Late Cenozoic environmental changes drove the diversification of a weevil genus endemic to the Cape Floristic Region

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ORIGINAL ARTICLE

Late Cenozoic environmental changes drove the diversification of a weevil genus endemic to the Cape Floristic Region

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Abstract

The Cape Floristic Region in the Republic of South Africa is a well-recognized hotspot of biodiversity. Although this region is mostly known for its high level of plant diversity and endemicity, it also hosts an understudied and likely diverse arthropod fauna. Here we investigate the evolutionary history and timing of diversification of the apterous weevil genus *Phlyctinus* (Curculionidae: Entiminae), which is endemic to the coastal area and adjacent mountain ranges of the Cape floristic region and generally associated with sunflower plants (Asteraceae). We use a diverse array of molecular analyses (phylogenetic inference, molecular species delimitation and dating analyses) to analyse a novel molecular dataset of 202 weevil specimens (including 170 *Phlyctinus* sampled in 60 sites), and sequenced for two mitochondrial and four nuclear gene fragments. Phylogenetic and dating analyses indicate that the genus started diversifying in the late Miocene, with contrasting diversification dynamics for the three inferred clades, which present disjunct distributions. Host plant records and the lack of relatedness of species living in sympatry indicate that the diversification of *Phlyctinus* was predominantly driven by allopatric (geographic) speciation. We hypothesize that the interplay between topography and recurring cycles of coastline-habitat fragmentation resulting from sea level oscillations spurred the diversification of the most speciose clade, whereas in the two remaining clades populations likely remained connected thus hampering allopatric speciation. Interestingly, this pattern echoes with the role of sea level oscillations as an important driver of the radiation of several lineages in the coastline ecosystems of the Cape Floristic Region.

KEYWORDS

allopatric speciation, banded fruit weevil, biodiversity hotspot, Entiminae, *Phlyctinus callosus*, sea level oscillations

1 | INTRODUCTION

The Cape Floristic Region (CFR) in the Republic of South Africa (hereafter referred to as South Africa) is a well-recognized hotspot of biodiversity (Linder, 2003; Mittermeier et al., 2004; Myers et al., 2000). It hosts about 9000 plant species with an endemism rate of about 70% (Goldblatt, 1997; Goldblatt & Manning, 2002), comparable to levels found on several islands (Linder, 2003). In vertebrates, endemism rates are comparably lower, but nevertheless remain high in several groups (between 22% and 41% in reptiles, amphibians and freshwater fishes; Mittermeier et al., 2004). This region of *ca.* 90,000 km^2 (Goldblatt, 1997; Linder, 2003) is traditionally divided into western and eastern subregions, and mostly corresponds to the southern part of the extant Western Cape Province. It is surrounded by several geographic barriers: the Great Escarpment mountain range in the North, semi-deserts in the North and Northwest (Nama Karoo and Succulent Karoo, respectively) as well as the Atlantic and Indian oceans on the coastlines (Compton, 2011; Linder, 2003). About half of the CFR is mountainous (Allsopp et al., 2014), as it hosts a 1300 km long fold-and-thrust mountain belt, the Cape Fold Belt, which can reach elevations of over 2000 m. The remaining half is made of low-land coastlines with the Cape Fold mountains in the background. The CFR consists of one main biome (Fynbos Biome) with two intertwined main vegetation types: fynbos and renosterveld (Allsopp et al., 2014; Cowling & Procheş, 2005). Fynbos is by far the dominant vegetation type in the CFR (Allsopp et al., 2014); it is a sclerophyllous, fire-prone shrubland characterized by specific plant assemblages dominated by Ericaceae, Proteaceae and Restionaceae (Cowling & Procheş, 2005). In the renosterveld, members of these three families are mainly absent, and this biome is mostly dominated by plants from the Asteraceae, Fabaceae, Iridaceae and Poaceae families (Grobler & Cowling, 2021); it is also characterized by the extraordinary diversity of geophytes (Cowling, 1990; Procheş et al., 2006).

During most of the Cenozoic and until the Middle Miocene, the CFR experienced a tropical/subtropical climate with no dry season (Linder & Hardy, 2004). About 11–14 Million years ago (Mya), the cold Benguela upwelling resulting from the separation of Antarctica and South America caused an extensive aridification leading to the extinction of tropical flora (Richardson et al., 2001). The glaciation of Antarctica that started approximately 8–10 Mya (Siesser, 1980) resulted in the accentuation of the cold upwelling waters on the southern Africa west coast, effectively blocking summer rainfalls (Linder & Hardy, 2004). Since then, the CFR experienced a Mediterranean-like climate with hot, dry summers and wet winters (Linder, 2003). Although the CFR underwent regular climatic fluctuations in the form of glacial cycles during the Pleistocene (Richardson et al., 2001), its rainfall regime was likely relatively stable during the last 3.5 Million years (Myrs) (Chase & Meadows, 2007; Maslin et al., 2012). The extant Cape Fold Belt has an old origin, as its emergence is ancient and occurred during the Mesozoic (Cowling et al., 2009). In the late Cenozoic, the Cape Fold Belt experienced a period of tectonic stability that lasted until the early Miocene (Cowling et al., 2009). A first major geomorphic uplift event, the 'Post-African I cycle', started *ca.* 22 Mya and lasted about 4 Myrs, causing uplifts reaching up to 150 m (for the western part of the CFR) and 200 m (for the eastern part of the CFR; Cowling et al., 2009). The second major geomorphic uplift event, the 'Post-African II cycle', started in the early Pliocene (*ca.* 5 Mya) and lasted about 2 Myrs; it caused greater uplifts reaching up to 150 m (western part of the CFR) and 300 m (eastern part of the CFR) (Cowling et al., 2009). Sea level variations were also important, as exemplified by the former coastline level in the Miocene, which was 150 m above the current coastline, flooding half of the Cape coastal plain (Cowling et al., 2009). This high sea level did not last long though, as the renewed glaciation of Antarctica (in the late Miocene) led to a rapid drop in sea level (Cowling et al., 2009). Since then, sea levels were transgressional, reaching modern levels during the Neogene (Miller et al., 2020). During the Pleistocene, sea levels also dropped several times during the past 2–3 Myrs, with lowering up to 130 m below the present coastline (Miller et al., 2020). Edaphic composition was impacted by these multiple changes of sea levels; noticeably, calcareous progressively started accumulating along the coast towards the late Pliocene, leading to the formation of large areas of calcareous sandy substrata along the coast in the Pleistocene (Cowling et al., 2009). The actual substrate diversity partially results from these sea level fluctuations but also from renewed erosion cycles and tectonic uplifts (Cowling et al., 2009; Hoffmann et al., 2015). Nowadays, a greater edaphic heterogeneity is found in low-land areas of the CFR, especially along the coastline (Verboom et al., 2015).

Most studies carried out on the origin of the CFR biodiversity have focused on plants, particularly on the richest families found in the CFR (*i.e.*, Asteraceae, Ericaceae, Fabaceae, Iridaceae, Proteaceae, Restionaceae; Linder, 2003; Hoffmann et al., 2015; Pirie et al., 2016; Grobler & Cowling, 2021). During the late Miocene and the Pliocene, rapid climate changes associated with the availability of new habitats resulting from geomorphic uplifts were likely instrumental in spurring the radiation of numerous plant lineages (Cowling et al., 2009; Richardson et al., 2001). During the Pleistocene, the suggested relative

stability of rainfall regimes possibly reduced the risk of extinction of species and populations (Potts et al., 2013). The high levels of environmental and topographical heterogeneity of the CFR (Cowling & Lombard, 2002; Linder, 2005; Procheş et al., 2009; van Santen & Linder, 2020; Verboom et al., 2015) effectively translate into diverse ecological niches that favoured the evolution of numerous endemic plant lineages with narrow ranges and poor dispersal abilities (Dynesius & Jansson, 2000; Linder, 2003). The oldest plant radiations likely occurred in mountain ranges whereas most recent radiations were hypothesized to have occurred more recently during the Pleistocene (Grobler & Cowling, 2021; Linder, 2008; Linder & Hardy, 2004; Verboom et al., 2009). Some authors also suggested that speciation processes in the CFR differ among lowland and montane plant lineages, with lowland taxa being more prone to ecological speciation whereas montane taxa are more subject to geographic (allopatric) speciation (Verboom et al., 2015). Though little is known on the precise timing and tempo of the diversification of the CFR fauna, it potentially echoes patterns inferred in Cape plants (Goldblatt & Manning, 2002). Insect diversity has long been considered poor compared with the plant diversity in the CFR (Giliomee, 2003). However, recent studies have revealed high levels of arthropod diversity in this region, especially in phytophagous insect groups (*e.g.*, Matenaar et al., 2018; Talavera et al., 2020). Though the latter may suggest a positive relationship between arthropod and plant diversity (*e.g.*, Kemp et al., 2017; Kuhlmann, 2009; Procheş et al., 2009; Wright & Samways, 1998), other studies instead posit that abiotic and environmental variables are more important, especially at broader scales (Procheş et al., 2009; Switala et al., 2014; van Schalkwyk et al., 2019).

With more than 62,000 known species, weevils (Coleoptera: Curculionoidea) are the most diversified phytophagous insect superfamily (Oberprieler et al., 2007). Thanks to their worldwide distribution and very diverse morphology and biology, weevils are often used as models to investigate diversification patterns in relation to biotic and abiotic drivers (*e.g.*, Baird et al., 2021; Condamine & Kergoat, 2021; Haran et al., 2021; Letsch et al., 2018; Toussaint et al., 2014). South Africa, and the CFR in particular, presents a high level of endemicity for weevil lineages, both at the generic and tribal level (Alonso-Zarazaga & Lyal, 1999; Meregalli et al., 2021; Oberprieler, 2014). Weevil diversity in this region reaches unusually high levels, as exemplified by the genus *Brachycerus* Olivier, which encompasses hundreds of species in the CFR while only 50 species are known in the southwestern Palearctic region (Oberprieler, 2014). A large part of the weevil diversity, however, is still undescribed in South Africa as shown by the description of entire new clades and a large number of new taxa in recent years (*e.g.*, Haran, 2021; Meregalli et al., 2021). Among them, Entiminae is the largest subfamily of Curculionidae with more than 12,000 described species worldwide (Marvaldi et al., 2014; Oberprieler et al., 2007). This subfamily is well-represented in the CFR, including several endemic lineages (Alonso-Zarazaga & Lyal, 1999). One of them is the genus *Phlyctinus* Schoenherr (Curculionidae: Entiminae: Oosomini), a broad-nosed apterous lineage endemic to the Western Cape Province of South Africa (Figure 1) consisting of six described species (Haran et al., 2020). Among this genus, *P. callosus* Schoenherr, also called the 'banded fruit weevil', is a major pest of vineyards and orchards and has been recorded on a wide array of introduced host plants at larval and adult stages (Pringle et al., 2015). Species in this genus generally have a restricted distributional range, with some species only known by a few localities (Haran et al., 2020). Populations found in natural habitats are only associated with a few genera of Asteraceae (noticeably *Athanasia* L., *Hymenolepis* Cass, *Osteospermum* L., and *Senecio* L.; Haran et al., 2020), thus suggesting the potential role of host plants in their diversification. Several species in the *P. grootbosensis* Haran species group (*P. grootbosensis*; *P.* 'sp. n. J1' and *P.* 'sp. n. J2'), for instance, remarkably mimics the seeds of their *Osteospermum* hosts when sheltering in the leaf litter during the day (J. Haran pers. obs.). As previously reported, the full picture of the diversity of this genus is far from being complete since preliminary investigations on the morphology and molecular diversity of recognized species have revealed potential cryptic lineages (Hansen et al., 2021; Haran et al., 2020).

In this context, exploring the diversity of the genus *Phlyctinus* can provide novel insights on the origin of the CFR phytophagous insect fauna. To this end, we used a molecular phylogenetic framework including molecular species delimitation and dating analyses, and discussed the results in light of morphology, ecology and distribution of lineages. We considered two main speciation patterns: allopatric by looking at geographical occurrences in light of late Cenozoic environmental changes, and sympatric by looking at host plant associations. More specifically, we aimed at: *(i)* investigating the evolutionary relationships of the genus *Phlyctinus*, and *(ii)* assessing the speciation processes that have led to the present diversity of the genus.

2 | MATERIAL AND METHODS

2.1 | Sampling

Between 2017 and 2019, about a thousand adult *Phlyctinus* specimens were collected in 60 distinct localities from the Western and Eastern Cape Provinces of South Africa (see

FIGURE 1 Examples of habitat, host plants and habitus *in natura* for *Phlyctinus* species. (a) Kogel Bay on the southern coast of the cape floristic region of South Africa with shale fynbos and shale band vegetation types, biotope for several *Phlyctinus* lineages. (b) *Osteospermum moniliferum* (top) host of *P.* 'sp. n. J2' (bottom). (c) *Dimorphotheca fruticosa* (top) host of *P.* '*littoralis* group 2' (bottom). (d) *Othonna arborescens* (top) host of *P.* 'sp. n. G' (bottom).

examples on Figure 1). *Phlyctinus* individuals were found along the coast and in lowland ranges up to 900 m above sea level. Specimens were sampled by beating/sweeping the vegetation by night – when adults are active – or through visual search by day in the leaf litter at the base of plants. Other South African provinces outside of the CFR (KwaZulu-Natal, Limpopo, Mpumalanga and Northern Cape) were also prospected but no *Phlyctinus* were found there despite extensive fieldwork (54 sites were sampled unsuccessfully). The examination of reference collections housed in various institutions (Ditsong Museum, Pretoria; Iziko Museum, Cape Town; South Africa National Collection of Insects, Pretoria; Stellenbosch University Insect Collection, Stellenbosch) further confirmed that the genus does not naturally occur outside the CFR (see also Haran et al., 2020). Specimens were identified by J. Haran, following Haran et al. (2020). Some of the specimens were directly assigned to species already described (*P. aloevorus* Haran, *P. callosus, P. grootbosensis, P. littoralis* Haran, *P. planithorax* Haran and *P. xerophilus* Haran) while others were considered as potential new species (coined as *P.* 'sp. n. G' and *P.* 'sp. n. H', *P.* 'sp. n. J1', *P.* 'sp. n. J2' *P.* '*littoralis* group 1' and *P.* '*littoralis* group 2') on the basis of morphology and of preliminary analyses of a cytochrome *c* oxidase subunit I (COI) gene fragment. All specimens were stored in 96% ethanol at ambient temperature. Map showing the geographic distributions of the sampled *Phlyctinus* specimens was made with QGIS v3.10.10 (QGIS Development Team, 2020. QGIS Geographic Information System. QGIS Association. http://qgis.org) with a raster of sea levels from GeoMapApp v.3.6.14 (GRTM Image v.4.0, http://www.geomapapp.org).

2.2 | Host plant records

Phlyctinus larval stages develop hidden in the ground on the root system of their hosts, a lifestyle characteristic of entimine weevils (Marvaldi et al., 2014). Adults are apterous but quite mobile at night; they can thus be quite eclectic in the choice of plants they use for food and shelter (Pringle et al., 2015; J. Haran & S. Hansen pers. obs.). As such, individual records obtained from beating the vegetation cannot be considered as a reliable indication of a trophic relationship. However, field observations of *Phlyctinus* show that – during the day – adults generally aggregate in small to very large (up to 200 individuals) at the base of their host plants, hidden in leaf litter (Haran et al., 2020; J. Haran & S. Hansen pers. obs.). Similarly to other apterous Entiminae, *Phlyctinus* adults are generally found near their host plants as long as resources (oviposition sites, food for adults) is available (J. Haran & S. Hansen pers. obs.). Here, we considered that a trophic relationship (*i.e.*, plant used for larval development) was only supported when multiple specimens (not limited to an individual plant) were exclusively and repeatedly found

at the base of a specific plant species, and/or repeatedly collected on a specific whilst feeding at night, in a given locality. Typical adult entimine feeding damage, such as notched leaves (Marvaldi et al., 2014) on individual plants on/by which weevils were collected also helped to confirm these plant species as hosts. The corresponding host plants were identified using the field guide of Manning (2007). In addition to their native asteraceous hosts, *Phlyctinus* have been shown to be able to shift onto diverse ornamental plants, crops and orchards (Haran et al., 2020; Pringle et al., 2015). In this study, only hosts recorded in natural and undisturbed habitats are discussed to avoid human-induced biases in the interpretation of the evolution of the genus.

2.3 | Molecular dataset

One to seven *Phlyctinus* specimens per locality were selected for the molecular analyses, with a total of 170 specimens analysed (Table S1). In the absence of a robust phylogenetic framework for Afrotropical Entiminae, outgroup selection was guided by the current morphological classification (Alonso-Zarazaga & Lyal, 1999; R. Borovec pers. com.). Closely related outgroups were sampled among genera from the tribe Oosomini (*Basotorhynchus* Borovec, *Bryochaeta* Pascoe, *Cladeyterus* Schoenherr, *Glyptosomus* Schoenherr, *Holcolaccus* Marshall, *Oosomus* Schoenherr, *Oxymorus* Borovec & Meregalli, *Porpacus* Schoenherr, *Pyctoderes* Schoenherr, *Rhysoderes* Marshall, genus nr. *Basotorhynchus*, genus nr. *Oxymorus* and an undetermined genus of Entiminae provisionally identified as Oosomini) whereas more distant outgroups were sampled among the tribes Embrithini (*Ellimenistes* Boheman, *Epibrithus* Marshall, genus nr. *Ellimenistes* and Embrithini genus undet.), Otiorhynchini (*Otiorhynchus* Germar), and Tanyrhynchini (*Afroleptops* Oberprieler, *Eremnus* Schoenherr, *Tanyrhynchus* Schoenherr and Tanyrhynchini genus undet.). In all, 32 individuals representing 22 genera of Entiminae were included as outgroups; all corresponding specimens were collected in South Africa except for *Bryochaeta* sp. and *Otiorhynchus meridionalis* Gyllenhal, from Tanzania and France, respectively.

Non-destructive DNA extractions were carried out on whole specimens or individual legs, using a Bio Basic plate DNA purification kit (Part#: BS437; Bio Basic). Amplifications and sequencing were conducted on two mitochondrial gene fragments: cytochrome *c* oxidase subunit I (COI; 658 bp sequenced), which is the standard marker for barcoding studies on animals (Hebert, Cywinska, et al., 2003; Hebert, Ratnasingham, & deWaard, 2003), and ribosomal 12S RNA (12S; up to 351 bp sequenced). Four nuclear gene fragments were also amplified and sequenced: arginine kinase (ArgK; 757 bp sequenced), carbamoyl phosphate synthetase 2 (CAD; 535 bp sequenced), internal transcribed spacer 2 (ITS2; up to 550 bp sequenced) and elongation factor 1-alpha (EF1; 517 bp sequenced). COI, ArgK, CAD and EF1 gene fragments are protein-coding genes, while 12S and ITS2 are non-coding genes.

All nuclear genes (ArgK, CAD, ITS2, EF1) were amplified with the primers listed in Appendix S1 and the protocol described in Haran et al. (2020), and further Sanger sequenced for both strands at Eurofins Genomics. The two mitochondrial gene fragments (COI and 12S) were sequenced using high-throughput sequencing. Amplicon libraries were constructed for these two genes following Galan et al. (2017), using specific primers (see Appendix S1) for the Polymerase Chain Reactions (PCR); for the COI, to obtain the whole 658 bp barcode fragment, two overlapping fragments were also targeted following Shokralla et al. (2015). In comparison with the settings of Galan et al. (2017), we made the following changes to reduce the proportion of chimeric fragments: for the first PCR step, the number of cycles was set to 35 whereas for the second PCR step, the extension duration was set to 120 s. The final library (containing three equimolar pooled libraries) was paired-end sequenced on an Illumina MiSeq flowcell using a MiSeq Reagent Nano Kit v2 at the AGAP laboratory (Montpellier, France).

Sequences of nuclear genes were checked manually using CodonCode Aligner v3.7.1 (CodonCode Co.). Illumina reads were processed using the FROGS pipeline (http://frogs.toulouse.inra.fr/; Escudié et al., 2018) on the Genotoul Galaxy server using demultiplexing, pre-processing, clustering and chimera removal tools. Remaining contaminants were further detected using the BLAST tool (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi) and removed manually. The two overlapping fragments of COI were merged using CodonCode Aligner. A total of 202 COI, 177 12S, 106 ArgK, 88 CAD, 158 ITS2 and 128 EF1 sequences were validated.

All mitochondrial and nuclear sequences were aligned using MAFFT v7 (Katoh et al., 2019) with default option settings and a gap opening penalty of 5.0, and further manually corrected using Mesquite v3.61 (Maddison & Maddison, 2018). For all protein-coding genes (COI, ArgK, CAD and EF1), coding frame and stop codons were checked with Mesquite to detect potential pseudogenes. This software was further used to concatenate all genes and for file conversion. The final concatenated dataset consisted of 202 specimens (170 *Phlyctinus* specimens and 32 outgroups) and 3613 aligned characters with *ca.* 35% of missing data (see Appendix S2).

2.4 | Phylogenetic analyses

Phylogenetic analyses were carried out under maximum likelihood (ML), as implemented in IQ-TREE v2.1.3 (Minh et al., 2020). The concatenated dataset was divided a priori into 14 partitions, with three partitions (one per codon position) defined for each coding gene fragment (ArgK, CAD, COI and EF1) and one partition defined for each non-coding gene fragment (12S and ITS2). Additional analyses were also conducted on each gene fragment, with three partitions implemented for the four coding genes. Best-fit substitution models and partition schemes were selected using the Bayesian Information Criterion (BIC) implemented in IQ-TREE (see Appendix S3).

Maximum likelihood trees were obtained using heuristic searches implementing 500 random-addition replicates with the following settings: random-starting tree, hill-climbing nearest neighbour interchange (NNI) search (*−allnni* option), a perturbation strength either set to 0.2, 0.5 or 0.8 (*−pers 0.x* option), partition-resampling strategy (*−-sampling GENE* option), best partition scheme allowing the merging of partitions (*−m MFP + MERGE* option; only when coding genes were analysed). For all analysed datasets (*i.e.*, concatenated dataset and the six individual gene datasets), only the best-scoring tree (out of the three distinct ML analyses with variable perturbation strengths) was kept for further analyses. Clade support for all analyses was assessed using 1000 replicates for both SH-like approximate likelihood ratio tests (SH-aLRT; Guindon et al., 2010) and ultrafast bootstraps (uBV; Minh et al., 2013). Nodes supported by ultrafast bootstrap values SH-aLRT values ≥80% and (uBV) ≥ 95% were considered as strongly supported following authors' recommendations.

2.5 | Molecular species delimitation analyses

Six distinct molecular species delimitation (SD) approaches were used in this study to better assess the reliability and repeatability of the proposed species delineations (see *e.g.*, Astrin et al., 2012; Dellicour & Flot, 2018; Luo et al., 2018).

First, we relied on the tree-based Poisson-tree-process (PTP) approach of Zhang et al. (2013). This SD method was carried out on the best-scoring ML tree resulting from the analyses of the concatenated dataset, and tentatively applied to the individual single-locus trees as well. All corresponding analyses were carried out with default settings on a dedicated webserver (https://species.h-its.org/). Second, we implemented another tree-based SD method, the General Mixed Yule Coalescent (GMYC) model of Pons et al. (2006). Similarly to PTP analyses, this approach was carried out on the best-scoring ML tree resulting from the analyses of the concatenated dataset, and tentatively applied to all single-locus trees. We relied on the default single threshold approach of Pons et al. (2006), but also on the more parameter-rich approach of Monaghan et al. (2009). The latter allows the use of multiple thresholds to account for the potential heterogeneity of evolutionary rates among lineages. General Mixed Yule Coalescent approaches require ultrametric trees (where all tips are equidistant from the root) as input. To generate them, we used treePL (Smith & O'Meara, 2012) with a standard smoothing rate of 100 and a root age arbitrarily set to 50 Mya. As a third SD approach, we used a distance-based method; the Automatic Barcode Gap Discovery (ABGD) model of Puillandre et al. (2012). This approach was only used on individual gene fragments to avoid known biases associated with missing data when working on concatenated datasets with distance-based methods (see *e.g.*, Mallo & Posada, 2016). Following Puillandre et al. (2012), the results of both initial and recursive partitioning strategies (referred to as ABGDi and ABGDr, respectively) were considered. Corresponding analyses were carried out using default settings with a standard Kimura 2-parameter model on a dedicated webserver (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). As a fourth SD approach, we used another distance-based method; the Assemble Species by Automatic Partitioning (ASAP) of Puillandre et al. (2021). This approach is similar to ABGD, but includes a scoring system to identify the best-fitting set of putative species. All corresponding analyses were carried out using default settings based on the K80 model on a dedicated webserver (https://bioinfo.mnhn.fr/abi/public/asap/#). For both ABGP and ASAP analyses, for each gene fragment, we assessed whether a barcoding gap could be detected; the rationale was to only consider the results of analyses on gene fragments exhibiting a clear barcoding gap. As a fifth SD approach, we used statistical parsimony analyses as implemented in TCS v.1.23 (Clement et al., 2000). This method collapses sequences into haplotypes and then constructs statistical parsimony haplotype networks formed at the 95% confidence level. This SD approach was carried out only on the concatenated dataset (following other authors; see *e.g.*, Vondráček et al., 2018, Tessens et al., 2021), considering each network as a putative species. As a sixth SD approach, we used a multilocus coalescent-based SD approach implemented in the program tr2 (Fujisawa et al., 2016). This method uses both single-locus trees and a guide tree as inputs, and relies on a Bayesian model

comparison framework with rooted triplets. All six single-locus trees were used (see Appendix S4) and the topology resulting from the analyses of the concatenated dataset was used as a guide tree.

Lastly, the congruence between *a priori* defined lineages and putatives species inferred by SD analyses was assessed for each methods and loci analysed using the match ratio (Ahrens et al., 2016): $2 \times N_{\text{match}} / \left(N_{\text{analysis}} + N_{\text{lineages}}\right)$. Here, N_{match}, is defined as the number of exact match between putative species inferred by SD analyses and *a priori* defined lineages, N_{analysis} is the number of putative species inferred by SD analyses, and N_{lineages} is the number of *a priori* defined lineages.

2.6 | Dating analyses

Divergence times were estimated using Bayesian relaxed clocks as implemented in BEAST v1.10.4 (Suchard et al., 2018) through the CIPRES Science Gateway v3.3 (Miller et al., 2010; www.phylo.org). We relied on secondary calibrations based on substitution rates from the study of Andújar et al. (2012) on Coleoptera; these rates have been often used for secondary calibration in diverse beetle groups (e.g., Caterino & Langton-Myers, 2018; Faille et al., 2013; Haran et al., 2021; Kamiński et al., 2022; Toussaint et al. 2017). Two clocks were used; one for the mitochondrial and one for the nuclear genes. Combinations of uncorrelated lognormal (UCLN) and strict clocks (SC) were tested, resulting in four distinct calibration strategies. The fit of each corresponding calibration strategy was compared using Bayes factors (BF); marginal likelihood estimations were carried out using path sampling procedures (Baele et al., 2012 for the rationale) with default settings (100 path steps, chains running for 1 million generation with a log likelihood sampling every 1000 cycles). The tree model was set to a birth–death speciation process, which is recommended for datasets with mixed inter- and intraspecific species sampling (Ritchie et al., 2017). A fixed topology corresponding to the best-scoring tree from the ML analyses of the concatenated dataset was used to limit the risk of over-parameterization. Analyses consisted of 50 million generations of MCMC with parameters and trees sampled every 500 generations. A 25% burn-in was applied and for each analysis, the maximum credibility tree, median ages and their 95% HPD were generated with TreeAnnotator v1.10.4, which is part of the BEAST software package. Convergence of runs was assessed graphically and by examining the effective sample size (ESS) of parameters under Tracer v1.7.2 (Rambaut et al., 2018), using the recommended threshold of 200.

3 | RESULTS

3.1 | Phylogenetic analyses

The best-scoring tree from the ML analyses of the concatenated dataset (Figure 2) has a likelihood score of −31,821.2. In the corresponding topology, the genus *Phlyctinus* is recovered monophyletic with maximum support (SH-aLRT and uBV of 100%). Node support for the relationships among the a priori defined *Phlyctinus* lineages (some of which with a species status) is high with 86% of SH-aLRT values ≥80% and 86% of uBV≥95%. *Phlyctinus* specimens are clustered into three well supported (SH-aLRT values comprised between 89.9% and 100% and uBV of 100%) geographic clades (Figure 2): (i) a first group consisting of *P. xerophilus* individuals, in inland valleys ('inland group'), (ii) a second group consisting of the two representatives of *P.* sp. n. 'G', restricted to the western Coast ('western coastal group'), and (iii) a third group consisting of all remaining specimens, restricted to the southern Coast of the CFR ('southern coastal group'). Within the southern coastal group, all lineages defined a priori (*P. aloevorus*, *P. callosus*, *P.* 'sp. n. J1', *P.* 'sp. n. J2', *P. grootbosensis*, *P.* 'sp. n. H', *P. littoralis*, *P.* '*littoralis* group 1', *P.* '*littoralis* group 2' and *P. planithorax*) are recovered monophyletic with a high support (see Figure 2). It should be noted that the few populations that deviate from this geographic pattern (some localities with *P. xerophilus* specimens near the coast, some *P. callosus* specimens inland and some *P.* sp. n. 'H' specimens on the western Coast) were sampled in disturbed habitats and probably originate from human-mediated introductions (J. Haran pers. obs.). The outgroups generally formed weakly supported clades (SH-aLRT < 80% and uBV < 95%), with the two sampled tribes sampled for more than one representative (*i.e.*, Embrithini and Oosomini) not being recovered monophyletic.

3.2 | Molecular species delimitation analyses

Outputs from molecular SD analyses made on ArgK, CAD and EF1 were not considered. For these three genes, both the high rates of polytomy in corresponding gene trees and the lack of genetic differentiation among specimens completely hindered PTP and GMYC analyses. Outputs of ABGD and ASAP analyses for ArgK, CAD and EF1 were also not considered as no barcoding gaps were recovered for these gene fragments. For the COI gene, the ABGD analyses (ABGDi and ABGDr) also yielded very contrasted results, transitioning abruptly from 1 to 30 putative species; this artefactual result was thus not taken

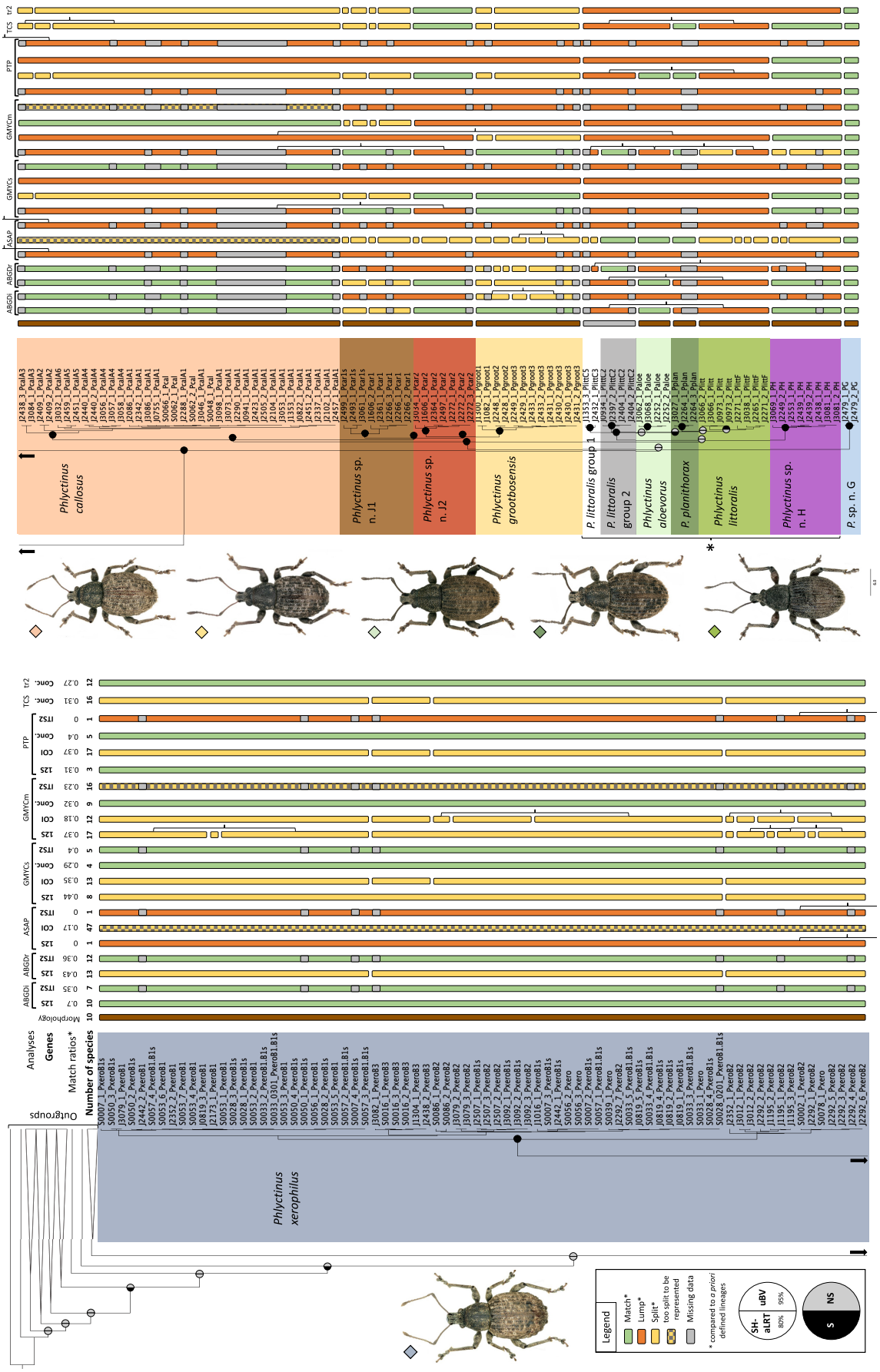
Phylictinus callosus
Phylictinus sp. n. J1
Phylictinus sp. n. J2
Phylictinus grootbosensis
P. littoralis group 1
P. littoralis group 2
Phylictinus aloevorus
P. planithorax
Phylictinus littoralis
Phylictinus sp. n. H
P. sp. n. G
Phylictinus xerophilus
Outgroups
Analyses
Genes
Match ratios*
Number of species
Morphology
Legend
Match*
Lump*
Split* too split to be represented
Missing data
* compared to a priori defined lineages
SH-aLRT 80%
uBV 95%
NS
S

FIGURE 2 Best-fit ML tree resulting from the analyses of the concatenated dataset, complemented by results of molecular species delimitation analyses. The ML ultrametricized tree resulting from the ML analysis of the concatenated dataset is shown on the left along with information on clade support for the major nodes. Results of molecular species delimitation analyses on the concatenated dataset, and on COI, 12S and ITS2 gene fragments alone are displayed on the right along with the match ratios and the number of putative species found in each analyse. Boxes are coloured in green when SD analyses are fitting a priori defined lineages, in yellow when SD analyses are splitting a priori defined lineages, and in orange when SD analyses are lumping a priori defined lineages. Lineages retained for the discussion of the diversification of *Phlyctinus* are highlighted with colours on the tree (see the discussion section for justification of lineages selection). '*' refers to the *P. littoralis* species group. Photographs of males from previously described species are from Haran et al. (2020).

into account. Limited congruence is found between a priori defined lineages and SD analyses with almost all match ratios <0.5. Depending on the methods and loci analysed between one and 47 putative *Phlyctinus* species are inferred by the SD analyses (Figure 2). Extant species are diversely supported overall: *P. aloevorus*, *P. callosus*, *P. grootbosensis*, *P. littoralis*, *P. planithorax* and *P. xerophilus* are respectively recovered in 19%, 57%, 57%, 5%, 19% and 86% of the SD analyses. Although there is a good consensus for *P. xerophilus* (86% of the SD analyses support its species status), it is worth underlining that some SD analyses did suggest the presence of three or four cryptic lineages (see *e.g.*, Appendix S5 for the results of the TCS analysis). For the potential new species, a similar pattern is observed (48%, 33%, 86%, 48%, 5% and 14% of support for *P.* 'sp. n. J1', *P.* 'sp. n. J2' *P.* 'sp. n. G', *P.* 'sp. n. H', *P.* '*littoralis* group 1' and *P.* '*littoralis* group 2', respectively).

3.3 | Host plant records

In natural habitats, all *Phlyctinus* lineages for which host plant data are available are exclusively associated with Asteraceae belonging to the tribes Anthemideae, Arctotideae, Calenduleae and Senecioneae (Figure 3, see Table S1 for details). However, lineages are generally associated with Asteraceae species belonging to multiple tribes. In most cases, in the same locality, *Phlyctinus* from distinct lineages are found sharing the same host (*e.g.*, *P.* 'sp. n. J1' codistributed with *P. callosus* or *P. littoralis*, all on *Osteospermum moniliferum*; *P.* 'sp. n. J1' and *P. littoralis* on *Athanasia trifurcata* L.; *P. grootbosensis* and *P.* 'sp. n. H' on *Senecio halimifolius* L.; Figure 3). Only in a few cases, distinct lineages are found on different host plants in the same locality (*e.g.*, *P. grootbosensis* on *Osteospermum moniliferum*; *P.* '*littoralis* group 1' on another unidentified Asteraceae). Species pairs (*i.e.*, *P. littoralis* and *P. planithorax*; *P. grootbosensis* and *P.* 'sp. n. J2') have distinct host plants but are not found in sympatry.

3.4 | Dating analyses

Out of the four distinct dating analyses, the one implementing a SC for both mitochondrial and nuclear genes is statistically recovered as the best-fit calibration procedure (Appendix S6), with ESS values ≥200. The corresponding age estimates are therefore presented in Figure 3, with a simplified topology that focuses on the main lineages discussed hereafter. The genus *Phlyctinus* is estimated to have originated during the late Miocene *ca.* 6.3 Mya (95% HPD: 4.9–8.1 Mya). The southern coastal clade containing almost all lineages originated during the Pliocene *ca.* 4.2 Mya (95% HPD: 3.3–5.4 Mya). All species (and putative new species) are estimated to have originated recently during the late Pliocene and the Pleistocene (see Figure 3, and for details Appendix S7).

4 | DISCUSSION

4.1 | Phlyctinus *diversity and phylogenetic relationships*

This study recovers the monophyly of the genus *Phlyctinus* with maximum support, thus confirming the phylogenetic consistency of its currently recognized concept. Within *Phlyctinus*, 12 distinct lineages are recovered as monophyletic (*P. aloevorus*, *P. callosus*, *P. grootbosensis*, *P. littoralis*, *P. planithorax*, *P. xerophilus*, *P.* 'sp. n. G', *P.* 'sp. n. H', *P.* 'sp. n. J1', *P.* 'sp. n. J2', *P.* '*littoralis* group 1' and *P.* '*littoralis* group 2'). However, the status of most of them is only partially supported by the results of the molecular SD analyses as highlighted by match ratios showing a low congruence between the inferred putative species and the a priori defined lineages. On the whole, the SD analyses yield a high range of putative species estimates, often under- or over-splitting species. This lack of consensus likely reflects the fact that molecular SD analyses can have trouble distinguishing between general population structures and species boundaries (Sukumaran & Knowles, 2017). In the case of *Phlyctinus*, we do have isolated populations and dating estimates indicating that the radiation of all major lineages is recent, hence the poor performance of molecular SD analyses is not unexpected. A similar outcome was also observed in wingless western Palearctic cryptorhynchine weevils, with a number of putative species recovered ranging from five to 568 depending on the data treatment (Astrin et al., 2012). In addition, several

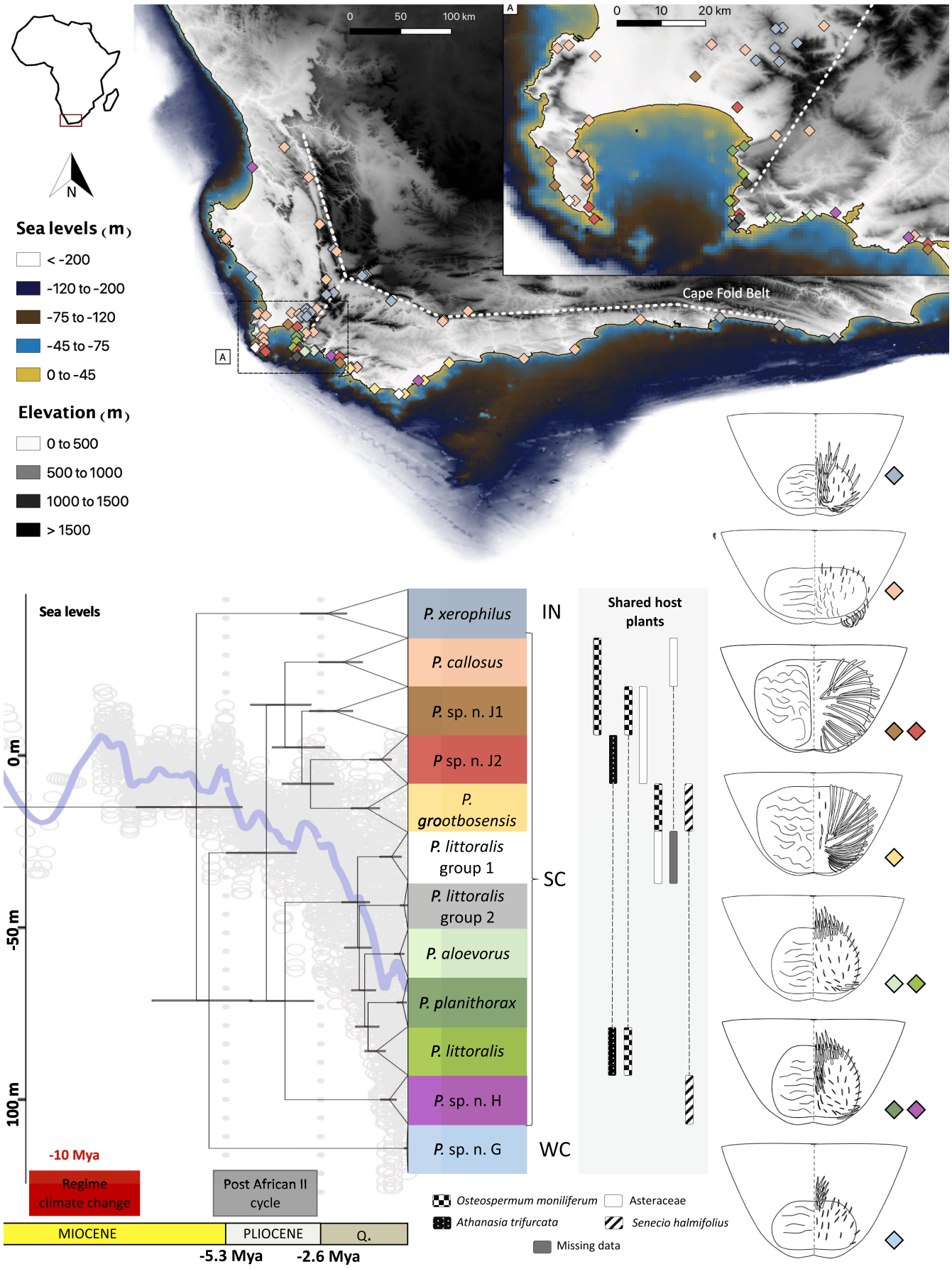
0 50 100 km
A
0 10 20 km
Cape Fold Belt
N
Sea levels (m)
< -200
-120 to -200
-75 to -120
-45 to -75
0 to -45
Elevation (m)
0 to 500
500 to 1000
1000 to 1500
> 1500
Sea levels
0 m
-50 m
100 m
-10 Mya
Regime climate change
Post African II cycle
MIOCENE
PLIOCENE
Q.
-5.3 Mya
-2.6 Mya
P. xerophilus
P. callosus
P. sp. n. J1
P sp. n. J2
P. grootbosensis
P. littoralis group 1
P. littoralis group 2
P. aloevorus
P. planithorax
P. littoralis
P. sp. n. H
P. sp. n. G
IN
SC
WC
Shared host plants
Osteospermum moniliferum
Athanasia trifurcata
Asteraceae
Senecio halmifolius
Missing data

FIGURE 3 Timing of diversification, distribution, host plant and diagnostic morphological features for the genus *Phlyctinus*. The top map shows the geographical distribution of the inferred *Phlyctinus* lineages (see Appendix S8 for details), with information on elevation and on the impact of past sea level regressions. The dated phylogeny (bottom left) provides median ages estimates for major nodes along with 95% HPD intervals. Background curve (raw data are indicated using grey circles) shows sea level oscillations (from Miller et al., 2005). The three major identified groups are underlined on the right side of the tree ('IN' for the inland group, 'SC' for the southern coast group and 'WC' for the western coast group). Host plants for species with overlapping distributional ranges are highlighted using vertical bars on the right of the tree ('shared host plants' panel). Line drawings of the main morphological features to distinguish lineages of *Phlyctinus* (arrangement of setae and cuticular cavity of ventrite 5 of males) are displayed on the bottom right.

factors could have biased the SD analyses. First, differences in effective population sizes are known for generating biases in analyses that rely on coalescent times, such as GMYC models (see Ahrens et al., 2016). Because effective population size is expected to be closely correlated with abundance (Palstra & Fraser, 2012), for *Phlyctinus* weevils, we could have had an effect for the two most abundant and widespread species (*i.e.*, *P. callosus* and *P. xerophilus*). However, if only considering natural and undisturbed habitats, their distributional range is actually quite restricted and their abundance/density is very similar to those of other *Phlyctinus* lineages. This however does not constitute a definitive evidence, especially in relation with biases related to GMYC models. For instance, for *P. xerophilus*, we recovered a tendency for over-splitting with the GMYC models (five out of eight analyses), and no lumping; hence, it could be possibly linked to the impact of differences in effective population sizes highlighted by Ahrens et al. (2016). Other factors – such as biases resulting from the ultrametricization procedure (see *e.g.*, Astrin et al., 2012) – could also be at play here. Another bias discussed in Hamilton et al. (2014) and Ahrens et al. (2016) is related to sampling. For this study, two of the authors conducted extensive field work, targeting with a similar sampling pressure all lineages, in all CFR regions and habitats (including perturbed ones). Hence, we think that the risk of geographic biases were limited; however, the rarity of some lineages could have had an effect (see also Ahrens et al., 2016). For several *Phlyctinus* lineages only encompassing a few individuals, we cannot exclude the hypothesis that we missed some of the genetic diversity for these lineages, and it could have impacted some of the SD analyses as well.

All these results emphasize the importance of assessing species boundaries within an integrative framework, in which not only molecular data but also morphological, life history and ecological information are considered (*e.g.*, Lukic et al., 2021).

The genus *Phlyctinus* harbours few morphological traits that can be used to distinguish closely related species. Relevant diagnostic characters are mostly found in males and include the convexity of eyes, the arrangement of setae and of cuticular cavities on ventrites 1 and 5, and the shape of the penis and its internal sclerites (Haran et al., 2020, J. Haran pers. obs.). The species status of the six previously described species (*P. aloevorus, P. callosus, P. grootbosensis, P. littoralis, P. planithorax* and *P. xerophilus*) is supported by clear morphological traits, by their monophyly and by some of the molecular SD analyses. It is also the case for two undescribed lineages (*P.* 'sp. n. G' and *P.* 'sp. n. H'), which will be described in the near future.

In the remaining *Phlyctinus* lineages, strong evidence is lacking to support a potential species status. For instance, the phylogenetic placement of the morphologically indistinguishable *P.* 'sp. n. J1' and *P.* 'sp. n. J2' lineages (in a clade also including the morphologically distinct *P. grootbosensis*) tends to support that they could constitute species on their own. The latter is partially supported by SD analyses (in 45% and 30% of the analyses, respectively) but clear morphological evidence is lacking as no differences can be found so far between *P.* 'sp. n. J1' and *P.* 'sp. n. J2' specimens (J. Haran pers. obs.). The fact that representatives of these two lineages also have overlapping distributional ranges (while also sharing the same host plants) is puzzling and calls for additional investigations, especially because we cannot exclude biases associated with the molecular markers that have been used. Based on the results of the phylogenetic analyses, representatives of *P. littoralis* 'group 1' and *P. littoralis* 'group 2' constitute distinct lineages, which could potentially correspond to species of their own. For these two lineages, the support from SD analyses is however extremely low (less than 15%) and no clear combinations of character states can be found to distinguish these specimens from other members of the *littoralis* group (also consisting of *P. aloevorus, P. littoralis, P. planithorax* and *P.* 'sp. n. H'). The fact that *P. littoralis* 'group 1' and *P. littoralis* 'group 2' are distributed in distinct bays of the southern coast of the CFR is consistent with the hypothesis of Haran et al. (2020), who postulated that the genus is likely prone to pocket speciation in isolated coastal habitats; however, whether these two lineages constitute potential new species or isolated populations from extant species remain to be further investigated. Although some level of genetic differentiation is suggested among *P. xerophilus*, the corresponding lineages are only unravelled by multiple molecular SD analyses. The latter does not allow us to conclude about the relevance of these lineages and also calls for more investigations.

4.2 | *Diversification of* Phlyctinus *weevils in the CFR*

The early diversification of *Phlyctinus* during the Miocene led to the emergence of three main groups currently restricted to specific regions (western coast, southern coast and inland groups) corresponding to CFR bioregions identified by Bradshaw et al. (2015). Interestingly a quite similar pattern of distribution is found in cicadas (Hemiptera: Cicadidae) belonging to the *Platypleura stridula* (L.) species complex (see Price et al., 2007); it is also partially consistent with the disjunct distribution of species belonging to the apterous stag-beetle genus *Colophon* Grey (Switala et al., 2014). Similar disjunct distribution patterns are also encountered in unrelated groups, such as freshwater fishes and crabs (Bronaugh et al., 2020; Daniels et al., 2006).

It is interesting to note that all *Phlyctinus* species are restricted to wet habitats, and that the genus as a whole is only distributed in CFR habitats receiving 300-500 mm/year of rainfall (Compton, 2011), while being surrounded by semi-arid deserts. We postulate that the progressive aridification of the CFR during the late Cenozoic (deMenocal, 2004; Richardson et al., 2001) strongly constrained the diversification of the genus *Phlyctinus* by 'trapping' populations in the most humid habitats of the CFR, which are the coastlines and the inland valleys. Another striking pattern in *Phlyctinus* is the contrasted number of extant species that are found in the three geographic groups (southern coastal, western coastal and inland). With up to 10 species and lineages, the southern coastal group is by far the most speciose, as both the western coastal and inland groups are only represented by a single species.

We hypothesize that this diversity pattern is best explained by the role played by the interplay between geographical barriers and sea level oscillations. Regarding geographical barriers, most *Phlyctinus* populations are surrounded by mountains covered with a vegetation type adapted to strong summer droughts and repeated cycles of fire (Kraaij & van Wilgen, 2014); such habitats are therefore unfavourable for insect groups restricted to wet habitats, such as *Phlyctinus* species. In flightless species, mountains also act as strong physical barriers to dispersal and gene flow, and therefore promote allopatric differentiation (Ikeda et al., 2012; Salces-Castellano et al., 2021). In this regard, the 'African uplift II' that occurred from the late Miocene to the Pliocene (Cowling et al., 2009) has likely been instrumental in generating allopatric speciation in *Phlyctinus*, especially for the southern coastal group located in bays that are strongly isolated by the abrupt southern hillsides of the Kogelberg mountain range (Figure 3). By contrast, the western coast shows little disruption of the coast line by extensions of the surrounding mountains; therefore, an increase in species diversity through allopatric speciation is not to be expected, which could possibly explain why only one species of *Phlyctinus* (*P.* 'sp. n. G') is found there.

The contrasted diversity richness observed between the inland and southern coastal groups also highlights the potential role played by sea level oscillations in the diversification of the genus. In the southern coastal group, speciation events occurred between the late Pliocene and the Pleistocene, a period associated with marked sea level oscillations (Miller et al., 2020) leading to cycles of fragmentation/reconnection of bays (Tolley et al., 2014). Sea level oscillations similarly impacted the diversification of several CFR lineages, such as coastal legless skinks for which cladogenesis also occurred between the late Pliocene and the Pleistocene (Engelbrecht et al., 2013). In plants, the role of sea level oscillations in the diversification of coastal lineages is particularly important, especially for the dune floras (Grobler et al., 2020), or for the species associated with calcareous substrates (Grobler & Cowling, 2021; Hoffmann et al., 2015). The fact that the inland group only consists of one species (*P. xerophilus*) tends to support the hypothesis that allopatric speciation likely did not play as a significant a role inland; a possible explanation for the latter is that the landscape was stable since the late Pliocene (*ca.* 2.6 Ma; Cowling et al., 2009). Because valleys of the Cape fold belt were similarly connected to each other in the past, it likely allowed gene flow between distant – but connected nonetheless – *Phlyctinus* populations, a pattern also found in populations of several plant lineages (Potts, 2017; Potts et al., 2013). Another potential factor that could explain the low level of diversity of inland *Phlyctinus* is their higher exposure to the aridification of the CFR during the late Pliocene and Pleistocene (deMenocal, 2004), which could have led to higher extinction rates in this group. By contrast, for coastal lineages, the proximity of the sea possibly acted as a buffer against changing climatic conditions through the maintenance of stable wet habitats, a condition that has possibly reduced extinction risk in coastal *Phlyctinus* populations.

Our results do not suggest a major effect of host plant associations on the diversification of *Phlyctinus* species. In natural habitats, *Phlyctinus* species from distinct lineages are generally found on the same host plant in the same locality, whereas the inferred species pairs are not found in sympatry. Such a pattern does not provide any support for ecological speciation in sympatry, where species pairs are expected to be found in sympatry on distinct plants. Instead, *Phlyctinus* species appear to be quite opportunistic (as typical for many Entiminae; Marvaldi et al., 2014), being able to develop on various hosts from the four aforementioned Asteraceae tribes. The specialization of these four tribes also cannot be associated with a pattern of phylogenetic niche conservatism of host use since these

tribes do not constitute a monophyletic group (Mandel et al., 2019). In anthropogenically disturbed habitats, such as gardens or orchards, some populations of *Phlyctinus* are also considerably more polyphagous as they are able to greatly expand their host range by shifting on non-native plants from diverse families (Aizoaceae, Amaryllidaceae, Asphodelaceae, Geraniaceae, Plantaginaceae, Plumbaginaceae, Rosaceae and Vitaceae; see Table S1 for details); this capacity indicates that host choice is likely not strongly driven by chemosensory cues, which again tends to argue against the role of sympatric (ecological) speciation in the genus.

5 | CONCLUSION

The integrative approach followed in this study provides support for the existence of additional cryptic *Phlyctinus* species, echoing the idea that insect diversity in the CFR is likely greatly underestimated. The distinct lineages we unravelled are clustered into three disjunct groups, which have contrasted species diversity as two of them only consist of a single putative species. Our molecular dating analyses indicate that cladogenesis events mostly occurred during the Plio-Pleistocene, a timeframe consistent with those inferred in other studies of species complexes endemic to the CFR. Overall, host plant association patterns provide no support for sympatric speciation in relation to host use, and allopatric speciation instead appears to be the main process of speciation for *Phlyctinus* weevils. This is especially apparent in the most speciose group, where the combined effects of topography and sea level oscillations apparently spurred allopatric speciation. By contrast, for the two other less speciose groups, we hypothesize that populations were likely more connected, and less subject to drastic reduction of gene flow thanks to either: *(i)* a long-lasting connectivity of inland valleys for the mountain group, or *(ii)* the lack of geographic barriers for the western coastal group. We also postulate that the relatively stable rainfall regime experienced during the late Pliocene and Pleistocene had a potential buffering role (*i.e.*, reducing extinction risks), especially for coastal areas where wet habitats were likely persistent. Overall, this study adds to the emerging corpus of research supporting the role of abiotic factors and allopatric speciation for generating arthropod species diversity in the CFR.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article. All datasets, main result files and scripts are available here: doi:10.6084/m9.figshare.20110430.

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