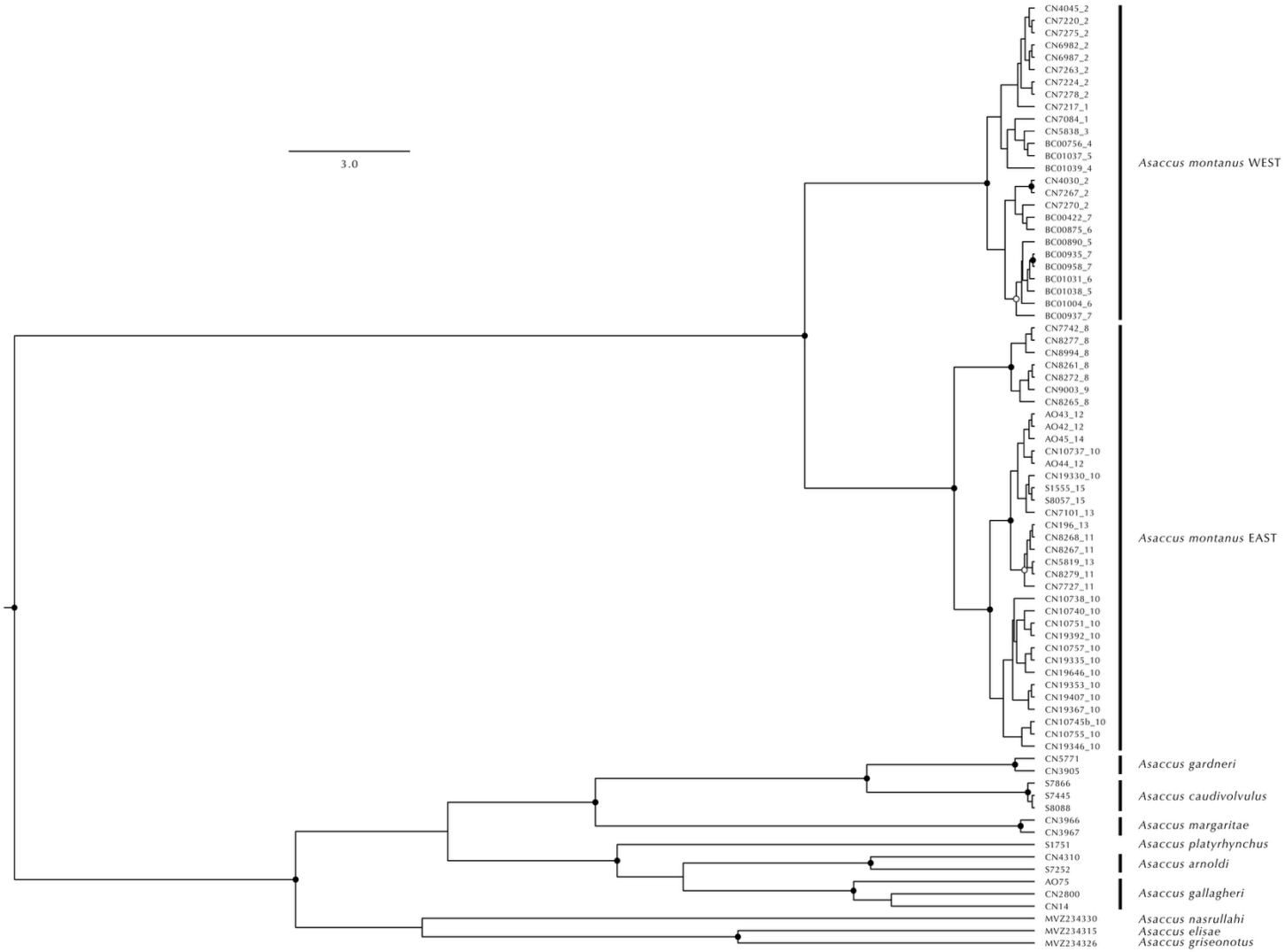
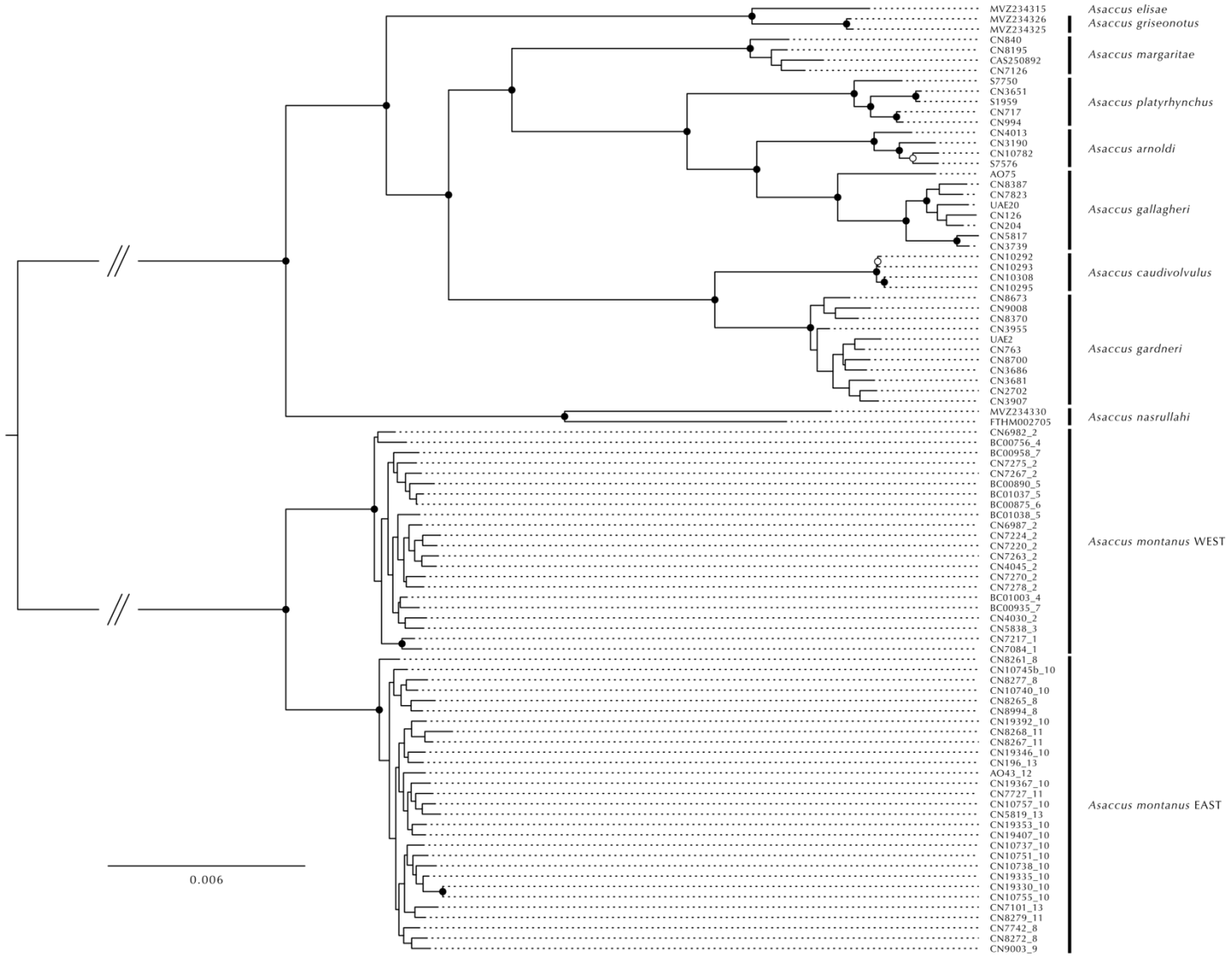
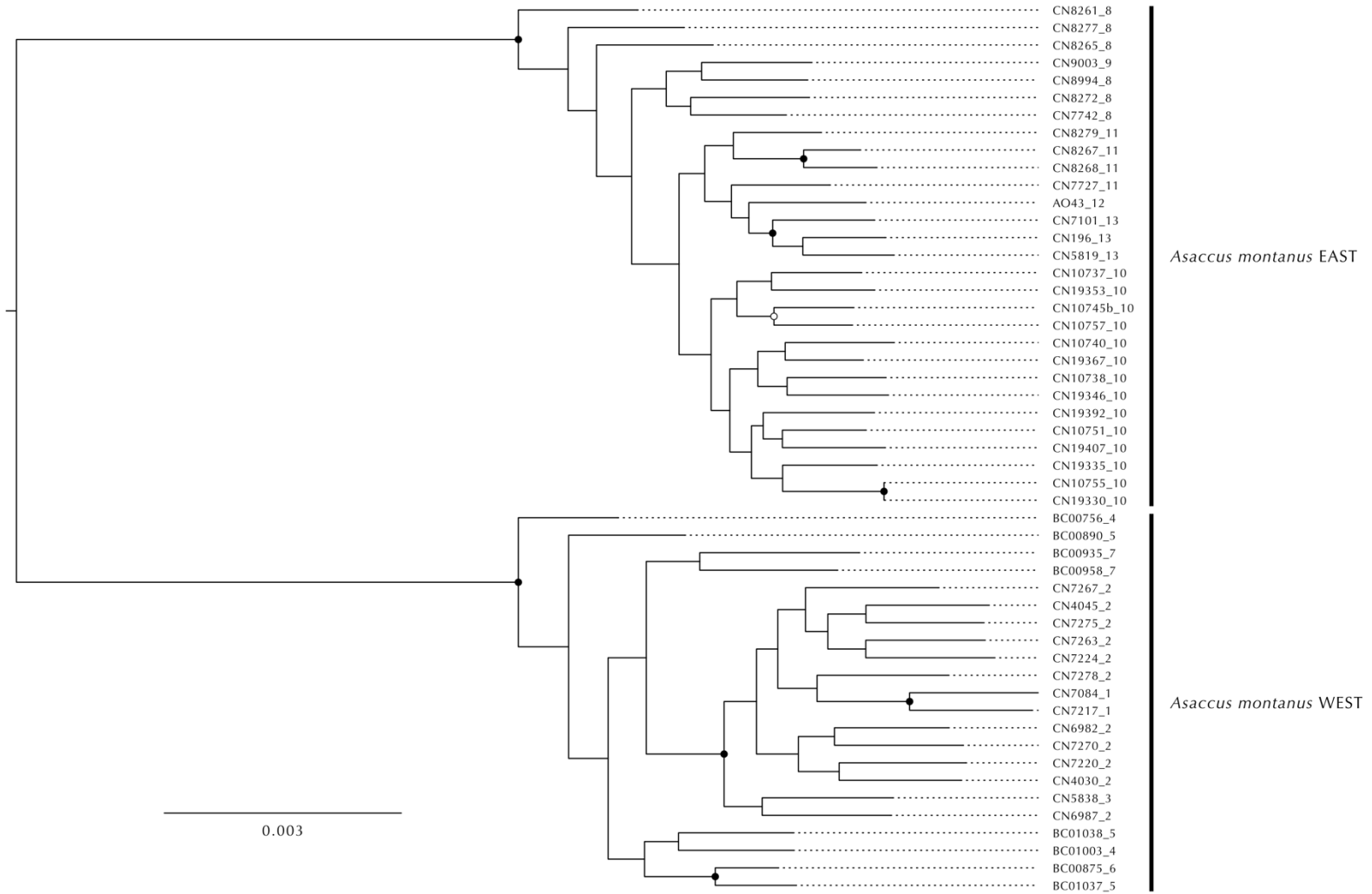
Supplementary Material



Supplementary Figure 1. Bayesian inference tree based on the *12S rDNA* mitochondrial gene from 51 *Asaccus montanus* specimens and 16 from other *Asaccus* species. Posterior probability (pp) values above 0.95 and pp > 0.85 are shown with black and white dots at each node, respectively; Numbers after the last dash correspond to locality numbers in Fig. 1&2a and Table 1.



Supplementary Figure 2. Maximum likelihood tree inferred with a concatenated dataset of 985 nuclear loci from 51 *Asaccus montanus* specimens and 41 from other *Asaccus* species. *A. montanus* was used to root the tree. Black dots at nodes represent bootstrap values above 85% and white dots values above 75%; Numbers after the last dash correspond to locality numbers in Fig. 1&2a and Table 1.



Supplementary Figure 3. Maximum likelihood tree inferred with a concatenated dataset of 7,326 nuclear loci from 51 *Asaccus montanus* specimens. *A. montanus* WEST was used to root the tree. Black dots at nodes represent bootstrap values above 85% and white dots represent values above 75%; Numbers after the last dash correspond to locality numbers in Fig. 1&2a and Table 1.