Museomics of Carabus giant ground beetles evidences an Oligocene origin and in situ Alpine diversification

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**Abstract:** The development of museomics represents a major paradigm shift in the use of natural history collection specimens for systematics and evolutionary biology. New approaches in this field allow the sequencing of hundreds to thousands of loci from across the genome using historical DNA. HyRAD-X, a recently introduced capture method using bench-top designed probes, has proved very efficient to recover genomic-scale datasets using natural history collection specimens. Using this technique, we infer at both the intra- and interspecific levels, the most robust phylogeny of Arcifera to date, an ecologically and morphologically diverse clade of *Carabus* giant ground beetles. We successfully generated a genomic dataset of up to 1965 HyRAD-X loci for all described species, permitting to infer a robust dated phylogenomic tree of this clade. Our species delimitation and population genomic analyses suggest that the current classification in Arcifera is in line with its evolutionary history. Our results suggest an origin of Arcifera in the late Oligocene followed by speciation events during the warm mid-Miocene unlinked to Pleistocene glaciations. The dynamic paleogeographic history of the Palearctic region likely contributed to the diversification of this lineage with a relatively ancient colonization of the proto-Alps followed by *in situ* speciation where most species of Arcifera are currently found sometimes syntopically likely as a result of post-glaciations secondary contacts.

**Keywords:** Arcifera; Beetle evolution; Carabinae; Historical DNA; HyRAD-X; Phylogenomics; Palearctic biogeography; Pleistocene glaciations
**Introduction**

Recent developments in museomics are opening new prospects allowing samples from natural history collections (NHC) to enter the era of genomics (reviewed in (Raxworthy & Smith 2021; Card et al. 2021). Specimens held in the collections are crucial for the study of systematics and taxonomy, but also for the study of ecology and evolution (Duchenne et al. 2020). Sampling from NHC specimens is also a major asset to study groups that are currently rare in the wild, for which authorizations to collect new specimens are difficult to obtain, or for which a comprehensive taxonomic and geographic sampling would require extensive fieldwork campaigns. Such a strategy is therefore very powerful when working on taxonomic groups presenting a wide geographical range. In extreme cases, and when species are believed to be extinct, NHC represent the only potential source of genetic data (Toussaint et al. 2021; de-Dios et al. 2023).

Innovative approaches are now making it possible to obtain genetic information from NHC specimens for which it has long been impossible to recover DNA. The DNA in these specimens, referred to as historical DNA (hDNA), is in low quantity, fragmented, has undergone chemical modifications over time and contains contaminants linked to the history of the collection (Raxworthy & Smith 2021). Improvements in extraction methods, sequencing technologies but above all the development of new capture methods has enabled an increasing amount of genetic information to be recovered. They allow to overcome the difficulties associated with highly degraded and fragmented hDNA from NHC samples, which prevents conventional amplification using standard molecular primers (Landry et al. 2023). Among these methods, Ultra Conserved Elements (Blaimer et al. 2016; Faircloth 2017) or anchored hybrid enrichment of conserved regions (AHE, Lemmon et al. 2012; Mayer et al. 2021), are based on the capture of informative loci previously designed from existing genomic data and generally target fairly conserved regions in order to perform large phylogenies. Applying these approaches to NHC specimens allows to integrate samples that are complicated to obtain in the field. In order to work on non-model species for which no prior genomic data is available, the HyRAD (Suchan et al. 2016) and HyRAD-X (Schmid et al. 2017) approaches enable probes to be designed directly from a few phylogenetically close fresh samples. These approaches, based on bench-top production of probes, also make it possible to dispense with the high cost of probe synthesis. Probes are designed using ddRADseq protocol (Peterson et al. 2012) allowing to target thousands of loci randomly distributed along the genome. This approach is suitable for integrating NHC samples into population-scale studies (Gauthier et al. 2020) or for phylogenetic studies of taxa that have recently diverged, such as within a genus (Gauthier et al. 2023). The HyRAD-X approach designs probes on fresh RNA extractions. By targeting only expressed gene loci, the HyRAD-X approach makes it possible to investigate phylogenetic questions at older evolutionary scales than the HyRAD approach (Toussaint et al. 2021). Using these probe sets, hDNA is then captured by hybridization and sequenced using NGS technologies. This allows only the targeted loci to be recovered while eliminating all unwanted fragments such as contaminants. After sequencing, the loci are reconstructed and aligned using appropriate bioinformatic pipelines in order to make phylogenetic inferences (Toussaint et al. 2021).
2021). Unlike random Whole Genome Sequencing (WGS) of all the extracted DNA, these targeted
approaches enable better recovery of loci and integration of a larger number of NHC samples into
the phylogenetic inferences made in fine (Toussaint et al. 2021). Although the efficiency of
HyRAD-X has been tested at higher taxonomic levels, an empirical investigation of its
performance at the interface between population and species levels is needed.

The genus *Carabus*, Linnaeus 1758 (Coleoptera: Carabidae), is a monophyletic highly
diversified lineage comprising ca. 970 species classified into 91 subgenera (Deuve 2019, 2021).
This genus, together with its sister genus *Calosoma* (cosmopolitan, 130 species) form the tribe
Carabini (Osawa et al. 2004; Toussaint Fls & Gillett 2018; Toussaint et al. 2021; Sota et al. 2022).
Within *Carabus*, the clade named Arcifera Imura, 1996 is sister to the very diversified clade
Eucarabi Deuve, 2013 (Deuve et al. 2012; Deuve 2021). This clade is mainly Palearctic, ranging
in the west from southwest England to Ukraine and Turkey in the east. The range of this group
notably encompasses the Carpathian mountains as well as the Swiss, Italian, Austrian and Dinaric
Alps. It currently includes four subgenera: *Carabus* (Hygrocarabus) Thomson, 1875, *Carabus*
(Platycarabus) Morawitz, 1886, *Carabus* (Chaetocarabus) Thomson, 1875 and *Carabus*
(Heterocarabus) Morawitz, 1886 (Deuve 2019, 2021). Within Arcifera, the subgenus *Carabus*
(Hygrocarabus) contains two species found from France to Ukraine, the status of which has been
extensively debated over the past decades due to reduced morphological differences and
inconsistent genetic admixture patterns (Müller-Kroehling et al. 2006; Müller-Kroehling et al.
2014); Matern et al. 2009, 2010; Mossakowski et al. 2020). These hygrophilous nocturnal species
live in river banks and hunt close or in the water of cold forest streams. The two species are in
relative allopatry with *Carabus nodulosus* Creutzer, 1799 being found from eastern France to
Austria and western Balkans, and *Carabus variolosus* Fabricius, 1787 from Slovakia to Ukraine
and Bulgaria (Kulijer 2019; Deuve 2021; Bekchiev et al. 2022; Hristovski et al. 2023). Despite
the protection status of their habitat (Annexes II and IV of the European Union’s Habitats
Directive), these two species appear to be declining due to anthropogenic activities and their
consequences (Tyszecka et al. 2023). The subgenus *Carabus* (Chaetocearabus) only contains two
allopatric species following Deuve (Deuve 2019, 2021), the widespread *Carabus intricatus* Linné,
1761 found from western France to Ukraine and Greece, and the Greek endemic *Carabus
arcadicus* Gistl, 1850. These two species are found in sympathy in Greece where hybrids are
known from example at the Katara Pass in the Epirus region. Additionally the status of several
subspecies in both taxa has been debated, and some authors recognize *Carabus arcadicus merlini*
Schaum, 1861 (Greece), *Carabus intricatus lefebvrei* Dejean, 1826 (southern Italy including
Sicily) and *Carabus intricatus krueperi* Reitter, 1896 (Greece) as separate species within which
additional subspecific taxa have been described (e.g., Cavazzuti & Ghiretti 2020). Perhaps the
most debated taxon of the three being *Carabus intricatus lefebvrei* found south of Umbria to
northern Sicily which is largely allopatric from the rest of the Italian populations found only in the
extreme north of Italy from Piemonte to Friuli (Cavazzuti & Ghiretti 2020). The subgenus *Carabus*
(Heterocarabus) contains a unique species, *Carabus marietti* Cristofori & Jan, 1837, that is found
in southern Bulgaria near the Black Sea and in Anatolia (Turkey), however its ecology and
relationships between the numerous described subspecies remain poorly known (Gueorguiev & Gueorguiev 1995; Hieke & Wrase 2008). Finally, the subgenus Carabus (Platycarabus) is composed of five currently accepted species: Carabus creutzeri Fabricius, 1801, Carabus cychroides Baudi, 1860, Carabus depressus Bonelli, 1811, Carabus fabricii Panzer, 1812 and Carabus irregularis Fabricius, 1792. These beetles are characterized by a flattened morphology, long legs, and elytra generally covered with small foveoli (except in Carabus depressus lucens Schaum, 1857). They are most widely distributed in Central and Eastern Europe, generally at high altitudes, in mountain forests and alpine pastures. The subgenus contains helicophagous species that exhibit different hunting techniques related to the morphology of their mandibles and prothorax (Casale et al. 1998). For instance, Carabus cychroides with a thin, elongated head and prothorax, is adapted to enter gastropod shells and has undergone a process known as “cychrization”. This species has a very restricted range in the Piedmont region of Italy, is endangered and the focus of reinforced conservation programmes (Anselmo & Rizzioli 2022a; b).

In contrast, the species C. irregularis presents a “licinization” resulting in a large head likely adapted to cracking snail shells (Casale et al. 1998). The relationships among species of the subgenus Carabus (Platycarabus) are still debated, and the various taxonomic divisions, both species and subspecies, have yet to be clarified (Casale et al. 1998; Deuve 2021). Natural hybrids have been suggested between Carabus fabricii and Carabus depressus, Carabus creutzeri and Carabus irregularis, Carabus creutzeri and Carabus depressus, and Carabus depressus and Carabus cychroides (Casale et al. 1998; Camard & Leplat 2004; Casale & Rapuzzi 2015), indicating the need for an in-depth study of possible hybridization in this group.

One of the earliest attempts to elucidate the phylogeny of Arcifera was conducted by Ishikawa (Ishikawa 1984), using 21 morphological characters. This study supported the monophyly of Arcifera and placed Carabus (Hygrocarabus) as sister to the rest of the group, in which Carabus (Chaetocarabus) was sister to Carabus (Heterocarabus) and Carabus (Platycarabus). The first placement of Arcifera members in a molecular phylogeny was based on a single mitochondrial fragment (i.e. ND5), and recovered Carabus (Chaetocarabus) and Carabus (Platycarabus) as sister lineages, close to Carabus (Limnocarabus) Géhin, 1876 and Carabus (Euleptocarabus) Nakane, 1956 (Imura et al. 1998). A subsequent study with the same gene fragment but increased taxon sampling recovered a paraphyletic Arcifera due to the placement of Carabus (Hygrocarabus) as sister to Carabus (Limnocarabus) and Carabus (Euleptocarabus) (Imura et al. 2000). In the same study, Carabus (Heterocarabus) was sister to Carabus (Chaetocarabus) and Carabus (Platycarabus). Using the same gene fragment, another study inferred Carabus (Platycarabus) as sister to Carabus (Chaetocarabus) and Carabus (Heterocarabus), within a largely unresolved Carabus clade (Su et al. 2003). A subsequent study using two nuclear gene fragments recovered Arcifera, represented by Carabus (Chaetocarabus) and Carabus (Platycarabus) as sister to the rest of the genus (=Eucarabi) (Sota & Ishikawa 2004). More recently, Deuve et al. (2012) used ten loci to recover Arcifera as sister to the Eucarabi and within Arcifera, they recovered Carabus (Hygrocarabus) as sister to Carabus (Chaetocarabus) and Carabus (Platycarabus). Phylogenetic relationships among Carabus (Platycarabus) were also
investigated using Sanger sequencing data (Casale et al. 1998), suggesting that *C. irregularis* is sister to the rest of the subgenus with *C. fabricii* and *C. depressus* being the most derived lineages in the tree. In parallel to a moderate refinement in the phylogenetic inferences of Arcifera, the estimation of divergence times in the clade has made some progress. Estimates for the origin of Arcifera based on few loci range from the mid-Miocene (ca. 14 Ma, Deuve et al. 2012) to the early Oligocene (ca. 30 Ma, Schmidt et al. 2023). No major improvement in our understanding of Arcifera systematics and evolution has been achieved in the past decade and there is a need to infer a robust evolutionary tree for this section of *Carabus* to better understand the morphological, ecological and geographical evolution of constituent lineages.

In this study, we take advantage of the HyRAD-X approach to integrate a large number of samples throughout the large geographical range of Arcifera. We rely on phylogenomic inferences, species delimitations and population genomics approaches to clarify the taxonomy and elucidate the evolutionary history of this complex group of species. In particular, we use this new genomic framework to test which abiotic factors may have fostered the diversification of Arcifera through space and time in the Cenozoic.

**Material and methods**

**Taxon sampling and DNA extraction**

The initial sampling was designed in order to sample major lineages within the Arcifera group comprising four subgenera *Carabus* (*Chaetocarabus*), *Carabus* (*Heterocarabus*), *Carabus* (*Hygrocarabus*) and *Carabus* (*Platycarabus*) (Deuve 2019). A total of 96 samples were initially collected, mainly from NHC samples (87 samples, i.e. 90% of the dataset) but also from a few fresh samples (9 samples, i.e. 10% of the dataset) when these were available (Supplementary Table 1). Multiple specimens of the same taxa and geographic populations were initially selected to mitigate the risk of hDNA degradation that can result in specimens not being processed. NHC specimens used in this study are kept at the Natural History Museum of Geneva (MHNG, 76 specimens) and Zoologische Staatssammlung München (ZSM-SNSB, 10 specimens). Eight specimens collected in 96% ethanol were also used and have been deposited in the MHNG collections. DNA was extracted destructively from a single leg using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany). Quantity and quality of the purified DNA were assessed with a Fragment Analyzer. Based on DNA quality and concentrations, 38 specimens were not included in the final samples selected for capture, enrichment and sequencing (ca. 40% of DNA extractions not processed). Overall, a total of 56 Arcifera specimens were sequenced de novo for this study, representing all Arcifera subgenera and species, several subspecies for the most widespread species as well as a good geographical representation of each species range (taking into account 12 specimens that were eventually not included in the decisive datasets, see Results). Commonly, early sampling erosion and discarded samples are not discussed in the framework of museomics studies but we believe that this is critical to understand the limitations and cost of such approaches in modern phylogenomic studies. The initial sampling in this study was specifically designed to accommodate a ca. 40–50% specimen loss during DNA quality/quantity assessment (e.g.,
Toussaint et al. 2021), and therefore the resulting sampling is well-suited to tackle the focal
taxonomic and evolutionary questions in Arcifera. The final taxon sampling was complemented
by eight samples of Carabus (including one of C. irregularis and one of C. variolosus) and one of
Calosoma sycophanta (Linné, 1758) retrieved from (Toussaint et al. 2021) (see Supplementary
Table 1 for more details).

HyRAD-X protocol

The HyRAD protocol was applied as in (Toussaint et al. 2021). For fresh specimens a shearing
step with NEBNext dsDNA Fragmentase (New England Biolabs) was performed before library
preparation. Shotgun libraries were prepared based on the protocol developed in (Tin et al. 2014).
Purified DNA was phosphorylated with T4 Polynucleotide Kinase. After heat-denaturation into
single-stranded DNA, G-tailing was performed with Terminal Transferase and second strand DNA
was synthesized with Klenow Fragment (3’-5’exo-) using a poly-C oligonucleotide. Blunt-end
reaction was performed with T4 DNA Polymerase and barcoded adapters were ligated to the
phosphorylated end with T4 DNA ligase. After adapter fill-in with Bst DNA Polymerase (Large
Fragment), PCR were run using Phusion U Hot Start DNA Polymerase (Thermo Scientific) and
indexed PCR primers. Libraries were pooled in equimolar quantities based upon their respective
concentrations. Hybridization capture for enrichment of shotgun libraries was based on the
MYbaits protocol (Arbor Biosciences) modified as in (Toussaint et al. 2021) to include a two-step
capture at different temperatures (Li et al. 2013). Final library sequencing was performed on
Illumina NovaSeq 6000 SP using a paired-end protocol (Lausanne Genomic Technologies Facility,
Switzerland).

Illumina sequencing data cleanup and processing

Raw reads were demultiplexed according to indexes and barcodes using CutAdapt2 (Martin 2011).
Reads were cleaned using CutAdapt2 (Martin 2011) and quality was assessed all along the process
using fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Cleaned reads were
individually mapped onto the loci catalog using BWA-MEM (Li 2013) (Supplementary Figure 1).
The GATK (GenomeAnalysisTK) IndelRealigner tool (McKenna et al. 2010) realigned the indels
and deamination were corrected using mapDamage2.0 (Jónsson et al. 2013). For each sample and
each locus, a consensus sequence was generated from the mapping file using samtools mpileup,
bcftools and vcfutils.pl (Li et al. 2009). Consensuses were generated keeping the majority allele at
each position. Twelve samples with too much missing data (more than 80% of N), were identified
using seqtk and removed (Supplementary Table 1). Two thresholds of minimum coverage
(min_cov) were applied to keep positions: min_cov=3 and min_cov=6. To test different levels of
missing data, decisive datasets were generated applying three thresholds for the minimum number
of samples per locus (min_sample): min_sample=10, min_sample=17 and min_sample=32. As a
result, six datasets were generated: Dataset A (min_cov=6, min_sample=10, 50 taxa, 1’481 loci),
Dataset B (min_cov=6, min_sample=10, 52 taxa, 1’965 loci), Dataset C (min_cov=6,
min_sample=17, 50 taxa, 1’014 loci), Dataset D (min_cov=3, min_sample=17, 52 taxa, 1’291
loci), Dataset E (min_cov=6, min_sample=32, 50 taxa, 366 loci) and Dataset F (min_cov=3, min_sample=32, 52 taxa, 478 loci). The consensus sequences were combined and aligned with MAFFT using the --auto option. Eight samples from (Toussaint et al. 2021) were integrated at the alignment step. The final datasets only differ at the taxon sampling level with respect to Carabus arcadicus merlini CBX0176 and Carabus cygroides CBX0082 that were included only in Datasets B, D and F (these two taxa were systematically discarded because of low genomic coverage when generating loci with a min_cov=6, i.e., in Datasets A, C and E).

MitoFinder (Allio et al. 2020) was used to identify mitochondrial genes among all sequenced loci. The kept genes were shared by at least half of the samples. The genes were aligned, and the sequences were cleaned in Geneious. Individual locus haplotype networks were built in SplitsTree v.4.19.1 (Huson & Bryant 2006). The networks were reconstructed using calculated uncorrected p-distances and the NeighborNet algorithm. All non-Arcifera outgroups were removed before analyses. In parallel, a SNP calling was performed on the mapping files from the Arcifera species using GATK (McKenna et al. 2010) in order to perform complementary population genomic analyses and compare the results with those obtained from the locus oriented approach, avoiding any bias linked to locus reconstruction (Dataset H).

Phylogenetic inferences
For each dataset, phylogenetic inferences were performed using IQ-TREE v2.0.5 (Minh et al. 2020) using the edge-linked partition model (Chernomor et al. 2016). First, the best partitioning schemes were estimated using PartitionFinder v2.1.1 (Lanfear et al. 2017) with the rcluster algorithm under the Akaike Information Criterion corrected (AICc), with a rcluster-max of 2,000 and a rcluster-percent of 20. The resulting partitioning schemes were then used in IQ-TREE to select corresponding models of nucleotide substitution using ModelFinder (Kalyaanamoorthy et al. 2017) and the AICc across all available models in IQ-TREE. To avoid local optima, we performed 100 independent tree searches for each dataset in IQ-TREE. To estimate branch support, we calculated 1,000 ultrafast bootstraps along with 1,000 SH-aLRT tests in IQ-TREE (Guindon et al. 2010; Hoang et al. 2018). We used the hill-climbing nearest-neighbour interchange topology search strategy to avoid severe model violations leading to biased ultrafast bootstrap estimations (Hoang et al., 2018). The best tree for each analysis was selected based on the comparison of maximum likelihood scores. Coalescent species trees were inferred using ASTRAL-hybrid (Zhang & Mirarab 2022). We first performed individual locus trees using IQ-TREE v2.0.5 (Minh et al. 2020) and branch supports were assessed using 1,000 ultrafast bootstraps. Best substitution model for each locus was estimated using ModelFinder (Kalyaanamoorthy et al. 2017). Species tree reconstruction was performed combining gene trees using the weighted-ASTRAL optimization algorithm (Zhang & Mirarab 2022) taking into account phylogenetic uncertainty by relying on branch length and branch support across locus trees. As a complement to the locus reconstruction approach, we performed phylogenetic inferences based on the SNP set used for the population genomic approaches. Bi-allelic SNP shared by at least four samples were extracted and all invariant sites removed. Species trees were inferred with RAxML-NG (Kozlov et al. 2019) using
GTR+G+ASC_LEWIS model for ascertainment bias correction and branch supports were assessed using 1,000 bootstraps.

Divergence time estimation

Divergence time estimation was performed in BEAST 1.10.4 (Suchard et al. 2018) based on a subset of loci selected using a gene-shopping approach to make these analyses tractable on a bioinformatic cluster. SortaDate (Smith et al. 2018) was used with default settings to select 100 loci sorted using the following criteria: clock-likeness, tree length, and least topological conflict with the IQ-TREE species tree on dataset E. The selected loci were then concatenated into a Dataset G for relaxed-clock Bayesian divergence time estimation. The best partitioning scheme and substitution models were determined with PartitionFinder2 (Lanfear et al. 2017) using the greedy algorithm with the parameter minsubset-size = 2000 and the Bayesian information criterion algorithm to choose between competing models. Clock partitioning was implemented by 1) a single clock for all partitions and 2) a clock for each partition (eight in total; see Results). A Bayesian lognormal relaxed clock model was assigned to the different clock partitions. Different tree models were tested using a Yule pure birth model (Yule 1925; Gernhard 2008), a birth-death model (Drummond et al. 2006; Gernhard 2008) as well as a Constant population size coalescent model (Kingman 1982). Since the fossil record of Carabus is scarce, we relied on secondary calibrations from a study focusing on Adephaga evolution based on 23 beetle fossil calibrations (Baca et al. 2021). According to this study, the separation between the genera Calosoma and Carabus occurred about 41.4 [37.1–46.1] million years ago (Ma). This age was used as a secondary calibration for the corresponding node in our topology (split Calosoma/Carabus, in this case the root). A second calibration was used to constrain the crown of Carabus. Following (Baca et al. 2021), this node was constrained to match the recovered age in their study at about 25.4 [22.8–28.2] Ma. The analyses were conducted for 50 million generations, sampling parameters and trees every 5000 generations. The maximum clade credibility tree for each analysis was generated in TreeAnnotator 1.10.4.

Species delimitation and hybridisation

We used a combination of species delimitation methods and population genomic approaches to test species and subspecies limits. For these analyses we excluded the six non-Arcifera outgroup specimens resulting in a dataset composed of 44 samples. We extracted the 44 Arcifera samples from Dataset E composed of 366 shared loci which present the lowest level of missingness (Table 1). First, BPP (Flouri et al. 2018) was used with the A11 option, using inverse-gamma distributed diffuse priors (α = 3) for the population sizes (θ) and root ages (τ0). Analysis was run for 100,000 generations, sampling every 100 generations after a burnin of 8,000 generations. Second, the multi-locus species delimitation using Bayesian model comparison implemented in the TR2 package (Fujisawa et al. 2016) has been applied on the same dataset. Locus trees generated with IQ-TREE v2.0.5 (Minh et al. 2020) and previously used for the
weighted-ASTRAL approach were used as well as the maximum likelihood IQ-TREE consensus tree on dataset A as guide tree. Outgroups were removed from gene trees and the guide tree.

From the SNP (Dataset H), population clustering was assessed using STRUCTURE 2.3.3 (Pritchard et al. 2000). Bi-allelic SNPs shared by at least 40% of the samples were extracted using VCFtools v0.1.12a (Danecek et al. 2011). Because markers are supposed to be unlinked, we extracted randomly only one SNP by locus. K-values from 1 to 15 were tested with no prior population information and performed three times for each of them to verify a convergence of estimations. A burn-in of 100,000 runs was used followed by 500,000 iterations. The most likely number of clusters was determined using the Evanno method (Evanno et al. 2005) implemented in Structure Harvester (Earl & vonHoldt 2012). The replicates were then combined and the figures generated using CLUMPAK server (Kopelman et al. 2015). To investigate putative admixture between species or subspecies we estimated Patterson’s D statistic (ABBA-BABA test) (Patterson et al. 2012) for all subspecies/species quartets using the Dsuite (Malinsky et al. 2021). The analyses were performed on bi-allelic SNPs shared by at least 40% of the samples composed of 6,743 SNPs. Z-scores and associated p-values were calculated to assess the significance of the results.

Results

Museomic approach efficiency

The combination of historical and fresh samples enabled to compare the effectiveness of museomics methods. The DNA concentrations obtained from a single leg are very variable between fresh samples (mean = 8.37 ng/µL; sd = 6.82 ng/µL) and NHC samples (mean = 1.18 ng/µL; sd = 2.45 ng/µL). There was a significant correlation between the quantity of DNA extracted and the age of the specimens (Figure 1A). For the NHC samples, this concentration was not homogeneous, with some samples nevertheless showing a high concentration. Forty samples with a concentration below the detection thresholds, were excluded from the rest of the capture process. It should be noted that some samples with very low DNA concentrations, such as Carabus fabricii CBX0094 captured in 1977 with a concentration of only 0.08 ng/µL, were reliably placed into the final phylogenetic inferences. For specimens with measurable DNA, the capture process worked efficiently, allowing the sequencing of an average of 8.4 million reads per sample (sd = 9.2 million). There was a large difference between the average number of reads obtained from fresh samples (mean = 23.8 millions; sd = 11.0 millions) and NHC samples (mean = 5.4 millions; sd = 5.0 millions). The age of the specimens also had an influence on the number of reads obtained, as there was a significant correlation between the age of the specimens and the number of reads obtained (Figure 1B).

After locus reconstruction, the difference between fresh and NHC samples persists, with an average of 1765 loci recovered in fresh samples (sd = 553) and 629 in NHC samples (sd = 447) (Figure 1C). This difference is of the same order when looking at shared loci (Figure 1D). There is a large heterogeneity in the number of loci recovered between NHC samples, largely linked to the age of the specimen. Samples with too few loci (< 150 loci), i.e. 12 samples, had to be excluded
from the final datasets. For 35 NHC samples, the number of loci recovered, on average 793 (sd = 400), was sufficient to include them in subsequent analyses. Although strict filtering steps reduced the number of NHC samples, they also ensured the reliability of the dataset for downstream inferences.

**Phylogenomic inferences**

We inferred the phylogenetic relationships among Arcifera using six different datasets under various taxon sampling and/or gene sampling strategies (Figure 2). The results of analyses based on a concatenation approach performed in IQ-TREE and on a coalescent species-tree approach conducted in wASTRAL are consistent except for the placement of *Carabus marietti*, the branching pattern within *C. (Chaetocarabus)* and relationships between *C. creutzeri* and *C. fabricii*. The subgenus *C. (Hygrocarabus)* is recovered as monophyletic in all analyses (including wASTRAL) and as sister to the rest of Arcifera, however all analyses failed to recover *C. nodulosus* and *C. variolosus* as reciprocally monophyletic. In all IQ-TREE inferences except the one based on Dataset E and all wASTRAL analyses except the one based on Datasets A and B, *C. marietti* is recovered as sister to *C. (Chaetocarabus)* with heterogeneous levels of branch support.

In the IQ-TREE analysis of Dataset E, this taxon is recovered as sister to the genus *Carabus* as a whole, whereas in wASTRAL analyses of Datasets A and B it is recovered as sister to Arcifera except *C. (Hygrocarabus)* with low branch support. The subgenus *C. (Chaetocarabus)* is always recovered as monophyletic but internal relationships differ between analyses. A minority of analyses recovered *C. arcadicus* and *C. intricatus* as reciprocally monophyletic (for instance no wASTRAL analysis recovered this relationship). The subspecies *C. intricatus* lefebvrei is recovered as sister to the nominal subspecies in all analyses. When *Carabus arcadicus merlini* is included (Datasets B, D and F only), it never groups with other specimens of the nominal subspecies resulting in *Carabus arcadicus* being consistently inferred as paraphyletic when this taxon is included (Supplementary Figure 2). The subgenus *C. (Platycarabus)* is recovered as monophyletic and with identical interspecific relationships across all IQ-TREE analyses but some contention in wASTRAL ones. The alpine endemic *C. cychroides* is recovered as sister to the rest of the subgenus in all analyses with strong branch support (IQ-TREE and wASTRAL). The species *C. depressus* is inferred as the next lineage branching off in *C. (Platycarabus)* across all IQ-TREE analyses and most wASTRAL analyses (except in Dataset A and E where it is recovered as sister to *C. irregularis* with low branch support). The subspecies *C. depressus lucens* is recovered as sister to the nominal subspecies in all analyses. The placement of the three remaining *C. (Platycarabus)* species is identical across all IQ-TREE analyses with strong branch support, with *C. creutzeri* being sister to *C. fabricii* and *C. irregularis*. The wASTRAL analyzes recover different relationships but with low branch support, with a weakly supported sister relationship between *C. creutzeri* and *C. fabricii* in analyses of Datasets D, E and F. The subspecies *C. fabricii malachiticus* is recovered as nested within the nominal subspecies in all analyses. The subspecies *C. irregularis montandoni* is recovered as sister to *C. irregularis bucephalus* and *C. irregularis*
irregularis in all IQ-TREE analyses whereas it is *C. irregularis bucephalus* that is inferred as sister to *C. irregularis irregularis* and *C. irregularis montandoni* in all wASTRAL analyses. Overall the IQ-TREE and wASTRAL inferences are highly compatible when collapsing the weakly supported relationships in wASTRAL species trees (gray and red asterisks in Figure 2). In particular, inconsistent relationships in wASTRAL compared to IQ-TREE always received poor branch support. We observe that branch support and overall phylogenetic resolution appears positively correlated to gene and taxon sampling (i.e., including less taxa and less loci to improve matrix completeness likely results in a loss of resolution).

**Divergence time estimation**

The BEAST dating analysis revealed consistent results for the four main nodes, i.e. the root, *Carabus*, Arcifera and *Carabus (Platycarabus)* nodes, according to the three models tested: Yule, Birth-Death model, and Constant population size coalescent (Figures 3 and 4). The coalescent model including eight Bayesian log-normal relaxed clocks received the best marginal likelihood as calculated using stepping-stone sampling in BEAST and was therefore selected hereafter. This inference suggests an origin of Arcifera at 26.07 Ma (95% HPD: 22.77 - 29.67 Ma) and 14.56 Ma (95% HPD: 12.52 - 16.76 Ma) for the *Carabus (Platycarabus)* subgenus.

**Species delimitation and putative hybridization**

The different approaches to species delimitation produced contrasting results. The analysis performed with BPP is the most consistent with morphology and the current classification. The two species *Carabus variolosus* and *C. nodulosus* are well separated even though *C. nodulosus* is not monophyletic in our phylogeny. TR2 approach proposes an oversplit of the three *C. nodulosus* samples. Conversely, the STRUCTURE approach groups the two species in a single cluster (Supplementary Figure 6). *Carabus marietti*, the only representative of *Carabus (Heterocarabus)* is delineated as a species in all three approaches. Within *Carabus (Chaetocarabus)*, the two species *C. arcadicus* and *C. intricatus* are delineated by BPP but are merged by TR2 and STRUCTURE, potentially for the same reasons as in *Carabus (Hygrocarabus)*. It should be noted that the two subspecies of *C. intricatus*, i.e. *C. intricatus lefebvrei* and *C. intricatus intricatus*, are never delineated as distinct species. The species *C. cychroides* was well discriminated in two of the three approaches, with only TR2 proposing an additional split of the most basal sample. The two subspecies of *C. depressus*, *C. depressus depressus* and *C. depressus lucens* are not grouped together in the BPP approach and are identified as two distinct species. The results of the three methods are fully consistent with the morphology for *C. creutzeri* and *C. fabricii*. For *C. irregularis*, the situation is similar for two of the three methods, i.e. BPP and Structure. Among the 85 trios analysed, high D-statistics values, > 0.25, with significant p-values were observed for three trios. For two of these, *C. cychroides* was observed in P1 and *C. arcadicus* in P3. Despite this, no f-branch signal significantly different from zero could be identified (Supplementary Figure...
These results suggest an absence of past introgression between the different species and subspecies.

Discussion

Using museomics to obtain an extensive dataset

The HyRAD and HyRAD-X methods are unique in that they allow in-house production of probes using a ddRAD protocol, either directly on the DNA of a few fresh samples (Suchan et al. 2016) or on their RNA (Schmid et al. 2017). These approaches allow to target several thousand loci and generate in turn high-resolution phylogenomic inferences (Young & Gillung 2020). In this study, we obtained 1,965 loci for the most extensive dataset. These loci were informative enough to resolve both the deep relationships between outgroups and the more recent relationships at the intrageneric and intraspecific scales. In addition, the identification of SNPs on these loci also enabled population genomic approaches such as the study of genetic structure and admixture.

In addition, the HyRAD-X approach made it possible to integrate samples with extremely low initial DNA quantities. However, out of 96 samples from which DNA was extracted, 40 had an undetectable quantity of DNA. In the context of museomics projects, it is therefore instrumental to plan for redundancy in the sampling, with several samples per targeted taxon, in order to compensate for any failures. Furthermore, the ability to generate genetic information from hDNA is not entirely predictable. The recovery of meaningful genomic data does not seem to be linked to the age of samples (Figure 1), in line with existing observations (Toussaint et al. 2021; Nunes et al. 2022). In that vein, large amounts of genomic data could be obtained from older NHC samples when almost none could be obtained from more recent samples. The quality and quantity of DNA that can be extracted from NHC specimens is linked to factors that we cannot control, such as the conditions of collection and preservation process (Post et al. 1993; Dillon et al. 1996; Ruppert et al. 2023).

Systematics and species delimitation in Arcifera

Our results provide a robust phylogenomic tree of Arcifera for the first time (Figure 2). Overall we support the view that Arcifera represents a monophylum within which all four subgenera form clades. The monophyly of Arcifera is also supported by the presence of a hook-shaped ligulum (i.e., arculus) at the base of the endophallus, a strong morphological character that unites all constituents of this lineage (Imura et al. 2000; Deuve et al. 2012). Our study is the first to provide strong evidence for these relationships while including all species of the group. Other studies based on reduced genomic sampling, often a single gene fragment, either failed to recover Arcifera as monophyletic (Imura et al. 2000; Osawa et al. 2004), or had too limited a taxon sampling to properly test the placement and otherwise monophyly of each subgenus (Su et al. 2003; Sota & Ishikawa 2004; Deuve et al. 2012). Except for a minority of analyses, our results strongly suggest
that Carabus (Hygrocarabus) is sister to the rest of Arcifera, with Carabus (Platycarabus) as sister to a clade formed by Carabus (Chaetocarabus) and Carabus (Heterocarabus).

Within Carabus (Hygrocarabus), we recover C. variolosus nested within C. nodulosus. This result contrasts with the ones of Mossakowski et al. (2020) where the two species were suggested to be well differentiated genetically. In their study, these authors argued based on the analysis of two gene fragments that both taxa form distinct clades although several specimens caused each species to be paraphyletic. Some tests of mating between the two candidate species were also performed in this study and suggested that the two lineages do not mate. However, the scale and conditions of these trials do not allow to conclusively rule out potential mating. We argue that in the current state of our knowledge it is not yet possible to definitively test species boundaries, past introgression and signature of hybridization between Carabus nodulosus and C. variolosus. A desired approach would be to combine a large geographical sampling as in Mossakowski et al. (2020) with a genomic scale dataset as developed in the present study to revisit the systematic conundrum within this subgenus at the population level.

Within Carabus (Chaetocarabus), we recover C. arcadicus as sister to C. intricatus in most analyses (Figure 2). These two species are allopatric, morphologically well-differentiated and little doubt exists with respect to their status as distinct species. Surprisingly our species delimitation analyses only partly support the two species hypothesis, with TR2 and STRUCTURE considering that Carabus (Chaetocarabus) is a unique species. Considering the low genomic coverage of some taxa included in the analyses (see below), the clear morphological and geographical split between these lineages and the support from BPP analyses, we argue that the validity of these two species is uncontroversial. Natural hybrids with an intermediate morphology and usually green dorsal pattern are known to exist along the limits of their respective ranges in northern Greece (i.e., at the Katara pass) where Carabus intricatus macedonicus (not sampled here) and C. arcadicus arcadicus co-occur. Both Carabus arcadicus and C. intricatus also comprise geographically restricted subspecies in Greece that have been considered valid species by some authors. In the south of Greece, the melanistic subspecies Carabus arcadicus merlini is endemic to the Peloponnese peninsula and allopatric from the nominal subspecies present in the north. One specimen of this taxon was sequenced but genomic coverage was low and therefore it was only included in the less stringent Datasets B, D and F. In the phylogenetic analyses of these datasets, the inclusion of Carabus arcadicus merlini systematically results in all three C. arcadicus specimens forming a phylogenetic grade within which C. intricatus is nested. We argue that this is an artifact possibly caused by missing genomic sampling and that both species are reciprocally monophyletic as recovered in all other analyses and as suggested by morphology. However, it is possible that Carabus arcadicus merlini represents a distinct evolutionary lineage since it is always recovered as sister to the rest of C. (Chaetocarabus). Additional taxon sampling is needed to test the placement of this morphologically distinct taxon within the subgenus. Across its range, Carabus intricatus is represented by the nominal subspecies from western France and UK to northern Greece. In the south of Italy and Sicily, this species is represented by the allopatric Carabus intricatus lefebvrei. The status of this taxon is debated and some authors consider it a
valid species. In our results, we recover this subspecies as sister to the nominal subspecies represented by specimens from France and Piemonte. Our phylogenetic inferences support the view of *Carabus intricatus* Lefebvrei as a possible distinct species but our species delimitation analyses reject this hypothesis. To properly test species boundaries within *Carabus intricatus*, additional taxon sampling is needed including a much denser geographical sampling of the nominal subspecies along with all described valid subspecies (Deuve 2019). In the Balkans, several subspecies of *Carabus intricatus* have been described and represented more or less isolated populations restricted to northern Greece. Despite our efforts we could not obtain DNA of good quality for *Carabus intricatus krueperi* endemic to eastern Thessaly and considered by some authors to be a valid species. Here as well, a denser taxon sampling is needed to properly test species boundaries in this group. The placement of *Carabus* (*Heterocarabus*) *marietti* as sister to *C. (Chaetocarabus)* receives support from most analyses in this study. Despite a relatively circumscribed geographic range in northern Turkey and southern Bulgaria, numerous taxa have been described in this subgenus even though currently a single species is considered valid (Turin et al. 2003; Deuve 2019). Increasing the taxon sampling for this group by covering all its geographical range would allow testing the match between morphological and genetic diversity and better understand the evolution of this unique lineage at the inter- and intraspecific interface.

Within *Carabus* (*Platycarabus*), we recover *C. cychroides* as sister to the rest of the subgenus. This result is unexpected because this species is a very narrowly restricted endemic to Piemonte mountain ranges where it lives in alpine meadows and scree >2000m. The species was only included once in a phylogenetic framework by Casale et al. (1998) who recovered it as a derived lineage close to *Carabus depressus* and *C. fabricii*. Interestingly, a sister relationship of this species to the rest of *Carabus* (*Platycarabus*) was suggested by the analysis of morphological characters in Casale et al. (1998). Indeed, this species is morphologically quite different from the rest of the subgenus in that it is one of the most extreme examples of cychrization in *Carabus*, a process by which the pronotum is narrowed to allow predation inside snail shells (= stenocephalic morphology). All species of the subgenus present a stenocephalic morphology, although less marked than in *Carabus cychroides*, except for *C. irregularis* which is macrocephalic. Our phylogenetic inferences are therefore important to understand the evolution of predation strategies and associated morphology across the genus *Carabus* in which both types of morphologies exist (Sota & Ishikawa 2004). Most malacophagous and helicophagous species in *Carabus* are macrocephalic and use their enlarged pronotum, head and robust mandibles to break snail shells. Cases of stenocephaly are most notably observed in *Carabus* (*Platycarabus*) but also in *C. (Damaster)* Kollar, 1836 and *C. (Macrothorax)* Desmarest, 1850. The fact that *Carabus irregularis*, the only *C. (Platycarabus)* macrocephalic species, is recovered as the most derived species in the subgenus, indicates that macrocephaly possibly evolved from a stenocephalic morphology unlike what was suggested in Casale et al. (1998). In the case of *Carabus cychroides*, it is not closely related to any other species of the subgenus as suggested by previous authors, and despite rare known natural hybrids with *C. depressus* in the Cottian Alps (i.e., Colle delle Finestre, Monte Morefreddo, Monte Albergian), these species do not share an immediate recent common
ancestry (Sturani 1962; Casale et al. 1998; Anselmo & Rizzioli 2022a; b). The rest of Carabus (Platycarabus) species and most sampled subspecies are found monophyletic (Figure 2). We recover the subspecies Carabus depressus lucens as sister to the nominal subspecies in all analyses and with robust branch support. This subspecies is morphologically quite divergent from the nominal subspecies and C. depressus bonellii as it completely lacks elytral foveoli. It is also allopatric from the rest of the C. depressus populations, being found in a small transalpine region between France and Italy (i.e., French Queyras to Italian Alpi Marittime), and its status as a valid species even though rejected by three out of four species delimitation analyses should be revisited with enhanced population sampling. Our taxon sampling within Carabus creutzeri does not allow testing subspecies monophyly and relationships in detail but species delimitation analyses unambiguously support a single species (Figure 3). Within Carabus fabricii, we recover the Carpathian populations of C. fabricii (ssp fassati = nominal ssp, and spp malachiticus) nested within Alpine populations of the nominal subspecies. This is unexpected to some extent as Carabus fabricii presents a disjunct distribution between the Alps and the Carpathians (i.e., it is not currently found in the Danube valley). Our results suggest that despite an allopatric range, gene flow has been maintained between all populations of this species, however branch supports for internal relationships in Carabus fabricii are moderate and an enhanced taxon sampling is needed to understand the past and present connectivity between populations. All species delimitation analyses support a unique species. One of the most interesting subspecific cases is recovered in Carabus irregularis. This species is the most widespread of the subgenus ranging from eastern France to Romania and Ukraine. It comprises three valid subspecies, one of which Carabus irregularis montandoni from the Carpathians, was suggested to be a valid species based on molecular evidence (Homburg et al. 2013). Our results support to some extent this view with C. irregularis montandoni being found sister to the rest of populations in all IQ-TREE analyses but not in wASTRAL analyses where the other subspecies C. irregularis bucephalus is found as sister to the rest of the clade. There seems to be a genetic differentiation between the three recognized subspecies of C. irregularis but our species delimitation analyses support the view of a single species.

Evolution of the Arcifera group

The divergence time estimation analyses all recover an origin of Arcifera ca. 26 Ma in the Oligocene. We did not perform a biogeographic estimation of ancestral ranges in the group because several species are very widespread and initial attempts resulted in unresolved patterns. The fact that species boundaries within Carabus (Hygrocarabus) are unstable also prevented a proper reconstruction. However, it is possible to discuss several phylogenetic splits in the framework of our results. The stem branch connecting Carabus (Hygrocarabus) to the rest of Arcifera is long, potentially representing periods of extinction in this lineage. Currently the two recognized species in the subgenus occur in temperate forests where adults live and hunt near and in good quality streams. The reconfiguration of such habitats in the past 25 million years due to climatic...
oscillations (Westerhold et al. 2020) may have extirpated populations and pushed others in their current ranges. Considering the specificity of these two lineages to their habitat, and predictions of global warming and their impact on such ecosystems (Capon et al. 2021; Bonacina et al. 2023), it is likely that they may be increasingly threatened in the future.

With respect to biogeography, one of the most interesting lineages in Arcifera is the clade composed of Carabus (Chaetocarabus) and Carabus (Heterocarabus). Because Carabus (Heterocarabus) marietti is restricted to eastern Bulgaria and western Turkey, and Carabus (Chaetocarabus) distributed in Greece (C. arcadicus is endemic to Greece), it is likely that the ancestors of this clade originated in the geologically highly complex Aegean area. The split between the two subgenera ca. 17 Ma predates the timing of the opening of the Aegean sea in the Tortonian ca. 8 Ma (i.e., opening of the Mid-Aegean Trench or Aegean barrier; van Hinsbergen & Schmid 2012), rejecting the hypothesis of geographic vicariance in the south as suggested in other lineages (Poulakakis et al. 2015). Interestingly, both subgenera have very marginally overlapping distributions in the Thrace basin with Carabus (Heterocarabus) currently distributed on the southern Black Sea coast where Carabus intricatus is also represented by the subspecies C. intricatus starensis (Gueorguiev & Gueorguiev 1995). At the time of divergence in the early Miocene (i.e., Burdigalian), the Thrace basin formed a connection between the eastern Balkan peninsula and Anatolia (Rögl 1997, 1999; Sachsenhofer et al. 2017; Erbil et al. 2021). It is possible that ancestral populations dispersed in the Balkan Peninsula and/or in Anatolia where they evolved independently. Under this scenario, the close geographic ranges of these two species would likely represent secondary contact associated with more recent colonization of the Thrace basin. A more robust population-level taxon sampling, especially of Carabus (Heterocarabus), might elucidate the fine-scale biogeographic history of this clade in the future. Within Carabus (Chaetocarabus), the two currently recognized species are mostly allopatric with only a short overlap in western Greece (e.g. Katara pass). There is no clear geological barrier that may have fostered vicariant diversification at the time of speciation ca. 7 Ma. Further diversification appears to be occurring at the population level with Carabus intricatus lefebvrei endemic of southern Italy and allopatric from the nominal subspecies. Similarly, Carabus arcadicus merlini endemic to Peloponnese is morphologically quite divergent from the nominal subspecies and might represent a case of ongoing speciation. The wide dispersal of Carabus intricatus across the western Palearctic region is likely recent and may be explained by the generalist habitat preference of this species. Additional geographical and taxon sampling will likely yield more robust inferences of evolutionary patterns and processes within this clade in the future.

The evolutionary history of the subgenus Carabus (Platycarabus) is also revealed by our analyses. We recover the narrowly endemic Carabus cychroides as sister to the rest of the subgenus. This is surprising as it was not suggested by the molecular inference of Casale et al. (1998). This placement has strong implications for our understanding of alpine biogeography in this group. Only Carabus irregularis has lowland populations and its derived placement in the phylogeny indicates that alpine specialization was likely ancestral in the subgenus with recent shift in that species to lower habitats. This phylogenomic pattern and the origin of the subgenus ca. 15
Ma during the warmest period of the Neogene seems to indicate that ancestors of Carabus (Platycarabus) may have been less specialized than nowadays and were distributed in mountain regions. In the mid Miocene, mountain ranges across the Alps had the same elevation as nowadays (Campani et al. 2012; Kršnik et al. 2021), however ecosystems were different due to significantly warmer climatic conditions. When the climate progressively turned colder these beetles adapted to ensuing conditions and became alpine specialists. It is possible that species of the subgenus diverged due to competition, niche filling and/or host specialization as observed in Carabus cychroides for instance. We hypothesize that in the latest sequence of their evolutionary history, Pleistocene glaciations played a limited role in speciation since all current species had already diverged (Figure 3).

Although natural hybrids are known between different species of the subgenus, our results recover no hybridization signal between them. The most significant case concerns the species C. fabricii and C. irregularis, whose ranges largely overlap in Switzerland, Austria and Slovakia. It is in these sympatric areas that several cases of natural hybridisation have been identified (e.g. at the Radstatt Pass in Austria, Mandl 1960). However, our genetic results do not show any hybridisation signals between the species, either on genetic structure, where the two clusters are well separated, or in the approach using Dsuite, which seeks to trace admixture signals in the lineages. These results suggest that these sporadic hybridization events are not conserved in populations and could imply a potential infertility of F1s (Casale et al. 1998). Furthermore, the networks obtained with the three mitochondrial genes (Supplementary Figure 4) group the samples of the C. irregularis and C. fabricii species in the same cluster. These mitonuclear discordance patterns are frequent in the literature and can be explained by the specific biological properties of mitochondrial DNA (uniparental inheritance and reduced recombination; Birky 2001) or differences in the evolutionary histories of nuclear and mitochondrial markers including incomplete lineage sorting and gene flow among species (Sota & Vogler 2001; Suchan et al. 2017). The results obtained with the nuclear loci are sufficiently robust to be able to consider that the hybridisations observed are either localised or do not induce lasting admixture between the species. A more detailed analysis of hybrids, local populations and the implications of hybridisation on the fitness of individuals could provide a better understanding of the mechanisms involved.

Integrating current species distribution, genetic isolation of these alpine species was already in place when glaciation cycles struck the Alps. As a result, dispersal of populations in peripheral glacial refugia as observed in C. irregularis (Homburg et al. 2013) did not result in genetic homogenization despite species being placed in secondary contact. It is also possible in the case of the more alpine-adapted species (all but C. irregularis) that dispersal occurred in nunataks rather than peripheral glacial refugia (Holderegger & Thiel-Egenter 2009; Schönswetter & Schneeweiss 2019; Kosiński et al. 2019), which would have resulted in an increased genetic differentiation among populations as suggested by our analyses. Coupling a more extensive geographic sampling of these five alpine species with niche modeling analyses may help testing more specifically the different scenarios that governed range and genetic evolution of these populations during Pleistocene glaciations.
Acknowledgements

We warmly thank Michael Balke for the loan of material from the ZSM-SNSB. We thank Elsa Ricossa for the digitization of specimens housed at the Natural History Museum of Geneva. We thank Céline Rochet for assistance in fieldwork. We thank the city of Geneva for an internal student grant awarded to MTP. We thank Conrad Gillett for allowing the use of his photographs and drawings in this article. We thank Ivan Rapuzzi for fruitful discussions and feedback on taxonomic aspects of this work.

Funding

This study was partly funded by a Master student grant awarded by the City of Geneva. EFAT is funded by a FNS grant 310030_200491.

Conflict of interest disclosure

The authors declare no conflict of interest.

Data, script, code, and supplementary information availability

Raw reads are available on the NCBI BioProject PRJNA1086379. The data underlying this article (final alignments and trees) and bioinformatic scripts are available on Github repository (https://github.com/JeremyLGauthier/Arcifera_phylogeny).

Figure captions

Table 1. Alignment statistics for each dataset, including the number of taxa, the number of loci, the minimum coverage, the minimum number of taxa, the alignment length, the percentage of missing data, the numbers and percentages of variable sites and of parsimony informative sites, and the GC content.

Figure 1. Statistical summary of locus recovery. Plots representing the relationship between the collection year and DNA concentration (A), number of sequenced reads (B), number of loci recovered for each sample (C), and number of shared loci in final dataset B. In each plot, ethanol-preserved samples are shown in green, samples from museums with an age < 30 years in blue and samples from museums with an age > 30 years in classic yellow. Correlations were tested with Spearman’s correlation tests and adjusted coefficients of determination R-squared were estimated using a linear model.
**Figure 2.** Summary of phylogenetic inferences across Arcifera based on HyRAD-X data. The presented topology is derived from a maximum likelihood analysis performed in IQ-TREE using Dataset A. Branch support from this analysis is shown for all branches. Branch support retrieved in different analyses is shown for major branches according to the inserted caption. Sample type is indicated according to the inserted caption. Abbreviations at the end of each taxon label correspond to the following countries: AT, Austria, CH, Switzerland, FR, France, GR, Greece, HU, Hungary, IT, Italy, KP, Carpathians (Slovakia to Romania), RO, Romania, SI, Slovenia, SK, Slovakia, TK, Turkey. An illustration of a male *Carabus* (*Platycarabus*) *cychroides* is presented (Drawing: Conrad Gillett).

**Figure 3.** Bayesian divergence time estimates for the subgenus *Carabus* (*Platycarabus*) and Arcifera group. Maximum clade credibility tree obtained from a BEAST analysis using eight Bayesian log-normal relaxed clocks and a Coalescent Constant Size tree model. Node estimates are postburn in median ages, with 95% credibility intervals. Histogram represents the number of loci recovered for each sample and sample type are indicated according to the inserted caption. The section on the right shows the results of species delimitations identified using the different methods indicated above. The shades of gray represent the concordance between the different approaches with black being a total consensus. Habitus of three representative species (1) *Carabus nodulosus nodulosus* (credit: Conrad Gillett), (2) *Carabus intricatus intricatus* (credit: Conrad Gillett) and (3) *Carabus irregularis irregularis* (credit: Conrad Gillett) are shown.

**Figure 4.** Comparison of divergence time estimation between competing tree models and relaxed-clock partitioning strategies. Box-plots indicate for each analysis (color-coding inserted as a caption on the right side of the figure) the median age of the focal node (see X axis) and associated 95% age credibility interval. BD, birth-death model; CS, constant population size coalescent model.

**Supplementary Table 1.** Descriptive statistics for each included and non-included sample, including historical sample data, molecular biology information (DNA concentrations), sequencing and loci reconstruction statistics.

**Supplementary Figure 1.** Schematic representation of the bioinformatic pipeline.

**Supplementary Figure 2.** Maximum likelihood trees for each dataset: Dataset A (min_cov=6, min_sample=10, 50 taxa, 1’481 loci), Dataset B (min_cov=6, min_sample=10, 52 taxa, 1’965 loci), Dataset C (min_cov=6, min_sample=17, 50 taxa, 1’014 loci), Dataset D (min_cov=3, min_sample=17, 52 taxa, 1’291 loci), Dataset E (min_cov=6, min_sample=32, 50 taxa, 366 loci) and Dataset F (min_cov=3, min_sample=32, 52 taxa, 478 loci). Node supports indicate SH-aLRT and UFBoot values.
Supplementary Figure 3. Species trees obtained with wASTRAL on each dataset: Dataset A (min_cov=6, min_sample=10, 50 taxa, 1'481 loci), Dataset B (min_cov=6, min_sample=10, 52 taxa, 1'965 loci), Dataset C (min_cov=6, min_sample=17, 50 taxa, 1'014 loci), Dataset D (min_cov=3, min_sample=17, 52 taxa, 1'291 loci), Dataset E (min_cov=6, min_sample=32, 50 taxa, 366 loci) and Dataset F (min_cov=3, min_sample=32, 52 taxa, 478 loci). Node supports indicate SH-aLRT and UFBoot values. Node supports indicate LPP values.

Supplementary Figure 4. Individual locus haplotype networks (A. CO1, B. CO3 and C. CYTB). Networks were generated in SplitsTree using calculated uncorrected p-distances and the NeighborNet algorithm. The colour coding for the different morphological groups is identical to the one used (photo credit: Marie Pauli).

Supplementary Figure 5. Structure plots estimated on unlinked shared SNPs for K=1 to K = 15. For each K, the Mean(LnProb) is indicated.

Supplementary Figure 6. F4-branch statistic plotted as a heatmap. The tree topology is plotted above, and on the left, every branch of the tree is displayed (including internal branches).

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<table>
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<tr>
<th>Dataset</th>
<th>No. of taxa</th>
<th>No. of loci</th>
<th>Mining cov</th>
<th>Min. taxa</th>
<th>Align length</th>
<th>Missing data</th>
<th>Variable sites</th>
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<td>19,061 (5.5%)</td>
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<td>3</td>
<td>10</td>
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<td>28,256 (6.2%)</td>
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<td>13,539 (52.0%)</td>
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</tbody>
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Figure 1.

A) DNA concentration (ng/µL) vs. date.

B) Number of reads vs. date.

C) Number of individual loci vs. date.

D) Number of final shared loci vs. date.

Adj R² = 0.12195, P = 0.00087868

Adj R² = 0.53268, P = 2.3986e−9

Adj R² = 0.56616, P = 4.2217e−10

Adj R² = 0.5572, P = 6.8036e−10
Carabus (Hygrocarabus)

Carabus (Heterocarabus)

Carabus (Chaetocarabus)

Carabus (Platycarabus)

Arcifera

Interspecific Cladogenesis

Pleistocene

Glaciations

Morphology

BPP TR2 STRUCT.

HyRAD-X loci / Dataset A

Geological Time (Ma)

Ethanol-preserved

Dry-pinned (< 30-year-old)

Dry-pinned (≥ 30-year-old)

Dry-pinned (Unknown age)