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Exploring the taxonomic status of the Palearctic cone weevil, *Pissodes validirostris*, inferred from morphometric and molecular data

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Abstract The Scots pine cone weevil, *Pissodes validirostris* Gyll. (Coleoptera: Curculionidae) widely distributed from Europe, across to North-Eastern China, consumes the cones of Mediterranean pines, as well those of the *sylvestris* section. The use of multiple host pine species with limited and patchy distributions, combined with low dispersal rates, likely contributed to significant genetic and morphological variation among *P. validirostris* populations in its native range. *P. validirostris* is being considered as a biological control agent for wilding pines in the southern hemisphere and for this programme to proceed, elucidating its intraspecific variability is necessary. This study aimed to explore the phylogeography of *P. validirostris* in its native range in Europe using mitochondrial and nuclear genetic markers as well as morphological characters. The integrative taxonomic analysis revealed high genetic structuring in *P. validirostris* populations, identifying three main phylogeographic clades: Clade A (Iberian Peninsula, associated with *Pinus pinaster* and *Pinus halepensis*), Clade B (Central Europe), and Clade C (Northern and Eastern Europe), both associated with *Pinus* species of the *sylvestris* section. The morphometric data showed that adults of the Iberic Peninsula clade were significantly larger than those of the two others. When compared to other Nearctic and Palearctic congeneric species, *P. validirostris* formed a monophyletic group, likely consisting of different evolutionary lineages within the sampled distribution. The findings highlight the necessity of genetic confirmation for insect taxonomy across wide distributions or multiple host

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trees. Clarifying whether host specificity or geography drives *P. validirostris* population structuring will facilitate the selection of a specific clade for wilding pine biological control.

Keywords Coleoptera: Curculionidae · *Pissodes* phylogeny · Phylogeography · Evolutionary lineages · Wilding *Pinus* · Insect morphology · Pine cone weevil

Introduction

Eight *Pinus* species are invasive in South Africa and are having major economic and environmental impacts in the country (Martin et al. 2025). Formal plantations with *Pinus* species cover about 660,000 ha of the country. However it is estimated that there are over 2.9 million ha invaded by populations that have escaped cultivation (van Wilgen and Richardson 2012). To address the continued spread of *Pinus* species, biological control (hereafter biocontrol) is being considered as the only viable long-term management option (Moran et al. 2000; Roques et al. 2004; Martin et al. 2025). Only three Mediterranean pines (*Pinus halepensis* Mill., *P. pinaster* Aiton, *P. pinea* L. (Pinales: Pinaceae)) with no commercial value are being considered for biocontrol to avoid potential conflicts of interest with the forestry industry. Furthermore, the programme intends to only prioritise seed feeding biocontrol agents to ensure there is no risk to valued *Pinus* timber (Hoffmann et al. 2011). *Pinus* species are able to invade new areas due to their ability to build up an abundance of seeds that produce trees that are able to outcompete native vegetation (Galloway et al. 2017). Targeting the seeds of pines is therefore regarded as a crucial step to begin curbing their spread (Moran et al. 2000).

The cone feeding weevil, *Pissodes validirostris* Gyll. (Coleoptera: Curculionidae), has been identified as the most promising candidate agent for biocontrol of invasive or wilding *Pinus* species in South Africa (Hoffman et al. 2011). Native to the northern hemisphere, weevils of the genus *Pissodes* have evolved a variety of life histories and adaptations for using conifers as feeding, ovipositing and developing resources (Zhang et al. 2007). *Pissodes validirostris* is the only species of *Pissodes* that does not feed on the cambium and phloem of Pinaceae but instead only feeds on the cones and seeds of *Pinus* and their whole life cycle occurs in the same host species (Lennox et al. 2009). Attacked cones react to weevil larvae by producing resin flows, which usually prevents shedding of undamaged seeds. Up to seven larvae may occur in a cone of Mediterranean pines, completely transforming the cone content, tissues and seeds into frass (Roques 1983).

Pissodes validirostris has a wide Palearctic distribution from Portugal and Scandinavia to North Eastern China and has been recorded attacking both the target Mediterranean pines as well as species in the section *sylvestris* (*Pinus sylvestris* L., *Pinus mugo* Turra, *Pinus uncinata* Mill., *Pinus nigra* Arnold and *Pinus leucodermis* Antoine), and some North American pines that have been introduced into Europe (*Pinus contorta* Doug. Ex. Loud) (Roques 1983; Delplanque et al. 1988). The taxonomy of *Pissodes* is complex (Lu et al. 2007; Wondafrash et al. 2016) and is largely unresolved for *P. validirostris.* The narrow, patchy distribution of host species, environmental variability across the range, low dispersal rates, and long adult longevity (up to three years) promote local adaptation of *P. validirostris* to its hosts. Furthermore, behavioural and morphometric studies have found significant differences across populations in the native range (Roques et al. 2004). Taken together, these factors all indicate that *P. validirostris* populations are highly structured and may even have speciated.

Molecular systematics can be applied to biocontrol programmes to guide control efforts by being used to clarify aspects such as taxonomy, cryptic species, host plant evolutionary relationships, and population structure (van Steenderen et al. 2023; Gaskin 2024). Biocontrol agents may vary in their efficacy or host use according to their evolutionary lineage. For example, multiple genotypically unique lineages of the agent *Tetramesa romana* Walker (Hymenoptera: Eurytomidae) were released in the USA to target *Arundo donax* L. (Poales: Poacaeae) and one lineage was found to be more successful in establishing and inflicting damage on this herbaceous plant (Goolsby et al. 2014). Molecular studies can also reveal cryptic species, for example the agent *Eccritotarsus catarinensis* (Carvalho) (Heteroptera: Miridae) on water hyacinth, *Pontederia crassipes* Mart. (Commelinales: Pontederiaceae) was collected from two isolated

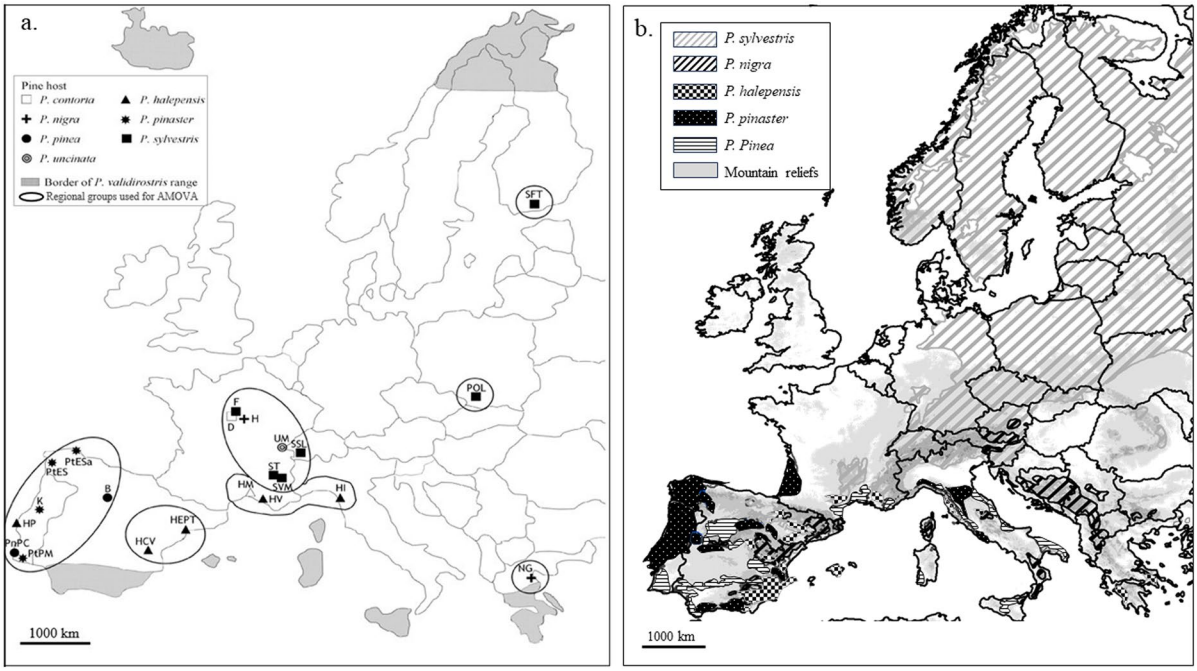


Fig. 1 **a** Sampling sites of *Pissodes validirostris* populations. Codes of the localities are given in Supplementary S1. The symbols denote the various host tree species, the shaded regions delineate the geographic distribution of *P. validirostris*, and the seven regional groups utilised for AMOVA analysis are specified. **b** Distribution of the five main host pine species used by *P. validirostris* (modified from Euforgen and Caudullo et al. 2017). (Color figure online)

lineages in the native range that were later found to be distinct species based on genetic and cross-breeding studies (Paterson et al. 2016). With these advancements in knowledge, it is now recommended that only one lineage should be introduced for biocontrol, instead of numerous individuals from many lineages that may differ in their ecologies due to local adaptations (Hufbauer and Roderick 2005).

With *P. validirostris* being considered as a biocontrol agent it is imperative to understand whether it is a generalist herbivore feeding on pine cones or whether it represents a complex of sibling species or lineages, each associated with particular species of *Pinus*. To address this, patterns of intraspecific polymorphism were measured on two markers, the mitochondrial COI gene and the nuclear ribosomal ITS2. In addition, morphometric data was collected on adult weevils to test whether there was variation across discrete lineages. Finally, to assess the validity of the taxonomic status of the pine cone weevil *P. validirostris,* a phylogenetic analysis was conducted with closely related species of *Pissodes* specialised on Pinaceae.

Material and methods

Cone sampling

Pine cones were collected from seven different pine species, including the target Mediterranean pines (*P. pinaster*, *P. halepensis* and *P. pinea*), European continental species (*P. nigra*, *P. sylvestris* and *P. uncinata*) and North American introduced species (*P. contorta*). Collections were carried out across seven Eurasian countries, to obtain a representative sampling of *P. validirostris* across its distributional range (Fig. 1). Cones were collected during late summer, just before adult emergence. Whenever possible, up to 100 cones were collected from ten different trees in each site. Cones were kept in separate boxes for each sample location until the adult weevils emerged. Emergent weevils were collected and immediately stored in absolute ethanol at − 80 °C until DNA extraction. A total of 22 populations were analysed for genetic and morphometric measurements (Fig. 1 and Supplementary Table S1). In addition, 26 specimens belonging to eight other *Pissodes* species were collected and

stored in absolute ethanol at − 80 °C (see Supplementary Table S2).

DNA protocols

DNA extraction

A total of 125 weevils (99 *P. validirostris*, 26 *Pissodes* spp.) representing two to seven individuals per population were used for the COI gene analysis and 50 weevils (34 *P. validirostris*, 16 *Pissodes* spp.) corresponding to one to two specimens per population were used for ITS2 (Supplementary Table S1). Total genomic DNA was extracted using the GenElute Mammalian Genomic DNA kit (Sigma) and was eluted in 200 µl and 100 µl of the elution solution provided in the kit for larvae and adults, respectively. Due to the presence of fungi and nematodes in the abdomen of pine cone weevil, only the head of the adults and the posterior segment of the larvae abdomen were used for DNA extraction.

Mitochondrial and nuclear DNA amplification and sequencing

The mitochondrial COI gene was amplified by PCR using two primers, C1-J-2220 (5′-TTGTAGTTG AGCCCATCAT-3′) and C1-N-2880 (5′-TTGGAA TCATTCGATAGAGG-3′), that were designed to be specific to *P. validirostris* using OLIGO© (Rychlik 2007). The full ITS2 region was amplified using standard primers ITS2F/ITS2R (Campbell et al. 1994) as well as an additional primer ITS2int (5′-AAACGCGAAAGCGACCCG-3′) to sequence the complete ITS2 fragment. Reactions were carried out in 50 µl total volumes, containing the 1 × reaction buffer provided with the polymerase, 1.5 mm MgCl2, 100 µm dNTPs, 0.2 µm of the COI or ITS2 primers, 1 unit of Red *Taq* polymerase (Sigma-Aldrich, France) and ~ 50 ng of template DNA. Amplifications were run in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, California, USA) with an initial denaturation step at 94 °C for two minutes followed by 30 cycles of 94 °C—30 s, annealing temperature of 50 °C (COI) or 52 °C (ITS2) for 60 s, 72 °C—90 s, followed by a final extension at 72 °C for five minutes. PCR products were purified using the GenElute PCR clean-up kit (Sigma) for the COI gene and the NucleoSpin® Extract II kit (Macherey Nagel) for the ITS2 fragment according to the manufacturer' protocol. Sequencing was performed using the BigDye Terminator Sequencing Kit (PE Applied Biosystems) and carried out with an ABI 3100 automatic sequencer. COI and ITS2 sequences were visualised using BioEdit (Hall 1999). The unambiguous middle part of the COI fragment corresponding to 542 bp was used for the final sequence alignment. The forward and reverse fragments were aligned using Clustal W (Thompson et al. 1994) as implemented in BioEdit. Additional *Pissodes* spp. sequences from GenBank were also used for phylogenetic reconstructions (Supplementary Table S2).

Genetic analysis

Mitochondrial and nuclear sequence data sets were analysed separately within and between *Pissodes* species. Within *P. validirostris*, maximum parsimony (MP) and maximum likelihood (ML) phylogenetic reconstructions were inferred for COI haplotypes using Paup 4.0b10 (Swofford 2003). MP analysis was performed using a heuristic search with a tree bisection-reconnection (TBR) branch-swapping option with all characters unordered and equally weighted. For ML analysis, the best substitution model was HKY (Hasegawa et al. 1985), including I = 0.7019 and G = 0.4276, inferred using Modeltest v3.7 (Posada and Crandall 1998). The relative level of support for particular phylogenetic groupings was assessed with the bootstrap analysis (500 and 100 interactions for MP and ML trees, respectively) (Felsenstein 1985). For *P. validirostris* ITS2 sequences, ML analysis was performed using PhyML implemented in SeaView 5.0.4 (Gouy et al. 2010). The COI and ITS2 sequences of *P. castaneus* were used as an outgroup as it is a closely related species.

Gene and nucleotide diversity indices of *P. validirostris* populations and clades were calculated for mitochondrial sequences using the Arlequin version 3.1 (Excoffier et al. 2005). Genetic structure was assessed by Analysis of Molecular Variance (AMOVA) using Arlequin version 3.1 (Excoffier et al. 2005). The populations were grouped either by geographical location as shown in Fig. 1 or by host species (see Supplementary Table S1).

Mitochondrial and nuclear *Pissodes* spp. phylogenies were reconstructed using ML analysis with GTR model and optimised options implemented

in Seaview 5.0.4 with *P. yunnanensis* and *P. punctatus* as outgroups. Uncorrected pairwise genetic distances were computed using MEGA5 (Tamura et al. 2011) within and among *Pissodes* species for both molecular markers.

Morphometric measurements

Adult morphology was examined using a MZ12 Leica® stereomicroscope equipped with a Leica DC100® digital camera. Using QWIN® V2.3 image processing and analysis software, a total of 303 individuals were measured for six morphological variables: (1) rostrum length, (2) pronotum width, (3) combined length of head and pronotum, (4) abdomen length, (5) abdomen width, and (6) total body length. Individuals from 18 out of the 22 populations analysed for mtDNA could be measured (Supplementary Table S3). Most specimens collected in Chera and Salamir (Spain), Figueira da Foz (Portugal) and Tuusula (Finland) were used as larvae for DNA analysis and no adults emerged from further cone rearing. Additional measures were carried out on seven other populations from Portugal (6) and France (1) which were not run in the molecular analysis. The number of measured specimens varied between two and 46 per population depending on adult emergence from cones. Morphological data were analysed by comparing means per host species and per host species within each genetic clade using ANOVA followed by a Tukey HSD post-hoc test for all pairwise comparisons whenever assumptions of normality were met. Tests were carried out under an error risk of 5% using Statistix 10®.

Results

Pissodes validirostris intraspecific genetic analysis

For the 95 *P. validirostris* that were successfully amplified and sequenced, a 542 bp fragment was obtained for the COI gene. We obtained 29 haplotypes due to 28 polymorphic sites of which 21 were informative (Table 1). They were distributed according to the populations' geographic origin (Supplementary Table S3). All 23 individuals from the west side of the Iberic Peninsula (all populations from Portugal, North-Western and central Spain), collected on *P. pinaster*, *P. halepensis* and *P. pinea*, shared a unique endemic haplotype named Port-EspNO. All other populations (except the Italian one that was monomorphic for all five individuals) were polymorphic and displayed several haplotypes. Uncorrected pairwise divergence ranged from 0.00 to 2.40%. Mean haplotype and nucleotide diversities were calculated for each population.

Regarding the nuclear marker, the boundaries of ITS2 were determined by the highly conserved flanking regions (Navajas et al. 1998). In total, 748 bp comprising 43 bp of the 5.8S, 695 bp of the ITS2 and 10 bp of the 28S were obtained for 32 individuals of *P. validirostris* from 16 localities (Supplementary Table S4 and Supplementary Figure S1). ITS2 fragment length was variable, from 683 to 701 bp, resulting from seven possible insertions or deletions (indels). Up to 24 apparent homozygous genotypes were defined by 11 variable sites, including one polymorphic site in the 5.8S and seven possible indels or variable microsatellite-like repeated motifs of two to four bp among the sequence. Following Gómez-Zurita and Vogler (2003), all microsatellites were coded so that each di- or tri-nucleotide repeat unit would be treated as a single mutational event.

Table 1 Sequence statistics and genetic diversity for the three lineages of *Pissodes validirostris*

	Source of variation	Number of individuals	Number of haplotypes	Transition/transversion ratio	Gene diversity (mean ± SD)	Nucleotide diversity (mean ± SD)
Clade A	Iberic Peninsula	33	5	6.600	0.044 ± 0.026	0.0000 ± 0.0001
Clade B	Central Europe	44	17	30.116	0.591 ± 0.179	0.0023 ± 0.0022
Clade C	North and eastern Europe	18	7	2.333	0.566 ± 0.108	0.0019 ± 0.0016

One sequence for each mtDNA haplotype was retained for *P. validirostris* phylogenetic reconstruction. The bootstrap consensus MP tree and the ML tree both agree in showing the separation of the haplotypes into three geographic clades (Fig. 2). A first group, named clade A, grouped the Iberian Peninsula populations together, a second group, clade B, the French, Swiss and Italian populations and a third group, clade C, grouped the Finnish, Greek and Polish populations together.

Mean genetic distances within *P. validirostris* increased with the geographical distances. MtDNA genetic distances ranged from 0.19% (within clades) to 4.50% between clades A and C (Supplementary Table S5). The genetic structure of *P. validirostris* reflects both host association and geographic structuring (Table 2). When populations were grouped by region, the most haplotype diversity (Hd) was found between groups (75.50%), this result being significant. When populations were grouped by host plant,

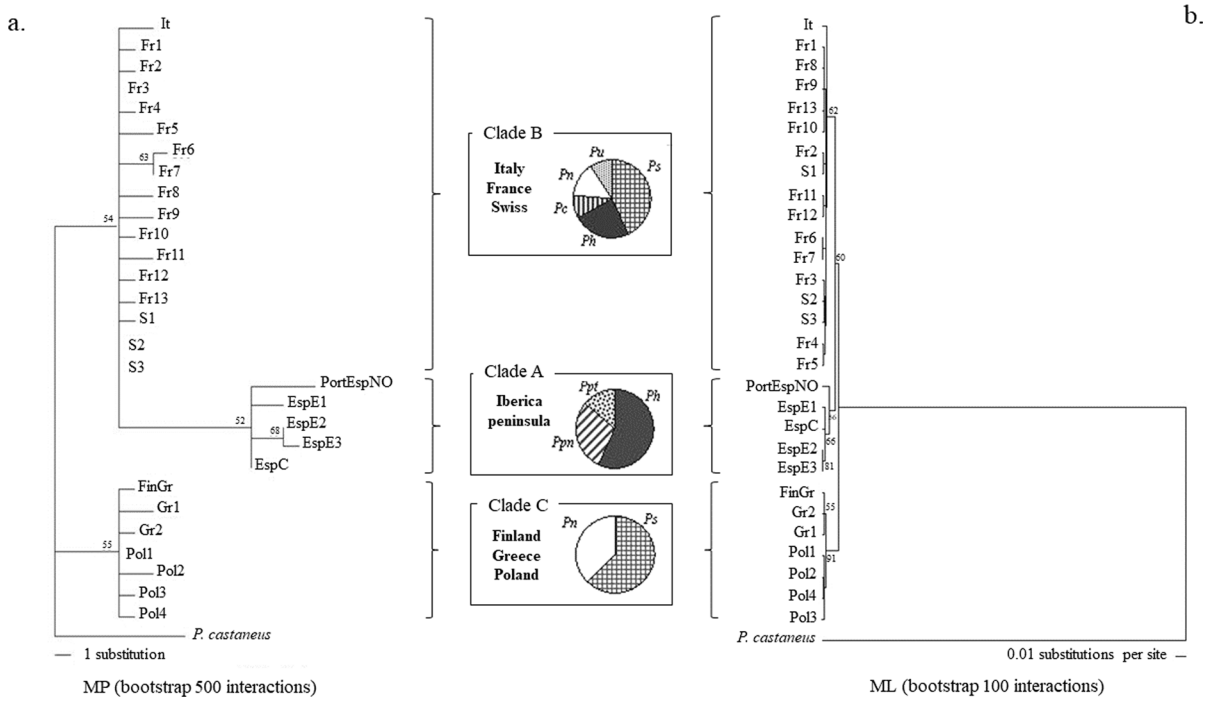


Fig. 2 Phylogenetic analysis of mitochondrial COI haplotypes in *Pissodes validirostris*: **a** Maximum parsimony and **b** maximum likelihood approaches based on the HKY+I+G model (Hasegawa et al. 1985). Bootstrap values are shown above branches of 500 and 100 replicates for MP and ML tree, respectively. At the centre, the boxes represent the names of the three geographic clades and the frequencies of the associated host pine species (Pc: *Pinus contorta*, Ph: *Pinus halepensis*, Pn: *Pinus nigra*, Ps: *Pinus sylvestris*, Ppt: *Pinus pinaster*, Ppn: *Pinus pinea* and Pu: *Pinus uncinata*)

Table 2 Analysis of molecular variance (AMOVA) among European populations of *Pissodes validirostris* grouping by geographic region and by host species

	Source of variation	Variance components	Percentage of variation (%)
Grouping by geographic region	Among groups	3.07027**	75.50
	Among populations within groups	0.63393**	15.59
	Within populations	0.36257**	8.92
Grouping by host species	Among groups	0.96235*	26.04
	Among populations within groups	2.37119**	64.15
	Within populations	0.36257**	9.81

*$p < 0.01$, **$p < 0.001$

64.15% of the Hd was significantly found among populations within groups and 26.04% between hosts.

The topology of ITS2 ML tree had weak support. However, it showed less obvious geographic but also host-species association. The tree clustered together most genotypes from *P. validirostris* collected on central and North-Eastern European *Pinus* species, belonging to the *sylvestris* section. In the same scheme, most genotypes from *P. pinaster* and *P. pinea* grouped together and genotypes found in weevils collected on *P. halepensis* tended to be more basal than those from northern pine populations. Nevertheless, these results should be considered with caution given the poor resolution of relationships between genotypes.

Pissodes validirostris morphometric analysis

Pissodes validirostris was found to vary across the populations sampled with significant differences in all measured morphological traits (Fig. 3, S7, Supplementary Tables S7 and S7). Independent of the location or the genetic clade, the rostrum was significantly longer in adults emerging from cones of *P. pinaster* and *P. pinea* than in those having developed in cones of native pines of the *sylvestris* section, while adults from *P. halepensis* showed intermediate values ($F_{9,311}=44.41$, $p<0.001$; $F_{6,311}=66.81$, $p<0.001$, followed by Tukey test, Fig. 3, S7). Adults emerging from the non-native *P. contorta* had a significantly shorter rostrum than all the other *P. validirostris.* Individuals emerging from *P. pinea* and *P. pinaster* cones also differed significantly from those emerging from other groups of host pines for pronotum width ($F_{9,318}=55.20$, $p<0.001$; $F_{6,318}=78.32$, $p<0.001$), combined length of head and pronotum ($F_{9,226}=50.61$, $p<0.001$; $F_{6,226}=38.77$, $p<0.001$), abdomen length ($F_{9,308}=47.07$, $p<0.001$; $F_{6,308}=55.71$, $p<0.001$), and abdomen width ($F_{9,310}=51.67$, $p<0.001$; $F_{6,310}=68.68$, $p<0.001$).

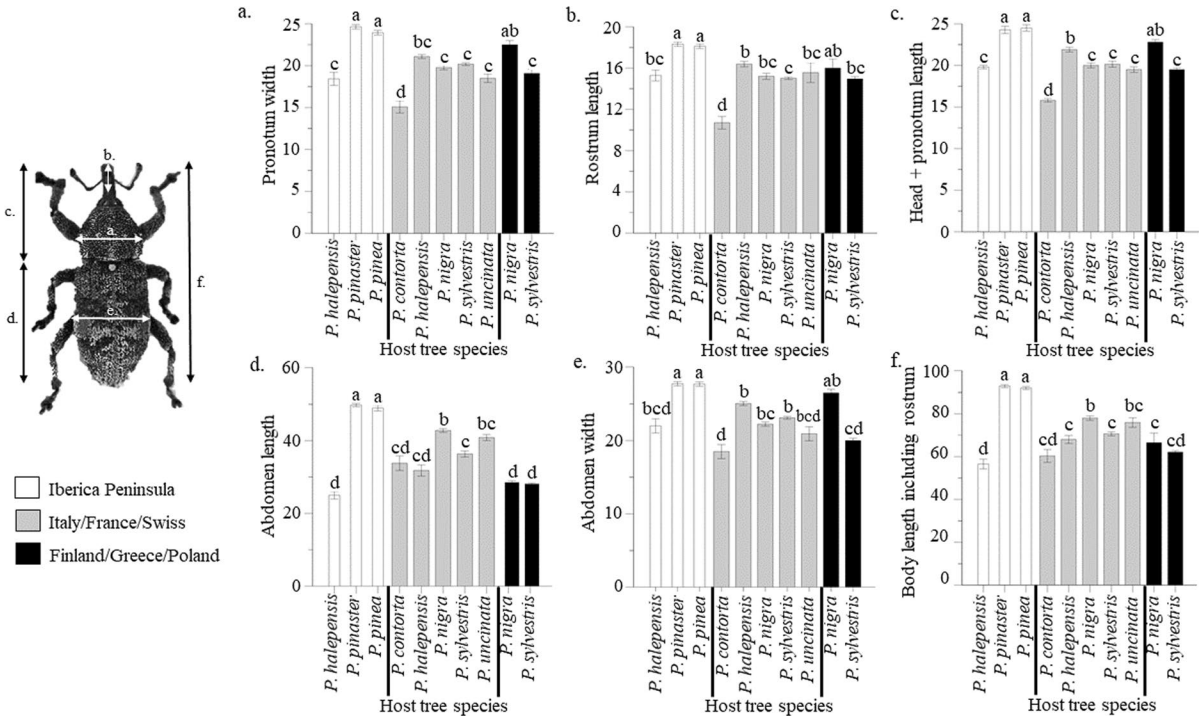


Fig. 3 Average length (in 1/10 mm ± SE) of **a** pronotum width, **b** rostrum length, **c** head and pronotum length, **d** abdomen length, **e** abdomen width, **f** total length of *Pissodes validirostris* (mm) reared from *Pinus pinaster, P. pinea, P. halepensis, P. nigra, P. contorta, P. sylvestris* and *P. uncinata* for each of the three clades (clade A in white: the Iberic Peninsula, clade B in grey: the Central Europe and clade C in black: the Eastern Europe). Bars with the same letter did not differ significantly from each other using Tukey HSD post-hoc test ($p<0.05$). (Color figure online)

As a result, total body length was significantly more important in individuals emerging from *P. pinea* and *P. pinaster* than in those emerging from pines of the *sylvestris* section, *P. halepensis* and *P. contorta* ($F_{9,270}=62.37$, $p<0.001$; $F_{6,270}=82.50$, $p<0.001$). Individuals emerging from the cones of *P. halepensis*, *P. nigra*, and *P. sylvestris* exhibited significant differences for the combined length of head and pronotum abdomen length, abdomen width or total body length depending on the genetic clade (Fig. 3).

Relationships among *Pissodes* species

A homologous 534 bp fragment of the COI gene was analysed for the 12 *Pissodes* species studied (Supplementary Table S2). No indels or features of pseudogene were observed. Within the genus, the ITS2 gene fragment was highly variable in the DNA sequence (38 polymorphic sites of which 27 are informative), as in its length sequence (ranging from 410 to 629 bp) due to numerous indels of nine microsatellite-like repeated motifs of two to four bp among the sequences.

Mitochondrial and nuclear phylogenetic analysis of different congeneric species belonging to the *Pissodes* genus strongly indicated the monophyly of *P. validirostris* haplotypes (Fig. 4). Mitochondrial analysis of the 12 *Pissodes* species for which COI sequences were available revealed ten monophyletic groups each associated with one species or with a complex of closely related species (*P. strobi* species complex and the two Chinese species complexes).

Uncorrected pairwise distances among all *Pissodes* species ranged from 7.0% to 16.5% for COI, and 2.0% to 16.4% for the ITS2. Sequence divergence between *P. validirostris* and the other *Pissodes* species ranged from 9.1% (with *P. piniphilus*) to 15.1% (with *P. yunnanensis*) for COI, and 2% (with *P. piniphilus*) to 15.2% (with *P. punctatus*) for the ITS2.

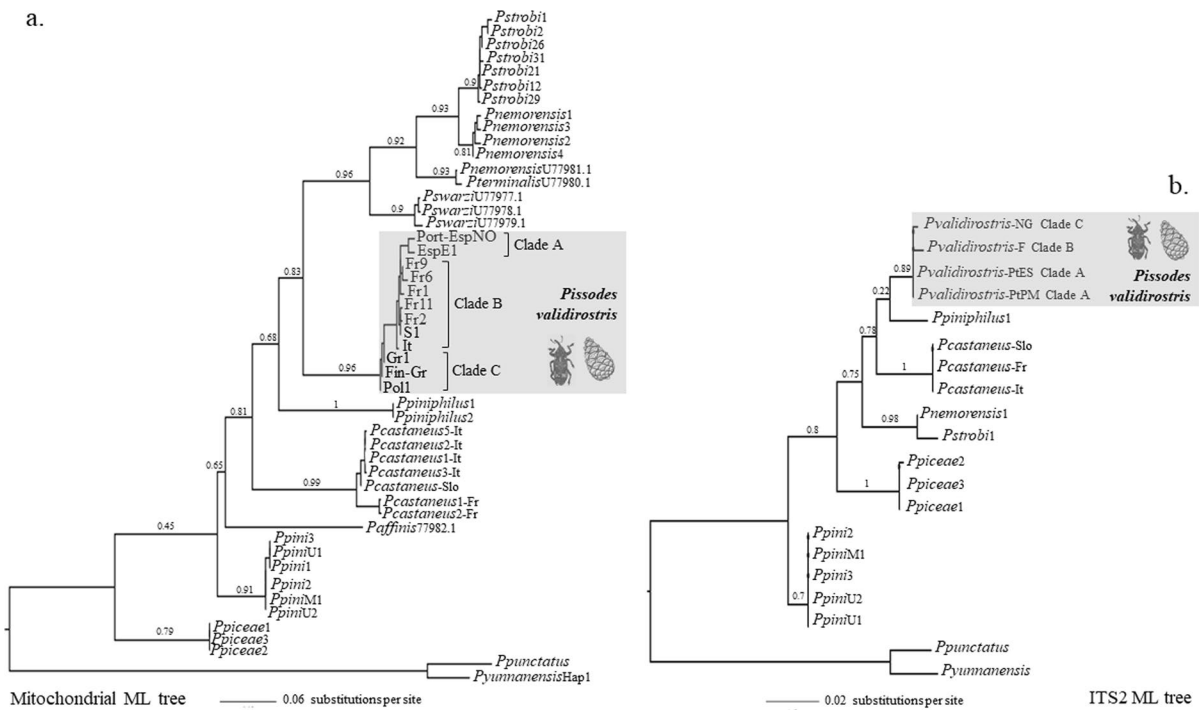


Fig. 4 Phylogenetic tree reconstructions of *Pissodes* species. **a** Maximum likelihood tree (ML) reconstructed using COI mitochondrial sequences. **b** Maximum likelihood tree reconstructed using ITS2 nuclear sequences. Branch support values are shown above branches. The grey rectangle highlights *Pissodes validirostris* clades. (Color figure online)

Discussion

Numerous species have been described in the genus *Pissodes.* However, several of these require more attention because of the occurrence of hybridisation, synonymy and cryptic species (Zhang et al. 2007; Wondafrash et al. 2016). The process of delineating species is challenging and generally requires multiple criteria to accurately define species boundaries (Wondafrash et al. 2016; van Steenderen et al. 2023). In the field of biocontrol, species delineation and phylogenetic relationships are critical, for example to ascertain which taxa are relatives of target pests for use in host range testing (Briese and Walker 2002). The integrative taxonomic approach taken in this study determined three distinct clades that may be considered as three evolutionary lineages—the Iberic Peninsula (clade A) on *Pinus* species belonging to the section *pinaster*, the central Europe (clade B) and the northern and eastern Europe (clade C) mainly on *Pinus* of the *sylvestris* section, with varying morphological characteristics and genetic diversity. The mtDNA revealed strong geographic structuring whereas the nuclear markers were differentiated according to taxonomic relatedness of the pine hosts.

Pissodes validirostris exhibited high genetic polymorphism, similar to its North American and Chinese congeneric species (Laffin et al. 2004; Zhang et al. 2007) and significant genetic structuring. This suggests restricted gene flow across populations, but this needs further testing using higher polymorphic genetic markers such as microsatellites. With its broad geographical and host ranges, the genetic variability observed is not unexpected, as has been found in other species with similar ranges (Avise 1994). The number of *P. validirostris* haplotypes (29 over a 542-bp mtDNA segment in 99 individuals from 22 localities) was comparable to that of *P. yunnanensis* (21 haplotypes over a 761-bp in 60 localities) and to that of *P. strobi* (36 haplotypes over a 826-bp segment in 130 individuals from 11 localities). This high intraspecific genetic variability reinforced the finding of other authors indicating that the mtDNA genome may evolve at a faster rate in *Pissodes* spp. compared to other insect groups (Langor and Sperling 1997; Zhang et al. 2007).

Within the three distinct phylogeographic clades that were recognised, all haplotypes were restricted to only few populations at relatively small spatial scales, except for populations from Southern Portugal and North-Western Spain sampled mainly on *P. pinaster*, which shared the same endemic haplotype (PortEspNO). This haplotype distribution indicates restricted gene flow within *P. validirostris* females or at least between populations that were geographically distant from each other. Geographic structuring has been found to result in genetically distinct populations in other *Pissodes* species (Phillips and Lanier 2000; Sperling et al. 2004; Zaleski et al. 2013). *Pissodes validirostris* have short-range host selection whereby they usually find their host trees by walking and adults generally stay up to three years on host trees and therefore would only experience a subset of local hosts during their lifespan (Dormont and Roques 2001). This strategy likely reflects the reason for this lack of gene flow between lineages and also may indicate the occurrence of host-association specialisation within these lineages. There are many examples of species that were considered generalists to a group of plants being found to be a complex of sibling species that have become specialised to a few host plants (Rafter and Walter 2020). For example, the Eastern Australian cicada *Pauropsalta annulata* Goding & Froggatt was considered a generalist across a wide native range. However, it has since been found to be a number of different species that are specialised to a different subset of trees (Popple and Walter 2010).

According to the phylogenetic reconstruction, all haplotypes from North and East Europe clustered together in a well-supported monophyletic sister group (clade C 96%) of all populations from central and Southern Europe (clades A and B). Genetic distances calculated from the COI sequences between *P. validirostris* lineages ranged between 1.0 and 4.5%, which is higher than those recorded within other *Pissodes* species (Pereyra et al. 2016). For example, *P. yunnanensis* was observed to have sequence divergences of 0.001% to 2.10% (Zhang et al. 2007) and *P. strobi* ranged from 0.12 to 2.54% (Laffin et al. 2004). The use of COI sequences in delineating species can be challenging and often cannot be used alone to recognise species taxa (Toon et al. 2016). For example, Toon et al. (2016) looked at random mating in two lineages of *Cryptolestes ferrugineus* (Stephens) with high sequence divergences and found no evidence of reproductive isolation and thus speciation. Comparison with sequence divergences of congeneric species

taxa can further assist in resolving taxonomic uncertainties (Cognato 2006; van Steenderen et al. 2023). Based on mitochondrial and nuclear molecular markers, phylogenetic reconstructions confirmed that *P. validirostris* lineages are monophyletic. The shift onto cone feeders appears as a single and relatively recent event within palearctic *Pissodes* species.

The use of an additional independent nuclear marker provides contrasting results compared to the highly structured *P. validirostris* mtDNA haplotypes. Nuclear DNA showed low variability among most of the sequence but revealed some highly repeated polymorphic regions. These variable microsatellite-like repeated motifs have been described in ITS of other beetle species (e.g., Gómez-Zurita and Vogler 2003) and Lepidoptera (Roe and Sperling 2007). *Pissodes validirostris* ITS2 sequences are amongst the longest in comparison to those reported for many arthropods: 499 or 491 bp in *Dioryctria* moths (Lepidoptera: Pyralidae) (Roe and Sperling 2007), 619 to 639 bp within the species complex *Timarcha goettingensis* L. (Coleoptera: Chrysomelidae) (Gómez-Zurita and Vogler 2003), but could be compared to that described for *Anopheles* species (Diptera: Culicidae) (600-716 bp; Mukabayire et al. 1999). Hence, such heterogeneity in the evolution rate within the same marker may provide biased information and could affect the phylogeographic pattern described. Nevertheless, we followed other authors in coding indel features as single mutational events in order to prevent branch lengthening (Gómez-Zurita and Vogler 2003; Simonato et al. 2007).

Pissodes validirostris was found to have significant differences in all measured morphological traits across the populations sampled. Adult weevils were significantly larger from populations sampled on *P. pinaster* and *P. pinea* compared to those collected on the *sylvestris* section, i.e., Mediterranean pines *vs.* north-central European pines. The level of morphological variation between populations in *P. validirostris* has also been observed in *P. strobi.* For example, the previously recognised *P. strobi* species, *P. schwarzi* and *P. nemorensis,* were found to be longer with more slender snouts and legs than *P. strobi* and *P. terminalis* (Williams and Langor 2002). Such variation has likely been driven by the diverse evolutionary forces across their geographic range (Pato et al. 2019) along with their low dispersal rates. Intraspecific variations can lead to speciation when these morphological divergences prevent or interfere with sexual communication, for example when changes occur in copulatory organs (Parzer et al. 2018; Vega-Sánchez et al. 2022). To determine if this is occurring in *P. validirostris* it will be necessary to conduct behavioural studies to determine whether or not reproductive isolation exists. Lastly, morphological traits were not consistently related to the molecular data. This may reflect insufficient sampling within these groups and also the influence of environmental factors. As such, morphological data cannot yet be reliably applied to assist in identifying between the phylogeographic groups.

Pissodes validirostris shares a number of parallels with *P. strobi.* Most notably both species have extensive native distributions, limited dispersal, significant genetic structuring and behavioural patterns in feeding on *Pinus* and *Picea* hosts (Sperling et al. 2004; Gagalova et al. 2022). Given these factors *P. strobi* was once considered to be a complex of four species. However, after extensive multi-faceted studies, including genetic, morphological and cross-breeding analysis, it is now regarded as a single species with distinct lineages (Gagalova et al. 2022). While this may also be the case for *P. validirostris,* these distinct clades could still be regarded as species in terms of their selection for biocontrol. According to Hey et al. (2003) races, biotypes and subspecies may represent "real" species in the sense that they are evolutionary entities or monophyletic groups. The three clades may vary in their host use and efficacy. Our study revealed that the greater preference of *P. validirostris* for Mediterranean pines (*P. pinaster* and *P. pinea*) was restricted to the western part of the Iberic Peninsula, whereas no such damage has been observed elsewhere despite extensive sampling in other locations in Spain and France. A study by Roques et al. (2004) collected *P. validirostris* from four different *Pinus* hosts and origins (*P. pinaster* in Portugal, *P. pinea* in Spain, *P. sylvestris* in France and *P. nigra* in France) and assessed their performance on six *Pinus* species and found significant differences in host use and subsequent damage to the *Pinus* species.

The biocontrol programme in South Africa is restricted to finding conospermatophagous agents that will be specific to the Mediterranean pines *P. halepensis, P. pinaster* and *P. pinea* (Moran et al. 2000). *P. validirostris* has been prioritised as the most promising agent to target these species based on its host specificity, climatic compatibility and damage

being restricted to only the plant's reproductive parts. Previous host specificity tests determined differences in feeding behaviour and host use by *P. validirostris* (Roques et al. 2004) and thus there was a need to determine if this variation was related to genetic structuring. This study confirmed that *P. validirostris* populations are genetically distinct and revealed the potential for the existence of three independent evolutionary lineages. The Iberian clade has been found to be most suited to the Mediterranean pines being targeted for biocontrol (Roques et al. 2004; Hoffmann et al. 2011). Future exploration for the programme can therefore target this geographic area and confirm that the correct clade is collected for further testing in South Africa.

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Declarations

Conflict of interest None to declare.

Research involving human and animal participants Not applicable.

Informed consent Not applicable.

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