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Taxonomic relationship among four European *Physokermes* species (Hemiptera: Coccomorpha) based on nuclear and mitochondrial DNA

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Introduction: Scale insects (Hemiptera: Coccomorpha) feed on a wide variety of agricultural crops and forest and ornamental trees worldwide. These pest insects damage plants not only by causing sap loss but also by reducing the plant's photosynthetic activity. This is because the honeydew they produce acts as a substrate for mold, which covers leaf surfaces. In the last decades, several outbreaks of *Physokermes spp.* (soft-scale insects) have occurred throughout Europe and have partly been attributed to unusual weather conditions or climate change, as some species seem to be expanding their distribution range. However, the small size of these insects and their large intraspecific morphological variation have hindered the identification of the species responsible for outbreaks.

Methods: In this study, mitochondrial DNA (cytochrome c oxidase subunit I, COI), ribosomal RNA (28S), and nuclear (elongation factor 1α, EF1α) DNA markers were used to reconstruct the phylogenetic relationships of four *Physokermes* species sampled throughout Europe in 2013–2015.

Results and discussion: The results allowed us to clearly distinguish *P. hellenicus* and *P. inopinatus* from each other, as these appeared in well-supported clades in the phylogenetic trees and from *P. piceae* and *P. hemicryphys*. However, *P. hemicryphus* appeared in a single clade in trees based on 28S and EF1α but among *P. piceae* in the COI tree. Further investigations are therefore required to determine the taxonomic status of *P. piceae* and *P. hemicryphys*, which seem to comprise a species complex.

KEYWORDS

Hungarian spruce scale, phylogeny, invasive species, forest pest, climate change

1. Introduction

Scale insects (Hemiptera: Coccomorpha) are important plant pests feeding on a wide range of agricultural crops as well as on forest and ornamental trees worldwide (Park et al., 2011). In addition to directly affecting host plants through sap loss, the honeydew produced by scale insects covers leaf surfaces, allowing mold development and thus reducing photosynthetic activity (Kozár et al., 2012). Because they are small and frequently found living within the plant, scale insects are often unseen. This contributes to their invasive potential and presents a huge challenge for plant quarantine (Andersen et al., 2010) and a major phytosanitary problem. The genus *Physokermes* comprises an important group of soft-scale insects infesting coniferous plants in the Holarctic region. In total, 13 species are currently recognized within *Physokermes* (Papanastasiou et al., 2021), but only four are

known to occur in Europe: the widespread *P. hemicryphus* (Dalman) and *P. piceae* (Schrank), the more easterly distributed *P. inopinatus (*Danzig & Kozár), and *P. hellenicus,* (Kozár & Gounari) described from *Abies cephalonica* (Pinaceae) in Greece (Kozár et al., 2012). Although commonly known as the Hungarian spruce scale, *P. inopinatus* caused a mass infestation in Sweden in 2010 (McCarthy and Skovsgaard, 2011), suggesting a considerable northward expansion of this species' distribution, possibly linked to climate change or the unrestricted transport of plants within Europe (Kozár et al., 2012). Indeed, the attack by *P. inopinatus* was triggered by a sequence of droughts that contributed to population expansions in the relatively dry summers of 2008–2009, leading to the severe mass infestation of 2010 (McCarthy and Skovsgaard, 2011; Olsson et al., 2012; Gertsson and Isacsson, 2014; Winde et al., 2018).

However, the identity of the species causing the 2010 outbreak in Sweden was not immediately determined. This was because morphological identification of scale insects requires a high level of expertise and is time consuming. It mostly relies on characters present only in adult females, which is difficult when the morphological variation observed between females of closely related species corresponds to natural intraspecific variation instead of interspecific variation (Malausa et al., 2011; Park et al., 2011). The limitations of this methodology have also raised concern about the possible non-identification of cryptic species complexes (Andersen et al., 2010), and thus, several DNA markers have been developed to distinguish between closely related species of scale insects, mainly mealybugs and armored scales (Kondo et al., 2008; Andersen et al., 2010; Gwiazdowski et al., 2010; Malausa et al., 2011 and references therein; Park et al., 2010; 2011; Abd-Rabou et al., 2012; Beltrà et al., 2012; Deng et al., 2012; Sethusa et al., 2014). Such markers include a set of gene regions providing reliable and rapid identification of scale insect species, even when only immature and male specimens are available. Although DNA barcoding based on *cytochrome c oxidase subunit I* (COI) sequence variation has become a popular tool for species delimitation in several insect groups (Hajibabaei et al., 2006), and therefore an ideal candidate for identifying scale insects (e.g., Kondo et al., 2008; Malausa et al., 2011; Park et al., 2011; Abd-Rabou et al., 2012; Beltrà et al., 2012; Deng et al., 2012; Sethusa et al., 2014), only 2,172 COI sequences of Coccidae are deposited in GenBank (http://www.ncbi.nlm.nih.gov; accessed November 8, 2022). Gene 28S has also been used to identify insect species (Campbell et al., 1994; Monaghan et al., 2006; Smith et al., 2008). Although lacking sufficient variation to delimitate some species (Park et al., 2011; Deng et al., 2012), gene 28S has been proposed as a complementary marker to COI in scale insects (Sethusa et al., 2014). This supports van Nieukerken et al. (2012) suggestion of including at least one nuclear marker in addition to COI in studies concerning cryptic lineages. Thus, in this study, three genes from three independent loci were amplified: COI (mitochondrial DNA), 28S (D2 and D3 regions, ribosomal RNA gene), and elongation factor 1alpha (EF1α, nuclear DNA). These genes represent a range of different rates of evolution: COI is a relatively fast-evolving mitochondrial gene (Simon et al., 1994), 28S is relatively conserved and represented in the nuclear genome as numerous tandem repeats (Hoy, 1994), and EF1α is a low-copy number nuclear protein-coding gene with conserved exons and variable introns (Hoy, 1994).

The DNA sequence analysis performed in this study for several *Physokermes* spp. populations across Europe aimed to (i) scan for cryptic species within taxonomically recognized *Physokermes* species; (ii) study the phylogenetic relationships between the identified taxa; and (iii) obtain a series of molecular markers suitable for the reliable identification of closely related *Physokermes* spp. in Europe, especially those with invasive pest status, thus contributing to more efficient pest management and quarantine procedures. Finding cryptic species will also provide the necessary background and molecular evidence to conduct thorough morphological studies on *Physokermes* specimens representative of several species. This will allow laying the foundations for a contemporary taxonomic revision and for the recognition of morphological characters important to species differentiation, similar to what has been done for *Chionaspis* spp. by Vea et al. (2012) following the work of Gwiazdowski et al. (2010). To date, only one molecular study of *Physokermes* spp. has been published, and it included COI sequence analysis of *P. hellenicus* from different Greek populations (Papanastasiou et al., 2018).

2. Materials and methods

2.1. Taxon sampling

In total, 203 *P. inopinatus*, *P. piceae, P. hemicryphus,* and *P. hellenicus* individuals were collected across their distribution ranges in Europe, i.e., from Turkey, in the southeast, to Sweden, in the northwest (Table 1). Sampling occurred in 2013–2015, and insects were immediately placed in 95% ethanol and kept at −20°C for DNA preservation. Taxonomic identity was determined by experienced scale insect specialists (see Acknowledgments) based on discriminating morphological characters published in reference studies (e.g., Kosztarab and Kozár, 1988; Kozár et al., 2012).

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was isolated from the 203 individuals using the QIAamp? DNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol for the purification of total DNA from insects. The nucleotide sequences including the barcoding region of COI, 28S, and EF1α were amplified *via* PCR using the 5′-tailed primers and profiles described in Table 2. Amplification reaction mixtures (total volume = 20 μL) contained 10 μl DreamTaq™ PCR Master Mix (Fermentas™, Waltham, MA, USA), 1 μl each primer, 6 μl DNA-free water, and 2 μl template DNA. To check for contamination, negative controls (sterile water instead of template DNA) were included in each reaction array. The resulting PCR products were then visualized under UV light in 1% agarose gels stained with SYBR™ Safe DNA Gel Stain (ThermoFisher Scientific, Waltham, MA, USA). Those representing successful reactions were prepared for sequencing by digesting 5 μl of the PCR mixture with 0.5 μl Exonuclease I, and 1 μl FastAP™ Thermosensitive Alkaline Phosphatase (both from Fermentas™) during a thermal cycle consisting of 15 min at 37°C and 15 min at 85°C. PCR products were then sequenced in

TABLE 1 Collection sites and the number of *Physokermes* spp. individuals examined in the present study, and the number of cytochrome oxidase I (COI), 28S, and elongation factor 1 α (EF1α) sequences obtained.

Species	Location	Country	Coordinates	Host	N	Sample code in Phylogenetic trees	Sequences (number)		
							COI	28S	EF1α
P. inopinatus	Södra Sandby	Sweden		*Picea glauca* var. *conica*	20	Pino1.n; Pino2.n	12	13	17
P. piceae	Målilla	Sweden	57.337920, 15.754570	*Picea abies*	30	Ppic1Mn-Pic6Mn	82	60	72
	Tilburg	The Netherlands	51.540504, 5.016668	*Picea abies*	2	Ppic4.n			
	Ancara	Turkey	Not available	*Picea excelsa*	20	Ppic5.n-Ppic6.n			
	Szabadság Square, Budapest	Hungary	47.528272, 18.954156	*Picea abies*	4	Ppic2.n			
	Villány	Hungary	Not available	*Picea abies*	8	Ppic3.n			
	Kaszó	Hungary	46.320881, 17.223469	*Picea abies*	4	Ppic21.n			
	Nagykanizsa	Hungary	46.401989, 17.053061	*Picea abies*	6	Ppic24.n			
	Bosárkány	Hungary	47.689524, 17.251699	*Picea abies*	6	Ppic27.n			
	Mosonmagyaróvár	Hungary	47.884160, 17.25471	*Picea abies*	7	Ppic28.n			
	Vágáshuta	Hungary	48.423353, 21.5394	*Picea abies*	7	Ppic38.n			
	Mikóháza	Hungary	48.466467, 21.588708	*Picea abies*	6	Ppic40.n			
	Székelyvaja	Romania	46.440833, 24.64866	*Picea abies*	6	Ppic42.n			
	Lukailencfalva	Romania	46.463667, 24.531167	*Picea abies*	5	Ppic43.n			
	Farád	Hungary	47.604667, 17.204583	*Picea abies*	6	Ppic47.n			
	Berzence	Hungary	46.206400, 17.151934	*Picea abies*	6	Ppic48.n			
	Balavásár	Romania	46.409167, 24.704167	*Picea abies*	7	Ppic41.n			
P. hemicryphus	Lund	Sweden	55.714360, 13.196340	*Picea glauca* var. *conica*	24	Phem1.n, Phem2.n, Phem3.n, Phem4.n, Phem2L, Phem5L, Phem6L	14	16	4
	Målilla	Sweden	57.337920, 15.754570	*Picea abies*	2	Phem1M, Phem2M			
	Gellért Hill, Budapest	Hungary	Not available	Not available	15	Phem5.n, Phem6.n			
P. hellenicus	Mount Parnitha	Greece	38.172806, 23.731111	*Abies cephalonica*	10	Phel1.1n	9	5	7
				Total	**203**		**117**	**94**	**100**

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TABLE 2 Primers and PCR profiles used to amplify cytochrome oxidase I (COI), 28S, and elongation factor 1 α (EF1α) gene fragments in *Physokermes* spp.

Region	Primer	Sequence	Source	PCR
COI	C1-J-2753ywr	GTAAACCTAACATTTTTYCCWCARCA	Gwiazdowski et al., 2010	1′ @ 95°C; 30′′ @ 95°C, 1′ @ 40-50°C, 2′ @72°C; annealing T was decreased 2°C every 2 cycles from 50°C to the final T of 40°C; 10′ @72°C
COI	C2-N-3662	CCACAAATTTCTGAACATTGACC	Gwiazdowski et al., 2010	
COI barcode	PcoF1	CCTTCAACTAATCATAAAAATATYAG	Park et al., 2011	2′ @ 95°C; 5x (40′′ @ 94°C, 40′′ @ 45°C, 1′ @72°C); 40x (40′′ @ 94°C, 40′′ @ 51°C, 1′ @ 72°C); 5′ @72°C
COI barcode	LepR1	TAAACTTCTGGATGTCCAAAAAATCA	Park et al., 2011	
D2-D3 28S	s3660	GAGAGTTMAASAGTACGTGAAAC	Morse and Normark, 2006	@ 95°C; 30′′ @ 95°C, 1′ @58°C, 2′ @ 72°C; annealing T was lowered 2°C every 3 cycles until final annealing of 42°C, which was used for 18 Cycles; 10′ @ 72C
D2-D3 28S	28b	TCGGAAGGAACCAGCTACTA	Morse and Normark, 2006	
EF1α	EF1-a(a)	GATGCTCCGGGACAYAGA	Morse and Normark, 2006	
EF1α	EF2	ATGTGAGCGGTGTGGCAATCCAA	Morse and Normark, 2006	

both directions, to ensure high-quality reads, using PCR primers (Table 2) and the BigDye Terminator Cycle Reaction Kit (Applied Biosystems, Foster City, CA, USA).

2.3. Phylogenetic analyses

Contiguous sequences were assembled in Geneious® 8.1.15 (Biomatters, Auckland, New Zealand), aligned using the default parameters and cost matrix of the Geneious® alignment algorithm as included in Geneious® 8.1.15 and edited by eye to maximize blocks of sequence identity. Sequences were checked for homology by comparison with those deposited in GenBank, using the Basic Local Alignment Search Tool (BLAST) available on the NCBI website (NCBI, http://www.ncbi.nlm.nih.gov/Genbank). Translation into amino acids was also performed to confirm data correctness.

Using PartitionFinder 2 (Lanfear et al., 2012), the best partitioning scheme and model of nucleotide substitution were selected for each dataset based on the Bayesian information criterion. All possible combinations of codon positions were considered as candidate partitions (i.e., from a single partition for the entire sequence to each codon position treated as a partition), but only the nucleotide substitution models supported by MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003) were included in the candidate model pool. This procedure was repeated five times to ensure correct estimation. The resulting models and partition schemes were used in phylogenetic reconstructions based on maximum likelihood (ML) and Bayesian Inference (BI), performed in Garli 2.1 (Zwickl, 2006) and MrBayes 3.2.6, respectively. Because considerable statistical inconsistency can be found when using the maximum parsimony method of phylogenetic reconstruction on this type of data (different rates of evolution), it was not performed in this study.

In Garli, clade stability was evaluated by non-parametric bootstrapping (five independent search replicates of 10,000 generations each). In MrBayes, and starting from a random tree, alternative tree topologies were evaluated through the estimation of posterior probabilities by Markov chain Monte Carlo re-sampling. These were performed in two runs of four chains each (with the default settings implemented in MrBayes) for 10×10^6 generations with a sampling frequency of 100 (average standard deviation of split frequencies <0.01). These samples were then used to generate 50% majority-rule consensus trees. Commands "sumt" and "burnin = 5,000" were set to discard trees that did not reach the likelihood score detected with the "sump" command. Only bootstrap and posterior probabilities values >80% were considered to support clades.

The *species delimitation* plugin implemented in Geneious® was used to evaluate different scenarios of lineage divergence within *Physokermes* spp. based on a neighbor-joining (NJ) tree constructed for COI sequences. The distance-based species divergence criterion relies on the postulate that intraspecific genetic variation is small relative to interspecific variation. In this study, two clusters containing a pair of nearest neighbors with >2% distance from each other were considered different species, as this threshold has been proven efficient to discriminate other insect groups (Foottit et al., 2009; Zhu et al., 2017). Only monophyletic clades showing bootstrap support >80% were tested.

3. Results and discussion

The BLAST of all sequences against the NCBI database revealed that some (most of them corresponding to 28S sequences) matched those of internal parasites, parasitoids, or microorganisms infecting *Physokermes* spp.; hence, these sequences were excluded from further analysis. This higher-than-expected ratio of non-specific amplification might be due to the binding of one or both PCR primers to a sequence other than the target one, which is frequently a problem in the amplification of conserved sequences, such as ribosomal DNA. The 117 COI sequences (total length = 712 bp) and the 94 28S sequences (total

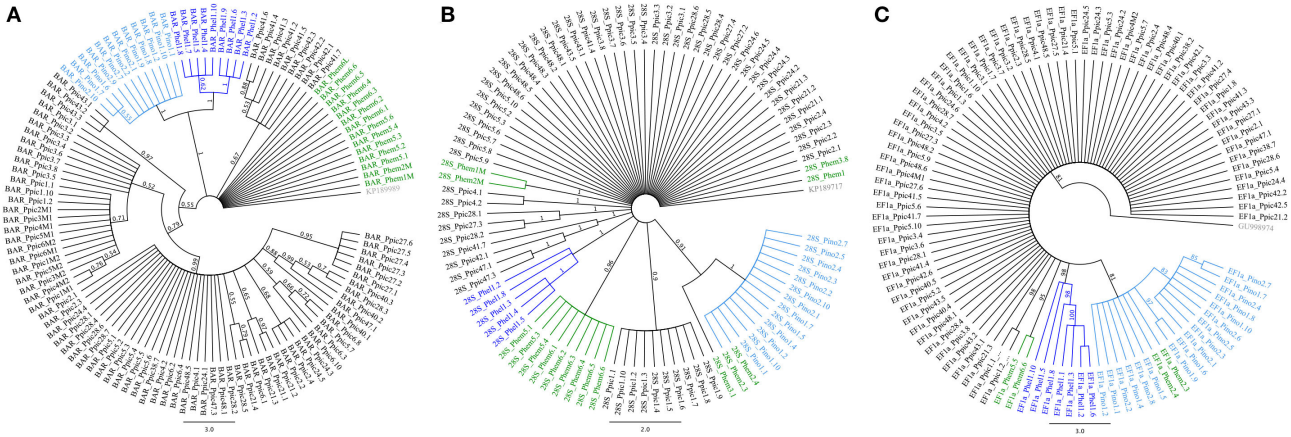

FIGURE 1
Bayesian inference phylogenetic trees constructed for *Physokermes inopinatus* (Pino), *P. hellenicus* (Phel), *P. piceae* (Ppic), and *P. hemicryphus* (Phem), based on cytochrome oxidase I [COI, **(A)**], 28S **(B)**, and elongation factor 1 α [EF1α, **(C)**] sequences, using the generalized time reversible (GTR) model with a gamma-distributed rate variation (G) among sites, the Hasegawa–Kishino–Yano (HKY) model with a proportion of invariable sites (I), and a partitioned model in which the first two positions evolved according to Felsenstein's 1981 model (F81) with G and the third according to HKY, respectively. Posterior probabilities (PP) are shown above branches; only clades with PP > 0.80 were considered well-supported. Numbers (and letters) next to species codes correspond to sample codes; numbers (and letters) before the dot to population codes; and the numbers (and letters) after the dot to individuals (see Table 1).

length = 842 bp) presented the highest sequence homology to *Physokermes jezoensis* Siraiwa isolate S2, whereas the 100 EF1α sequences (total length = 1,056 bp) presented the highest sequence homology to *Paralecanium* sp. (Coccomorpha) isolate D0764B. These BLAST hits are probably the result of the very low number of *Physokermes* species nucleotide sequences deposited in GenBank ($n = 22$, Available online at: http://www.ncbi.nlm.nih.gov; accessed November 8, 2022). In addition, the handling of specimens for morphological identification might have damaged the DNA quality leading to amplified fragments shorter than ideal for BLAST hits. Nevertheless, the most similar sequences were used as outgroups in the subsequent phylogenetic analyses. The sequences obtained and analyzed in the present study are deposited in GenBank under accession numbers OQ433782–898 (COI), OQ470427–526 (EF1α), and OQ657360–453 (28S).

According to PartitionFinder, the best nucleotide substitution model for *Physokermes* spp. COI evolution was a generalized time reversible (GTR) with a gamma-distributed rate variation (G) among sites, whereas 28S evolved according to the Hasegawa–Kishino–Yano (HKY) model with a proportion of invariable sites (I). A partitioned model of evolution was found for EF1α sequences, with the first two positions evolving according to Felsenstein's 1981 model (F81) with G and the third according to HKY. The ML and BI analyses performed for all datasets (Figure 1) revealed similar results, although BI usually presented higher support values, and therefore, these are the trees presented here. One clade comprising *P. hellenicus* and another comprising *P. inopinatus* were clearly evidenced and well-supported in all trees. However, only 28S and EF1α BI trees evidenced clades comprising *P. hemicryphus* alone (Figures 1B, C, respectively), with this species appearing mostly among *P. piceae* and *P. inopinatus*. In addition, *P. hemicryphus* samples from Hungary (Phem5.n and Phem6.n) appeared in a different clade from *P. hemicryphus* collected in Sweden (all other Phem codes). These populations do not seem to constitute host races, because they were both sampled from *Picea abies*, but might have been isolated by distance. Thus, the 28S nuclear gene is complementary to COI in *Physokermes* spp. identification, as proposed by Sethusa et al. (2014).

The NJ tree produced for the COI sequences (Figure 2) also evidenced well-supported clades containing *P. hellenicus* and *P. inopinatus*, and three other clades corresponding to *P. piceae* from Kaszó in Hungary (Ppic21.1 and Ppic21.2) and from Balavásár (Ppic41.2 and Ppic41.3) and Lukailencfalva (P.pic43.1, Ppic43.2, and Ppic43.3) in Romania. All these clades were monophyletic and their distance to the nearest neighbor varied from 3.5% (between *P. piceae* clades from Romania) to 6.3% (between *P. inopinatus* and *P. hellenicus*). These results support the distinction between *P. inopinatus* and *P. hellenicus*, as indicated in the ML and BI analyses. Moreover, the genetic distance found is similar to the congeneric 5.8% average mitochondrial DNA divergence (Pons et al., 2006) and within the 0.0–7.95% range (Hebert et al., 2004) reported for insects. The clade support and genetic distances found within *P. piceae* from Hungary and Romania (>2%) suggest the existence of cryptic species. Similar to other scale insects, *Physokermes* spp. females are flightless, males have a short lifespan, and both sexes have low dispersal capacity, all contributing to population differentiation in different areas and/or host species and promoting cryptic speciation, as observed in *Aspidiotus nerii* in Australia (Andersen et al., 2010) and in *Chionaspis heterophyllae* and *C. pinifoliae* in North America (Gwiazdowski et al., 2010). Because all the genetically differentiated *P. piceae* were sampled from *P. abies*, host species does not appear to be the factor contributing to such differentiation. Although explanations for the recent outbreaks remain elusive, particularly given the limited dispersal ability of *Physokermes* spp., changes in climate or weather conditions inducing rapid reproduction of endemic (and thus escaping detection) populations are more likely than sudden range expansions.

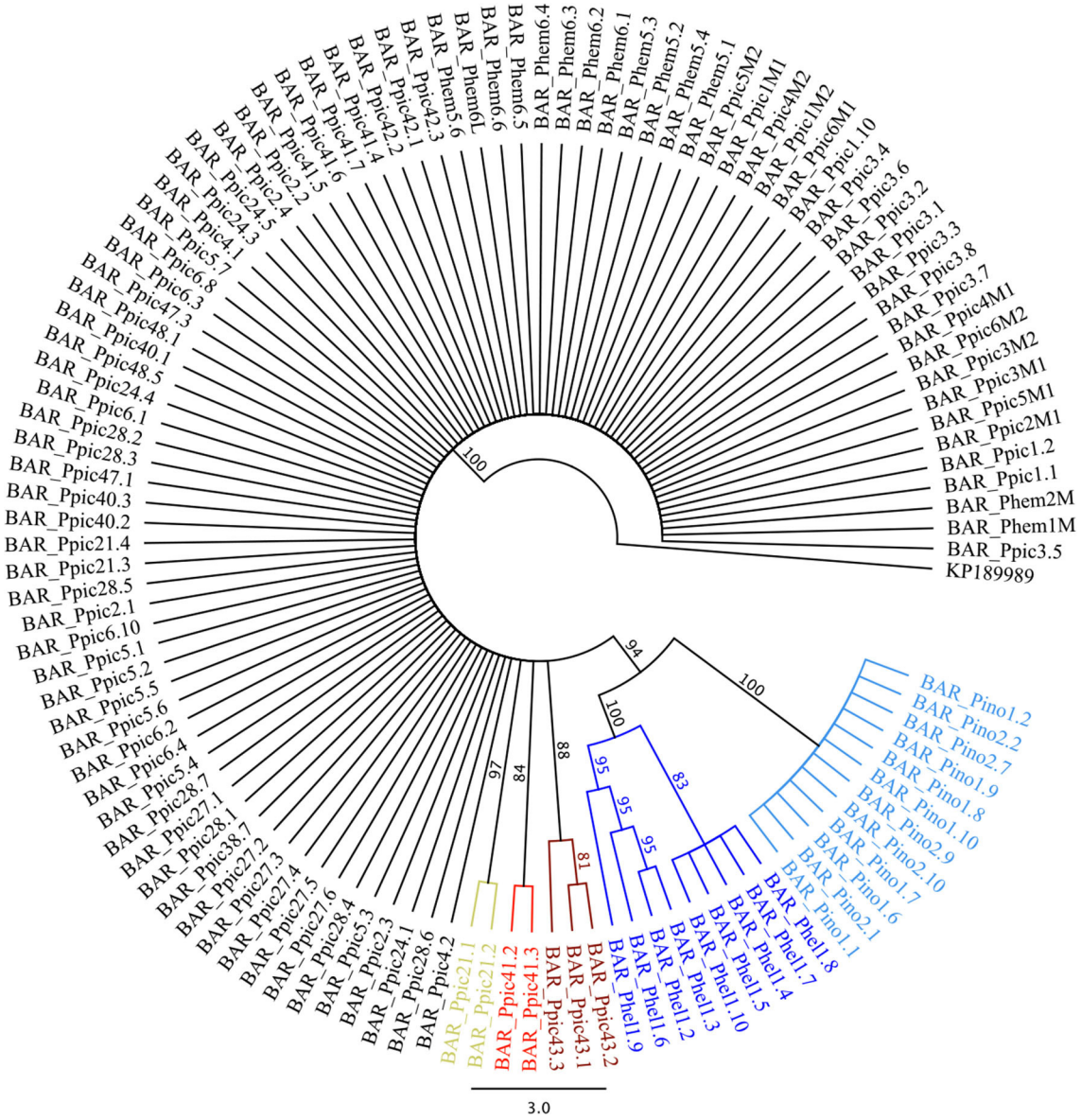

FIGURE 2
Neighbor-joining tree produced for the cytochrome oxidase I (COI) sequences obtained for *Physokermes inopinatus* (Pino), *P. hellenicus* (Phel), *P. piceae* (Ppic), and *P. hemicryphus* (Phem) to test different scenarios of species divergence, using the *species delimitation* plugin implemented in Geneious® 8.1.15. Clades with bootstrap support >80% were tested (presented in different colors). Numbers following species codes correspond to population codes (see Table 1). *Physokermes jezoensis* (GenBank accession KP189989) was used as an outgroup.

Overall, the results obtained here (i) clearly distinguished the closely related *P. hellenicus*, *P. inopinatus*, and (*P. piceae* + *P. hemicryphus*); (ii) revealed the existence of intraspecific variation and, possibly, of unknown complexes of species; and (iii) confirmed the value of using 28S in addition to COI for identifying scale insects. However, the differentiation of *P. hemicryphus* suggested in the nuclear data (particularly in 28S) analyses was not supported in the *species delimitation* analysis, as *P. hemicryphus* was not genetically distant from *P. piceae*. However, the sample size of *P. hemicryphus* and its geographic range was much smaller than that of *P. piceae,* and thus, further studies are needed to confirm the taxonomic status of *P. hemicryphus*.

Data availability statement

The gene sequences for this study can be found in GeneBank at https://www.ncbi.nlm.nih.gov/nuccore/?term=OQ433782:OQ433898[accn] for COI; https://www.ncbi.nlm.nih.gov/nuccore/?term=OQ4704273:OQ470526[accn] for EF1α; https://www.ncbi.nlm.nih.gov/nuccore/?term=OQ657360:OQ657453[accn] for 28S.

Author contributions

OA, AJ, and IW secured funding. IW ensured access to specimens. JM performed the genetic analyses and wrote

the first draft of the manuscript. All authors conceived the study, contributed to the manuscript, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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