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ORIGINAL ARTICLE

Co-phylogeny and biogeography of the myrmecophilous beetle *Paussus favieri* (Carabidae, Paussinae) and its host ant *Pheidole pallidula* (Hymenoptera, Myrmicinae)

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Abstract Strict patterns of co-phylogeny have seldom been observed, except among organisms and their symbionts with limited dispersal abilities. In this study, we investigate potential signs of co-phylogeny at the population level between an obligate myrmecophile, the beetle *Paussus favieri*, and its host ant, *Pheidole pallidula*. While neither species is physically dependent on the other, as both are fully winged and capable of independent dispersal, *Paussus favieri* relies entirely on *Pheidole pallidula* throughout its life cycle. These predatory beetles feed on ants and reproduce in their nests, where they lay eggs and their larvae develop. Therefore, the beetle cannot survive or reproduce without its host, making this an interesting system to explore potential co-phylogenetic patterns. In this paper, we infer population-level phylogenies for both species based on molecular sequence data and apply distance-based and event-based co-phylogenetic methods to search for signs of co-phylogeny, codivergence, or evidence of frequent host population shifts. Molecular phylogenetics reveals significant co-phylogenetic signals, but not phylogenetic congruence, using distance-based methods, as would be expected if the populations of both species shared a similar evolutionary or biogeographic history, without a strict evolutionary dependency. Co-phylogenetic signal without phylogenetic congruence is further explained by event-based methods with a history of codiversification and host population switching, typically occurring among nearby, closely related populations. We discuss the putative mechanisms that might have driven the co-phylogenetic signal between these strictly myrmecophilous beetles and their host ants with particular emphasis on a shared biogeographic scenario within the complex biogeographic history of the Mediterranean Basin.

Key words ants; co-phylogenetic signal; host–parasite; myrmecophile; Mediterranean; phylogeography

Introduction

The intricate relationships between parasites and their hosts offer a fascinating glimpse into evolutionary and ecological dynamics. According to Fahrenholz's rule (Fahrenholz, 1913), the evolutionary history of a specialized parasite mirrors that of its host. As populations of the host diverge from one another, so will the populations of the parasite, which leads to co-divergence (Lagrue *et al.*, 2016; Drábková *et al.*, 2019). Over evolutionary time, the iterative process of co-divergence between hosts and parasites leads to topological similarities in their phylogenies (Legendre *et al.*, 2002; Hutchinson *et al.*, 2017). However, parasites can also disperse to other hosts, or fail to

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diverge with their hosts, and in these cases, lead to varying degrees of topological similarity and discordance between host–parasite phylogenies (De Vienne *et al.*, 2013). Furthermore, the relationship between parasites and hosts is shaped not only by evolutionary pressures but also by ecological factors such as geographical distributions, ecological niches, and host availability. As parasites may move between different host species or adapt to new environments, their phylogenetic trajectories may diverge significantly from those of their hosts. This ecological flexibility in some parasites contrasts with the stricter evolutionary dependency seen in others, making the study of host–parasite co-evolution complex and multi-faceted (Perez-Lamarque & Morlon, 2024).

Many studies have investigated these patterns between ants and their symbionts (Mehdiabadi *et al.*, 2012; Kellner *et al.*, 2013, 2018; Levitsky, 2013) however, very few have focused on host–nest parasite associations (Jansen *et al.*, 2011; Murray *et al.*, 2013), especially at the population-level (Kellner *et al.*, 2018). Here, we take advantage of an unusual opportunity to conduct a phylogeographic study of a rarely collected obligate nest parasite, the beetle *Paussus favieri* Faimaire, 1851, and its host ant, *Pheidole pallidula* (Nylander, 1849), from throughout their range in the Western Mediterranean.

Species of the carabid genus *Paussus* L. (Carabidae, Paussinae, Paussini) are thought to be entirely dependent on their host ants throughout all stages of their life cycle (Geiselhardt *et al.*, 2007; Moore & Robertson, 2014; Moore *et al.*, 2022). The Western Mediterranean species *Paussus favieri* Faimaire, 1851 is by far the most studied *Paussus* species in the world (Cammaerts & Cammaerts, 1989, 1992; Cammaerts *et al.*, 1990; Di Giulio *et al.*, 2009, 2011, 2012, 2014, 2015; Maurizi *et al.*, 2012; Fattorini *et al.*, 2021; Moore *et al.*, 2022; Muzzi & Di Giulio, 2019). From this body of work, we have learned that *P. favieri* uses a combination of behavioral, chemical, and acoustic strategies to enter the ant's nest and feed on its hosts. These parasites rise in the social hierarchy within the colony by mimicking the stridulations produced by queen, minor, and major workers (Di Giulio *et al.*, 2011). Adult *P. favieri* even interacts directly with the queen without eliciting an alarm response within the colony (Maurizi *et al.*, 2012; Moore *et al.*, 2022). Eggs are laid inside the nest, and the immature beetles feed on ant brood and possibly elicit trophallaxis from the adult ants (Di Giulio *et al.*, 2015).

Despite the intriguing nature of this host–parasite interaction, research on *Paussus* and its association with ants remains limited, with only a few species being documented through rare field collections and laboratory observations (Moore *et al.*, 2022). *Paussus* species are usually collected outside of their host ant's nest with Malaise traps or at lights during their dispersal flights. However, only when the beetles are collected directly from the nest is it possible to estimate the degree of specialization between the parasite and its host. Even then, it is necessary to establish infested ant colonies under controlled laboratory conditions to estimate fecundity, record host–parasite interactions (chemical, behavioral, mechanical, etc.), and document the development of immature stages, all of which would inform us of the impacts *Paussus* has on host colonies. Difficulties in finding Paussini (and myrmecophiles in general) within the ant nests in the field and in rearing them in the laboratory limit our ability to understand their ecology and behavior.

Fig. 1 *Paussus favieri* and *Pheidole pallidula*. *Paussus favieri* adult depicted with its only recorded host ant *Ph. pallidula*. All life stages of *Ph. pallidula* are shown except for the larvae. Photo credit: Andrea Di Giulio.

Unlike most species of *Paussus* for which the degree of host ant specificity is unknown, *P. favieri* is well-documented to integrate only into *Pheidole pallidula* (Nylander, 1849) colonies (Casale *et al.*, 1982; Nagel, 1987; Di Giulio *et al.*, 2011) (Fig. 1). Even in northern Africa, where 10 species of *Pheidole* co-occur, *P. favieri* has only been collected in association with *Ph. pallidula* (Di Giulio, personal observation). *Pheidole pallidula* is a dominant ant in the Mediterranean region. It is an polygynous species that creates huge nests, easily adapts to new environments, and prefers humid microclimates within arid landscapes. At some sites in Morocco, Tunisia, and Spain, *P. favieri* is quite frequent, being present in almost every *Ph. pallidula* nest, sometimes in relatively large numbers. However, even highly infested colonies show no signs of collapse (Di Giulio, personal observation).

It is possible that our current concept of *Ph. pallidula* might represent a cryptic species complex (Seifert, 2016), in which case the distribution of the host and its nest parasite might be more similar. But based on our current understanding, the distribution of *Ph. pallidula* is much wider than that of *P. favieri* (Figs. 2 and 3), which would

not be surprising given that there are greater constraints on the successful dispersal of the obligate nest parasite relative to its host. Although *P. favieri* are fully winged, they are reluctant to fly, and depend on finding a mature ant colony to invade immediately after dispersing. Therefore, successful dispersal events of *P. favieri* into a new area must occur only after its host's colonies have become established, creating a major constraint on the ability of *P. favieri* to successfully invade new regions. After dispersing, *Paussus* must land near their host's nest, potentially using their ability to sense and follow the "nest odor" plume. The ants then actively drag their predators inside the nest (Nagel, personal observation), much like the myth of the Trojan Horse (Moore & Robertson, 2014).

Unlike many parasitic relationships (such as those between birds and lice), *Paussus* beetles and their host ants are not physically bound to each other, as they are both fully winged and capable of dispersal. This case presents an intriguing form of parasitism in which both the parasite and host can disperse independently, distinguishing it from other systems where the parasite or symbiont is physically bound to the host, often leading to co-phylogenetic patterns.

Past studies conducted at deeper phylogenetic scales suggest both *Ph. pallidula* and *P. favieri* dispersed to the Mediterranean region from sub-Saharan Africa (Economo *et al.*, 2014; Moore & Robertson, 2014; Robertson & Moore, 2017), therefore indicating a possible shared biogeographic history between these species. Here, we aim to shed light on the possible processes that have led to the evolution of this unique host–nest parasite association by investigating signs of co-phylogenetic signal, co-divergence, and host population switching in this system.

Materials and methods

Taxon sampling

Forty *Ph. pallidula* and 37 *P. favieri* from 19 localities in Europe and northern Africa were used in this study (Tables 1–3). Most specimens were collected between 2005 and 2019. Beetles and ants collected from the same colony were preserved in 99% EtOH and stored at −20 °C (Table 1). We also included several specimens of *Ph. pallidula* collected outside the range of *P. favieri* (Table 2), and several specimens of *P. favieri* collected without *Ph. pallidula* (Table 3). All specimens were identified to the species-level morphologically. Outgroups were selected based on previously published studies (Economo *et al.*, 2014; Moore & Robertson, 2014). One pinned specimen of *Paussus biflagellatus* (Luna De Carvalho) was included as an outgroup to root the *P. favieri* phylogeny.

DNA extraction

Total genomic DNA of *P. favieri* and *Ph. pallidula* was extracted using a DNeasy Blood & Tissue Kit (QIAGEN) following manufacturer protocols. One or two *Ph. pallidula* workers from the same colony were used for each extraction. *Paussus favieri* specimens were dissected, and single abdomens were used for extraction without grinding. After digestion with proteinase K, the abdomens were reassociated with the rest of the body. DNA of *P. biflagellatus* was extracted from a pinned specimen collected in 1985 (voucher DRM5426) at Oregon State University using the Qiagen QIAmp Micro Kit using the standard protocol (with RNA carrier added) in a laminar flow hood within a room dedicated to DNA extraction from specimens with degraded DNA.

Gene sampling, amplification, and sequencing

Eleven gene fragments were explored for this study: a mitochondrial protein-coding gene, the cytochrome c oxidase subunit 1 (COI); a nuclear ribosomal gene, the 28S; and 9 nuclear protein-coding genes found to be evolving at the fastest rates in beetles: Top1, Wg, Glus, CG4933, Hmgs, Lar, Ndae1, rols, and Sur-8 (Che *et al.*, 2017). Target fragments were amplified using previously published primers and standard PCR methods (Hebert *et al.*, 2003; Che *et al.*, 2017). Additional tables provide all primer and PCR details (Supporting information 1).

Purified PCR products were quantified, normalized, and sequenced in both forward and reverse directions using Sanger sequencing methods at the University of Arizona Genetics Core using an Applied Biosystems 3730 DNA Analyzer. Chromaseq v. 1.51 package (Maddison & Maddison, 2019) in Mesquite v. 3.5 (Maddison & Maddison, 2019) was used with the programs Phred v. 0.020425.c (Green & Ewing, 2002) and Phrap v. 0.990319 (Green, 2009) to assemble the chromatograms into contigs and make final base calls.

DNA from the old, pinned specimen of *P. biflagellatus*, was quantified using a Qubit Fluorometer (Life Technologies, Carlsbad, CA) with a Quant-iT dsDNA HS Assay Kit, and DNA fragment length distributions with a 2100 Bioanalyzer (Agilent Technologies) using the High Sensitivity DNA Analysis Kit. As expected, the DNA was fragmented, so we proceeded with direct Illumina sequencing, rather than PCR amplifica-

Table 1 Taxon sampling for specimens of *P. favieri* and *Ph. pallidula* collected together from the same nests.

Locality	Number	DNA	Nest	Taxon	COI	28S	Topo1	WG
Morocco: Tizi n'Test (30.8833° N 8.3468°W)	1	5039	nest 1	*P. favieri*	PV761154			
		4477	nest 1	*Ph. pallidula*	PV761181			
Morocco: Oukaimeden (31.2219°N 7.8288°W)	2	4290	nest 9	*P. favieri*	PV761143			
		4291	nest 9	*Ph. pallidula*	PV761183		PV815403	
	2	4579	nest 3	*P. favieri*	PV761144		PV815394	PV815367
		4580	nest 3	*Ph. pallidula*	PV761185		PV815404	PV815379
		4292	nest 2	*P.favieri*	PV761147			
		4293	nest 2	*Ph. pallidula*	PV761179			
Tunisia: Thala (35.5454°N 8.6838°E)	3	4123	nest 1	*P. favieri*	PV761151			
		4124	nest 1	*P. favieri*	PV761152			
		4125	nest 1	*P. favieri*	PV761153			PV815370
		4393	nest 1	*Ph. pallidula*	PV761197		PV815407	PV815382
	3	4584	nest 3	*P. favieri*	PV761142	PV834282	PV815392	PV815366
		4583	nest 3	*Ph. pallidula*	PV761199	PV834260	PV815408	PV815383
France: Banyuls sur Mer (42.4757°N, 3.1115°E)	4	4553	nest 2	*P. favieri*	PV761155			
		4555	nest 2	*Ph. pallidula*	PV761201			
	4	4554	nest 3	*P. favieri*	PV761156	PV834247		
		4557	nest 3	*Ph. pallidula*	PV761203			
	4	4549	nest 17	*P. favieri*	PV761157	PV834249		
		4558	nest 17	*Ph. pallidula*	PV761205	PV834263		
	4	4551	nest 19	*P. favieri*	PV761158	PV834250	PV815397	PV815371
		4556	nest 19	*Ph. pallidula*	PV761207	PV834264	PV815410	PV815385
	4	4552	nest 1	*P. favieri*	PV761160	PV834283		
		4560	nest 1	*Ph. pallidula*	PV761210	PV834265		
	4	4550	nest 18	*P. favieri*	PV761161	PV834251	PV815398	PV815373
		4559	nest 18	*Ph. pallidula*	PV761212	PV834266	PV815411	PV815386

(to be continued)

Table 1 (Continued).

Locality	Number	DNA	Nest	Taxon	COI	28S	Topo1	WG
Portugal: Grandola (38.1083°N, 8.5686°W)	5	2211	nest 1	*P. favieri*	PV761162	PV834252		
		4587	nest 1	*Ph. pallidula*	PV761214			
	5	2213	nest 2	*P. favieri*	PV761163	PV834279		PV815374
		4588	nest 2	*Ph. pallidula*	PV761216	PV834267	PV815412	PV815387
	5	2215	nest 3	*P. favieri*	PV761164			
		4589	nest 3	*Ph. pallidula*	PV761180	PV834268		
Spain: Tarifa (36.0723°N, 5.5157°W)	6	4540	nest 1	*P. favieri*	PV761166	PV834253	PV815399	PV815375
		4564	nest 1	*Ph. pallidula*	PV761186		PV815405	PV815380
	6	4547	nest 3	*P. favieri*	PV761167	PV834284		
		4568	nest 3	*Ph. pallidula*	PV761188			
	6	4541	nest 12	*P. favieri*	PV761168	PV834254		
		4565	nest 12	*Ph. pallidula*	PV761190			
	6	4546	nest 52	*P. favieri*	PV761169	PV834255		
		4567	nest 52	*Ph. pallidula*	PV761194			
	6	4542	nest 61	*P. favieri*	PV761170	PV834256	PV815400	PV815376
		4566	nest 61	*Ph. pallidula*	PV761196		PV815406	PV815381
Spain: Granada, Loja (37.1671°N, 4.1821°W)	7	4537	nest 1	*P. favieri*	PV761173	PV834280	PV815401	PV815377
		4561	nest 1	*Ph. pallidula*	PV761204	PV834269	PV815409	PV815384
	7	4538	nest 2	*P. favieri*	PV761174	PV834258		
		4562	nest 2	*Ph. pallidula*	PV761208	PV834261		
	7	4539	nest 4	*P. favieri*	PV761175	PV834259		
		4563	nest 4	*Ph. pallidula*	PV761211	PV834262		

Note: Number, locality number on maps in Figs. 2 and 3; DNA, the W. Moore DNA voucher number; Nest, refers to the number temporarily assigned to specific *Ph. pallidula* nests in the field. GenBank accession numbers for gene fragments are provided.

Table 2 Taxon sampling for outgroup and specimens of *P. favieri* collected without their *Ph. pallidula* hosts.

Taxon	Locality	Number	DNA	COI	28S
Paussus biflagellatus	Togo		DRM5426	DRM5426	
P. favieri	Morocco: Al Haouz (30.8729°N 8.3620°W)	1	2039	PV761150	PV834281
	Morocco: Oukaimeden (31.2219°N 7.8288°W)	2	4151	PV761145	
	Morocco: Oukaimeden (31.2219°N 7.8288°W)	2	4152	PV761146	
	Morocco: Oukaimeden (31.2273°N 7.8227°W)	2	4543	PV761148	PV834248
	Morocco: Oukaimeden (31.2288°N 7.8244°W)	2	2154	PV761149	PV834246
	France: Banyuls sur Mer (42.4757°N, 3.1115°E)	4	2002	PV761159	
	Portugal: Grandola (38.1083°N, 8.5686°W)	5	2413	PV761165	
	Spain: Puerto del Saltillo (36.8639°N 5.0530°W)	7	2156	PV761171	PV834285
	Spain: Puerto del Saltillo ((36.8639°N 5.0530°W))	7	2164	PV761172	PV834257
	Spain: Murcia, El Algar (37.6494°N, 0.8671°W)	8	1153	PV761176	KM407206.1
	Spain: Murcia, El Algar (37.6494°N, 0.8671°W)	8	1946	PV761177	
	Spain: Murcia, El Algar (37.6494°N, 0.8671°W)	8	2037	PV761178	PV834286

Note: Number, number on maps in Figs. 2 and 3; DNA, the W. Moore DNA voucher number. GenBank accession numbers for gene fragments are provided.

tion followed by Sanger Sequencing. The DNA extraction was treated with NEBNext FFPE DNA Repair Mix (New England BioLabs) prior to library construction. Libraries were prepared using a NEBNext DNA Ultra II kit (New England BioLabs), following optimization recommendations from Sproul and Maddison (Sproul & Maddison, 2017). Illumina sequencing was performed at the Oregon State University Center for Genomic Research and Biocomputing (OSU CGRB) on a HiSeq 3000 platform. Reads were processed in CLC Genomics Workbench version 9.5.3. Low-quality ends (limit = 0.05), and adapter sequences were removed from the reads. De novo assemblies were generated using Genomics Workbench from paired, trimmed reads using an automatic word and bubble size, with the minimum contig length set to 200. The de novo assemblies were converted to BLASTable databases in Geneious Prime. The COI sequence for *P. biflagellatus* was identified using a COI sequence of *P. favieri* as the query sequence.

Gene selection and data matrices

All sequences for each gene fragment were aligned using MAFFT (Katoh & Standley, 2013) with the L-INS-I iterative refinement method. Aligned matrices were then scanned by eye to detect genetic variability. Only the COI matrices for both *Ph. pallidula* and *P. favieri* contained enough variability to be phylogenetically informative. Therefore, we proceeded with single gene analyses. The GenBank accession numbers for the sequence data generated by this study are provided in Tables 1–3.

Phylogenetic analyses

Maximum-likelihood analyses and bootstrap analyses were conducted using IQ-TREE version 1.6.10 (Nguyen *et al.*, 2015), as orchestrated by the CIPRES Science Gateway (Miller *et al.* 2010). The ModelFinder feature within IQ-TREE was used to find the optimal model

Table 3 Taxon sampling for outgroups and specimens of *Ph. pallidula* collected without association with *P. favieri*.

Taxon	Locality	Number	DNA	COI	28S
Ph. liengmei				KJ141920.1	
Pheidole sp. UG01				KJ141922.1	
Pheidole sp. TZ02				KJ141927.1	
Pheidole sp. KE28				KJ141928.1	
Pheidole sp. KE03				KJ141925.1	
Ph. pallidula	Morocco: Igunane (31.2405°N 7.8099°W)	2	4478	PV761192	
	Morocco: Igunane (31.2405°N 7.8099°W)	2	4480	PV761193	
	Tunisia: Thala (35.5454°N 8.6838°E)	3	4581	PV761213	PV834277
	Tunisia: Thala (35.5454°N 8.6838°E)	3	4582	PV761215	PV834278
	Spain: Soria, Medinaceli (41.1816°N, 2.3990°W)	9	4590	PV761182	
	France: Laroque des Albères (42.5118°N, 2.9358°E)	10	4569	PV761184	
	France: Montpellier (43.5442°N, 3.7924°)	11	4571	PV761187	PV834270
	France: Miramas (43.5739°N, 4.8860°E)	12	4570	PV761189	PV834271
	France: Marseille (43.5883°N, 5.1897°E)	12	4572	PV761191	PV834272
	Italy: Sicily, Filicudi	13		MT606366	
	Italy: Sicily, Filicudi	13		MT606364	
	Italy: Sicily, Floresta (38.0086°N, 14.8823°E)	13	4577	PV761187	
	Italy: Sicily, Milazzo (38.2195 N 15.241 E)	13		MT606363	
	Italy: Sicily, Stomboli	13		MT606365	
	Italy: Sicily, Ucria (38.0431°N, 14.8696°E)	13	4578	PV761209	PV834276
	Italy: Sicily, Vulcano (38.3898 N 14.9609 E)	13		MT606367	
	Italy: Calabria, Civita (39.8227°N, 16.2889°E)	14	4574	PV761202	PV834275
	Italy: Sardinia, Lodè (40.5430°N, 9.6059°E)	15	4586	PV761198	
	Italy: Sardinia, Ollolai (40.1609°N, 9.1313°E)	15	4576	PV761195	PV834273
	Italy: Abruzzo (42.5656°N, 13.9616°E)	16	4573	PV761200	PV834274
	Italy: Riomaggiore (44.09899°N 9.7382°E)	17		KJ141904	
	Locality not reported in GenBank			EF518381	

Note: Number, number on maps in Figs. 2 and 3; DNA, the W. Moore DNA voucher number. GenBank accession numbers for gene fragments are provided.

of character evolution (Kalyaanamoorthy *et al.*, 2017). Searches for maximum-likelihood tree and bootstrap analyses were both conducted with 1000 replicates.

Co-phylogenetic analyses

To evaluate different evolutionary scenarios that may have led to the degree of specialization shown by *P. favieri* and *Ph. pallidula*, we used both distance-based and event-based co-phylogenetic methods. Distance-based methods explore the overall topological similarity between host and parasite phylogenies. If phylogenies are more similar to each other than expected by chance, this can be interpreted as a co-phylogenetic signal (Legendre *et al.*, 2002; Hutchinson *et al.*, 2017; Perez-Lamarque & Morlon, 2024). Event-based methods, on the other hand, evaluate different processes like co-divergence, duplication (i.e. divergence in the parasite that is not followed by divergence in the host, or vice versa), duplication with lineage switch, loss, and failure to diverge to reconcile similarities and incongruences between host and parasite phylogenies (Conow *et al.*, 2010; De Vienne *et al.*, 2013).

For co-phylogenetic analyses, matrices were trimmed to include only those sequences obtained from *Ph. pallidula* and *P. favieri* specimens that were collected from the same nest (Table 1, sites 1−7 in Figs. 2C and 3C).

PACo (Hutchinson *et al.*, 2017), a distance-based method, was used as implemented in R-package ape (Paradis *et al.*, 2004). The input for PACo consists of two patristic distance matrices calculated from the ML trees and a matrix of the association between the two. In PACo, patristic distance matrices are transformed into Principal Coordinates matrices. We used a Cailliez correction method for this transformation to avoid negative eigenvalues. Extended Principal Coordinates matrices are then calculated based on the order of the association matrix. Finally, Procrustes analysis was used to infer the best-fit superimposition between the extended Principal Coordinates matrices and calculate the residual sum of squares ($m^2 \times y$) as a measure of global-fit between host and parasite phylogenies (Balbuena *et al.*, 2013). The significance of the fit was inferred via 1000 permutations. Since it is unclear if and how *Ph. pallidula* is affected by *P. favieri*, we used the "r0" algorithm to explicitly test the dependence of the nest parasite phylogeny upon the host phylogeny.

Once overall similarity was found, PACo was used to assess the degree to which each host–parasite link contributed to the overall similarity between host and parasite phylogenies. We used a jackknife approach in PACo to estimate the contribution of each individual host–parasite association to the global fit in the form of squared residual values. Host–parasite associations with low squared residuals are those that contribute the most, and they can be interpreted as populations undergoing co-divergence.

A tanglegram was built in Dendroscope (Huson *et al.*, 2007) to visually compare host and parasite phylogenies. The tanglegram also depicts the jackknife squared residual values, to show the contribution of each host–parasite association to the global fit as calculated by PACo.

Event-based methods were employed to infer the possible evolutionary scenarios that led to congruence and incongruence between host and nest parasite phylogenies. Jane 4.0 (Conow *et al.*, 2010) was used to reconcile the parasite tree on the host tree by minimizing costs. Possible evolutionary events used to assess congruence and incongruence included: co-divergence, duplication, duplication with lineage switch, loss, and failure to diverge. We conducted our analyses with two scheme costs. We used the default scheme costs (0 co-divergence, 1 duplication, 2 duplication and lineage switch, 1 loss, 1 failure to diverge) and an alternative with cost of 1 for duplication and host switch, given the likelihood of this event as shown in many previously published studies (De Vienne *et al.*, 2013). The parameters of the genetic algorithm were left as default settings (100 population size, 100 number of generations). The significance of the retrieved total cost was tested via 1000 permutations using the random tip mapping method.

Results

The maximum likelihood (ML) tree indicates *Ph. pallidula* dispersed from ancestral populations in Morocco and/or Italy to Tunisia and mainland Europe on several occasions (Fig. 2B). ML trees of *P. favieri* populations also suggest that Moroccan lineages represent ancestral populations and that *P. favieri* dispersed from there to Tunisia and mainland Europe once (Fig. 3B). When non-parasitized populations of *Ph. pallidula* are removed from the analyses, the phylogeographic structure of parasitized populations of *Ph. pallidula* appeared to be very similar to that of *P. favieri* (Figs. 4−6).

Although not variable enough to be phylogenetically informative, *wingless* supported the inferred phylogeographic structure observed for *P. favieri*. Mutations at two nucleotide positions differentiate Moroccan sequences and all other sequences, one of which led to an amino acid change. An additional pdf file of this region of *wingless* alignment is provided (Supporting information 2).

PACo detected significant co-phylogenetic signal between *Ph. pallidula* and *P. favieri* populations ($m^2 \times y$:

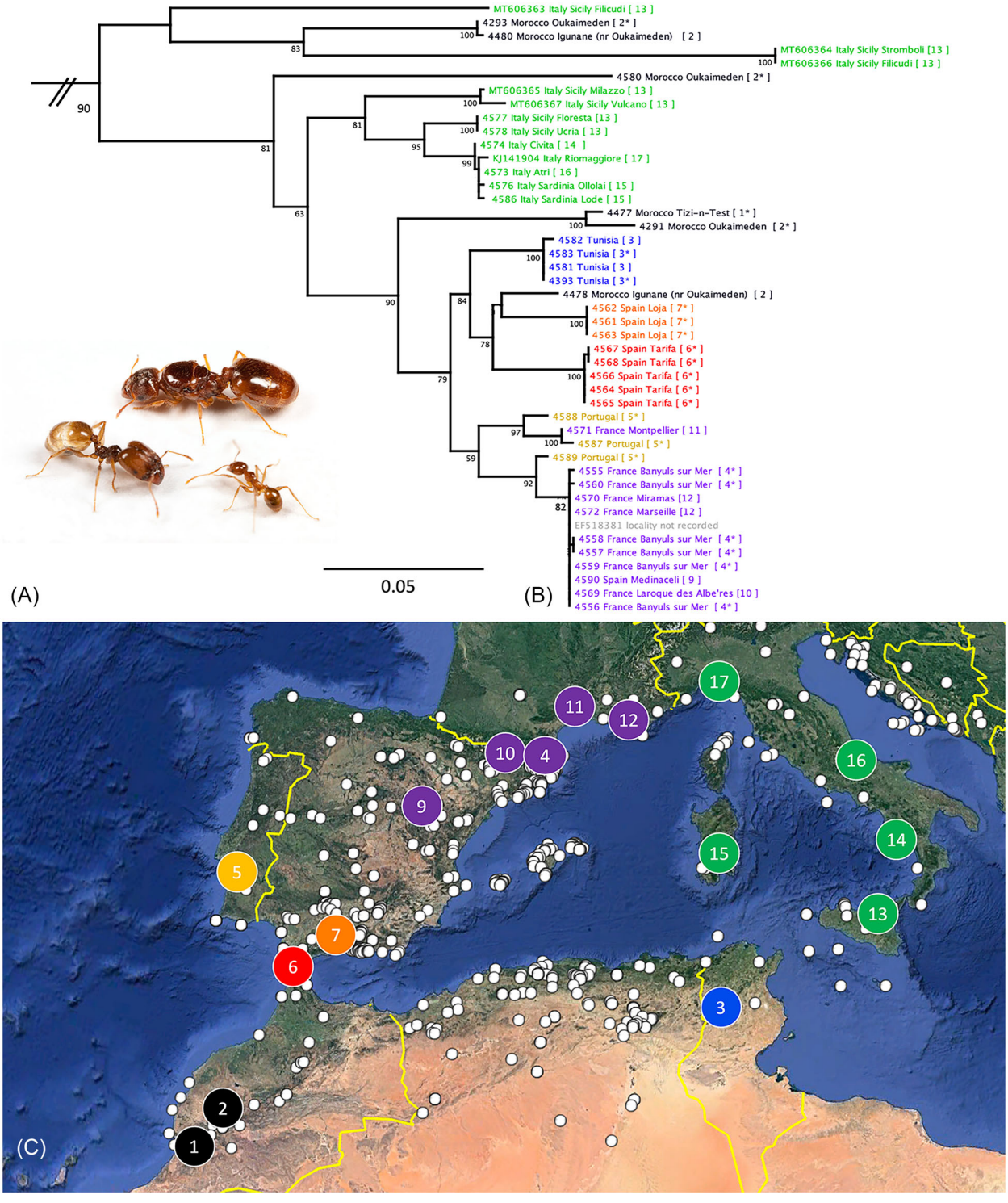

Fig. 2 Lateral views (A), maximum likelihood tree (B), and distribution map (C) for *Pheidole pallidula* (host). Numbered and colored waypoints on the map correspond to sampling locations of specimens in the phylogeny. Specimens collected with *Paussus favieri* (parasite) were included in our co-divergence analysis and are indicated on the tree with an asterisk after the locality number. White dots indicate locations where *Ph. pallidula* occurs in the region depicted on the map. The actual distribution of the species extends slightly to the north and to the east. Branch lengths are shown proportional to relative divergence, as estimated by IQTree. Outgroup taxa have been pruned from the tree. Numbers below branches in the phylogeny are bootstrap support values. Photo credit: Hugo Darras.

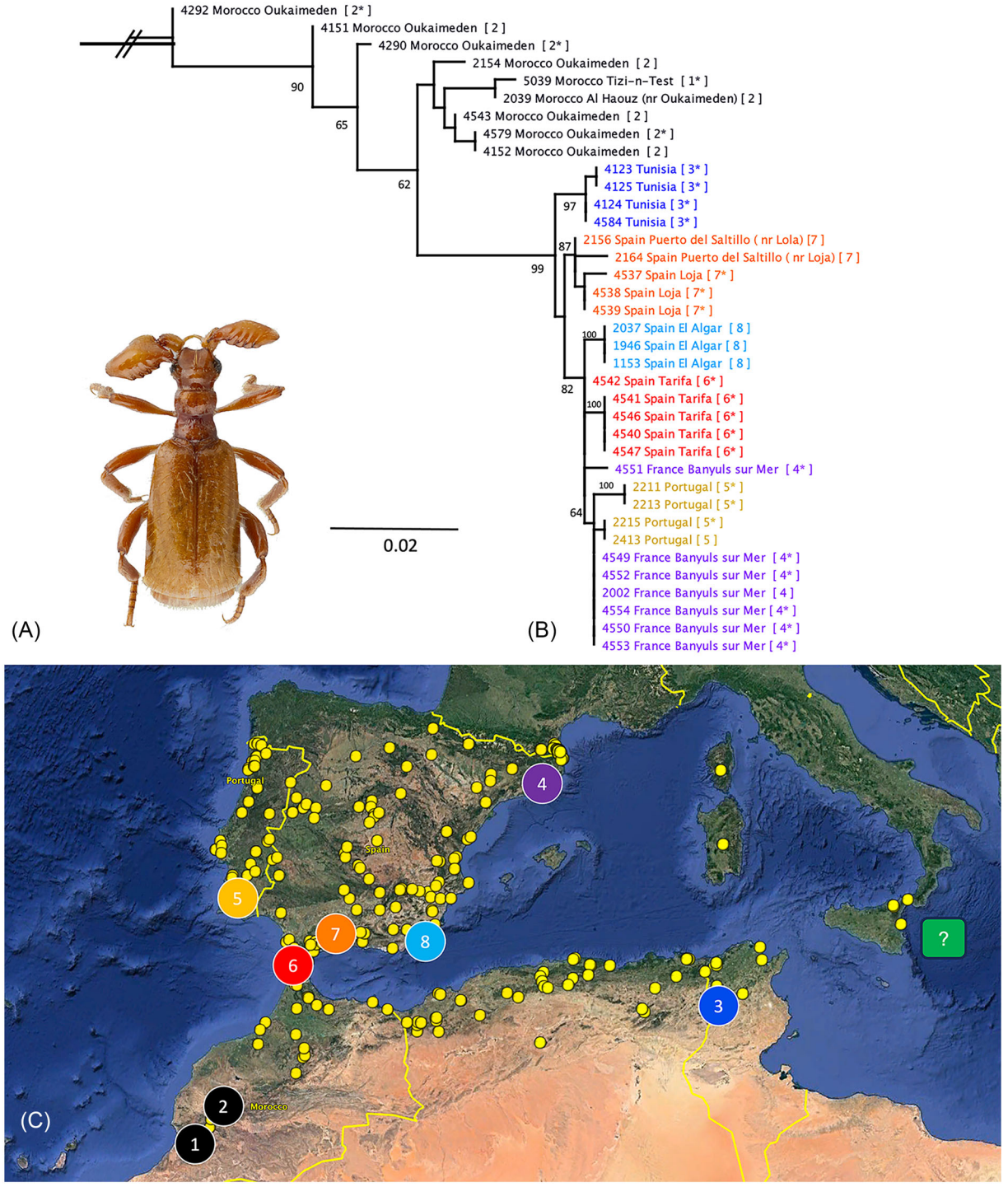

Fig. 3 Dorsal view (A), maximum likelihood tree (B), and distribution map (C) for *Paussus favieri*. Numbered and colored circular waypoints on the map correspond to sampling locations of specimens in the phylogeny. Specimens collected with *Pheidole pallidula* were included in co-divergence analyses and are indicated on the tree with an asterisk after the locality number. Yellow dots indicate all locations where *P. favieri* are known to occur. Branch lengths are shown proportional to relative divergence, as estimated by IQTree. Outgroup taxa have been pruned from the tree. Numbers below branches in the phylogeny are bootstrap support values. Photo credit: James Robertson.

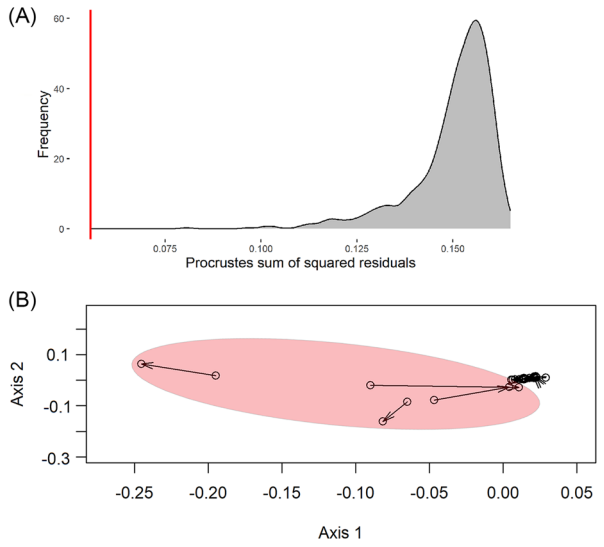

Fig. 4 Results of PACo analyses support congruence between the *Ph. pallidula* and *P. favieri* phylogenies. (A) In this density plot, the grey area represents the null distribution of the Procrustes sum of squared residuals values generated through 1000 random permutations of the association matrix. The observed value (red line) is much lower than any of the permutation values ($P = 0.001$), supporting a degree of congruence between the *Ph. pallidula* and *P. favieri* phylogenies higher than expected by chance. (B) Procrustean superimposition plot of *Ph. pallidula* and *P. favieri*. The dots at the base of the arrows represent Principal Correspondence Coordinates of patristic distances of the beetle. Their configuration has been rotated and scaled to fit that of the ants (dots pointed by the arrow tips). The length of the arrow approximates the residual of each ant-beetle link. The links that have contributed less to the global fit between host and parasite are those with longer arrows. In this case, these are highlighted by the pink circle which contain all four Moroccan associations.

0.056, *P*-value = 0.001). When examining the contribution of each host–parasite association to the measure of global fit, most showed low squared residuals indicate a possible co-divergence scenario for some of these associations. Moroccan host–parasite associations were those with the highest jackknife squared residuals and contributed least to the measure of global fit (Figs. 4−6) (Supporting information 3).

When host–parasite phylogenies were reconciled, four equally parsimonious non-isomorphic solutions were found using default scheme costs (see Supporting information 4 for a representation of the most common reconciliation found in this analysis). The total cost of these solutions was 30 and included 10−11 co-divergence events. The remaining events inferred were one duplication, 12−13 duplications and lineage switches, and three losses. Re-running the analysis using a scheme with a lower cost for duplication and lineage switches (0 co-divergence, 1 duplication, 1 duplication and lineage switch, 1 loss, 1 failure to diverge) resulted in twelve equally parsimonious non-isomorphic solutions, each with total cost 17 and between 7−10 co-divergence events. The number of alternative events also varied for duplications and lineage switches (14−16) and losses (0−3), while only one duplication event was inferred for all of them (Supporting information 5). Final costs, using the default and alternative scheme costs, were significantly lower than those generated through random tip mappings ($P = 0.001$).

Discussion

This study suggests that *P. favieri* first became established in northern Africa and subsequently dispersed from there to Europe (Fig. 2B, B). The phylogeny of *Ph. pallidula* populations suggests multiple dispersal events between northern Africa and Europe (Fig. 2B), with some Moroccan populations more closely related to Italian populations and other Moroccan populations more closely related to Iberian, French, and Tunisian populations (Fig. 2B, C). In contrast, *P. favieri* dispersed from Morocco to Iberian, French, and Tunisian areas only once (Fig. 3B).

These dissimilarities between phylogenies lessen when considering only those *Ph. pallidula* populations that were parasitized by *P. favieri* (Table 1). Except for Tunisian populations, a similar phylogeographic pattern was inferred for *Ph. pallidula* and *P. favieri* phylogenies (Fig. 6). This similarity is also found in our co-phylogenetic analyses. All analyses conducted in PACo recovered significant overall similarity between ant and beetle phylogenies, suggesting co-phylogenetic signal (Figs. 4−6). This scenario is also supported by the low squared residual values of most host–parasite links as inferred by PACo (Fig. 5). Such co-phylogenetic signal without phylogenetic congruence is not unusual, as random processes like incomplete lineage sorting might result in dissimilarities between phylogenies of hosts and their symbionts, even in stricter symbiotic associations (e.g., Symula *et al.*, 2011; Garrick *et al.*, 2017). Alternatively, cophylogenetic signal might arise from conserved traits or biogeographic events, even if host and parasite diversification are not linked (Perez-Lamarque & Morlon, 2024). Similarly, event-based methods revealed several co-divergence events, depending on the cost scheme selected for this analysis. Co-divergence events were found at both shallow and deep levels of the trees, sug-

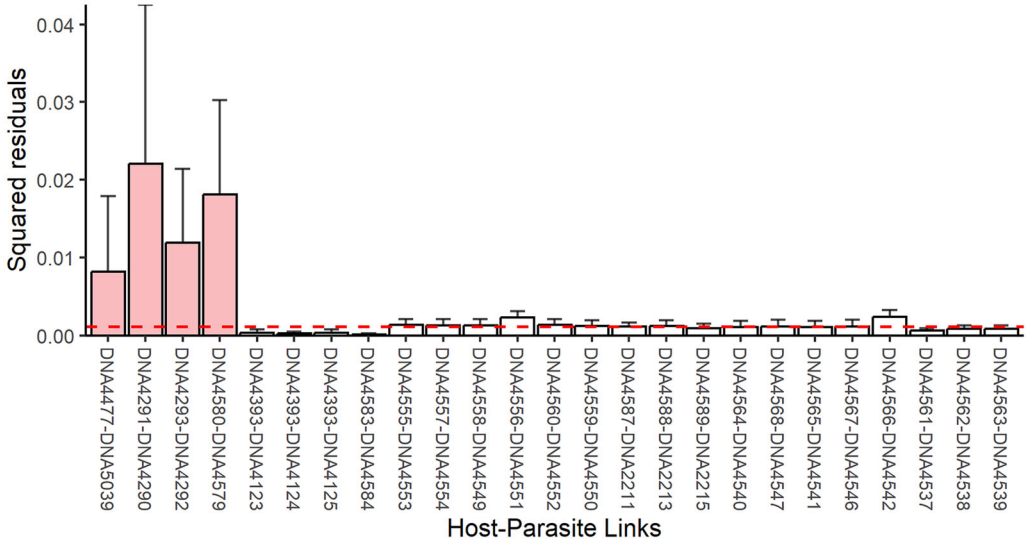

Fig. 5 Contribution of each ant-beetle link to the global fit between *Ph. pallidula* (first DNA number) and *P. favieri* (second DNA number). The squared residual contribution of each link, with upper 95% confidence intervals, was calculated using a jackknife approach in PACo. The dashed line represents the median squared residual. Low squared residuals can be interpreted as possible co-evolutionary links. The four links showing the highest squared residuals are associations among Moroccan taxa (pink bars).

gesting that the beetle and ant populations regularly co-diverge. However, some topological discordances also exist which are most likely explained by lineage switching events ("duplication and lineage switching events" in event-based methods). Since the number of host switching events is greater than the number of codivergence events, our study provides evidence of cophylogenetic signal, without phylogenetic congruence, following the framework of Perez-Lamarque & Morlon (2024).

However, in most reconstructions found by the event-based method with the default scheme costs, most lineage switches occurred between nearby populations of *Ph. pallidula* and at a shallow phylogenetic scale in areas of the trees with low bootstrap support (Supporting information 4). These reconstructions suggest *P. favieri* most commonly moves among closely related ant populations in the same area, a process known as preferential host switching. Although rarely found outside of ant nests, *P. favieri* can move between colonies and/or follow the queen and workers during colony budding using the chemical trails of its host ant (Cammaerts & Cammaerts, 1989, 1992; Cammaerts *et al.*, 1990). Given the poor dispersal capabilities of *P. favieri*, shifts between distant localities may be explained by the greater dispersal capabilities of the host ant. For example, one parasitized population of *Ph. pallidula* may be replaced over time by new lineages dispersing from distant localities. In this case, a lineage shift may result from *P. favieri* persisting within that population by parasitizing the "new" incoming lineage. Preliminary behavioral experiments revealed that Moroccan *P. favieri* are easily accepted by European colonies of *Ph. pallidula* (Di Giulio, personal observation) which supports the possibility of lineage switches in this host–parasite system. Wider distributions of the hosts, relative to those of their parasites, have also been found to be one of the main causes of discordance in another co-phylogenetic study of ants and their nest parasites (*Myrmica* ants and *Maculinea* butterflies) (Jansen *et al.*, 2011).

It is also possible for co-phylogenetic signal to arise simply from a shared biogeographic history rather than co-divergence (De Vienne *et al.*, 2013; Martínez-Aquino, 2016). Given that *P. favieri* requires established ant colonies to survive, the parasite cannot become established in a new location before its host. Although we accept that similarities in the phylogenies indicate that the beetles and the ants are following the same dispersal corridors, this does not necessarily imply that their dispersal occurred simultaneously. It is also possible that present-day distributions may be relicts of once much larger panmictic populations that were subsequently fractured by the same series of vicariant events over time.

Several well-documented geo-climatic events are known to have greatly influenced the evolutionary history and distribution of species in the Mediterranean Basin. For example, during the Messinian Salinity Crisis (5.96 to 5.33 Ma), closure and desiccation of the Mediterranean Basin led to faunal exchanges between North Africa and

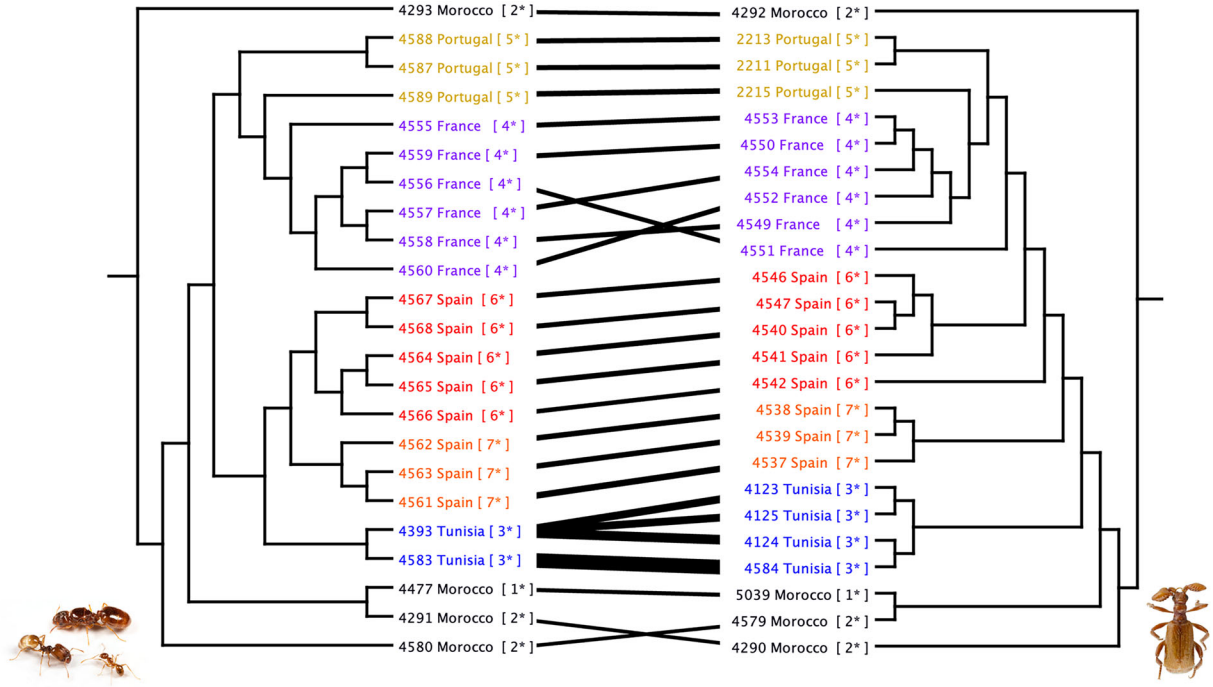

Fig. 6 Tanglegram depicting associations between *Ph. pallidula* (left) and *P. favieri* (right). Line thickness is proportional to the relative contribution each association makes to the congruence between the phylogenies, as inferred in PACo. Tunisian sequences are those that contribute the most to the overall congruence.

Europe through the Strait of Gibraltar and the Strait of Sicily (Husemann *et al.*, 2014; Achalhi *et al.*, 2016), and the subsequent refilling of the Mediterranean Sea led to isolation and differentiation of European and north African populations for several taxa (e.g., Kindler *et al.*, 2018; Todisco *et al.*, 2018; Trájer *et al.*, 2021). However, based on dated species-level phylogenies that include the ant (Economo *et al.*, 2014) and the beetle (Moore & Robertson, 2014), this event is too old to have influenced the distributions of *P. favieri* and *Ph. pallidula* populations.

Pleistocene glacial cycles might be evoked as more plausible events to have influenced the population-level structure and distribution of the species in our study. During glacial phases, *Ph. pallidula* and *P. favieri* may have found refuge in different areas of southern Europe and northern Africa. Then, their populations may have expanded to new regions during post-glacial cycles as has been found for other Mediterranean species (Hewitt, 2004; Schmitt, 2007; Mas-Peinado *et al.*, 2018). Our phylogeographic reconstructions are consistent with the refugial role of Maghreb (Morocco and Tunisia), the Iberian Peninsula, the Italian peninsula and major islands for several organisms (Fattorini & Ulrich, 2012; Sousa *et al.*, 2012; Dapporto *et al.*, 2014; Veríssimo *et al.*, 2016). In line with the refugia-within-refugia hypothesis, the Italian and the Iberian peninsulas may have harbored multiple refugia. As inferred for a wide variety of taxa including amphibians, reptiles, plants, and insects (Gómez & Lunt, 2006), the Betic Ranges probably played an important role in the survival and differentiation of *Ph. pallidula* and *P. favieri* populations in the Iberian Peninsula. Populations from this region form four different subclades of *P. favieri* (Tarifa, Loja, Puerto del Saltillo, and El Algar) (Fig. 3B, C), two of which are shared with *Ph. pallidula* (Fig. 2B, C). Short branch lengths separating the remaining European populations may suggest a northward expansion from the Betic Ranges (Fig. 3B, C). However, taxon sampling of *Ph. pallidula* populations from Portugal, central Spain, and France suggests the presence of a nearby coastal Atlantic region where both species may have survived glacial phases. The refugia-within-refugia hypothesis cannot be excluded for Morocco: the geographic variability of the High Atlas Mountains might have contributed to further habitat fragmentation and lineage divergence during glacial cycles, therefore influencing the high number of lineages found in this study (e.g., Habel *et al.*, 2012).

While the phylogeographic structure of parasitized populations of *Ph. pallidula* is similar to that of *P. favieri*,

the pattern becomes more complicated when sequences from host populations that are not parasitized are included in the analyses. Moroccan populations are recovered as polyphyletic and scattered throughout the phylogeny, suggesting multiple colonization events that may have occurred between Africa and Europe in one or both directions. Again, this would support a higher dispersal capability of *Ph. pallidula* compared to *P. favieri*. Alternatively, given the occurrence of this species even in urban environments and its invasive potential (Seifert, 2016), we cannot exclude the possibility that humans may have introduced *Ph. pallidula* to some locations, influencing its current distribution and phylogeographic signal.

Finally, *P. favieri* is also known from Sicily (number 13 on map, Fig. 2C) and Sardinia (see number 15 on map, Fig. 2C). However, its occurrence on these islands is only documented by museum specimens collected over 100 years ago. Despite multiple recent expeditions to both islands dedicated to recollecting this species, we found none. If *P. favieri* still exists on these islands, its inclusion in future studies would help disentangle the relative importance of co-divergence and lineage shifts in this system. For example, if *P. favieri* from Italy would attach to the tree of *P. favieri* within the Moroccan grade (Fig. 3B), this would bring further support to a co-divergence scenario, as Italian populations would therefore share the same biogeographical history as their host (Fig. 2B).

In this paper, using a molecular approach, we have unveiled the evolutionary history of the ant *Ph. pallidula* and the ant-nest beetle *P. favieri*. When considering all *Ph. pallidula*, idiosyncratic relationships emerged. Our results suggest that *Ph. pallidula* colonized Europe several times, but *P. favieri* did so only once. When only the populations of *Ph. pallidula* parasitized by *P. favieri* were considered, similar phylogeographic patterns were recovered: both originated in Northern Africa and subsequently colonized Europe. In line with these findings, significant co-phylogenetic signal was found. However, our results do not support a univocal co-phylogenetic scenario driven by co-divergence alone. Rather, our findings suggest that other processes like a shared biogeographic scenario might have shaped the similarity between *P. favieri* and *Ph. pallidula* evolutionary histories.

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Disclosure

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supporting information 1 Primer sequences and PCR cycling conditions.

Supporting information 2 The only variable portion of the *Paussus favieri wingless*.

Supporting information 3 The jackknife squared residual contribution of each host–parasite link with upper 95% confidence.

Supporting information 4 One evolutionary scenario proposed by Jane 4.

Supporting information 5 Most parsimonious solutions found by Jane 4.