



## Household and Structural Insects

# Molecular identification of the invasive subterranean termite *Reticulitermes grassei* (Blattodea: Rhinotermitidae) outside its known distribution: introduction routes and implications for pest management strategies

David Hernández-Teixidor<sup>1,\*</sup>, Sónia Duarte<sup>2,3</sup>, Ahmed Taheri<sup>3,4</sup>, Paulo A.V. Borges<sup>4,5</sup>, Lina Nunes<sup>2,4,6</sup>

<sup>1</sup>Island Ecology and Evolution Research Group, Instituto de Productos Naturales y Agrobiología (IPNA-CSIC), La Laguna 38206, Canary Islands, Spain, <sup>2</sup>Structures Department, LNEC, National Laboratory for Civil Engineering, Av. do Brasil, 101, Lisbon 1700-066, Portugal, <sup>3</sup>Laboratory of Plant Biotechnology, Ecology and Ecosystem, Faculty of Sciences of El Jadida, Chouaib Doukkali University, P.O. Box 20, El Jadida 24000, Morocco, <sup>4</sup>cE3c—Centre for Ecology, Evolution and Environmental Changes/Azorean Biodiversity Group/CHANGE—Global Change and Sustainability Institute, University of Azores, School of Agricultural and Environmental Sciences (FCAA), Rua Capitão João d'Ávila, Pico da Urze, Angra do Heroísmo 9700-042, Azores, Portugal  
\*Corresponding author, mail: [davidhdez@ipna.csic.es](mailto:davidhdez@ipna.csic.es)

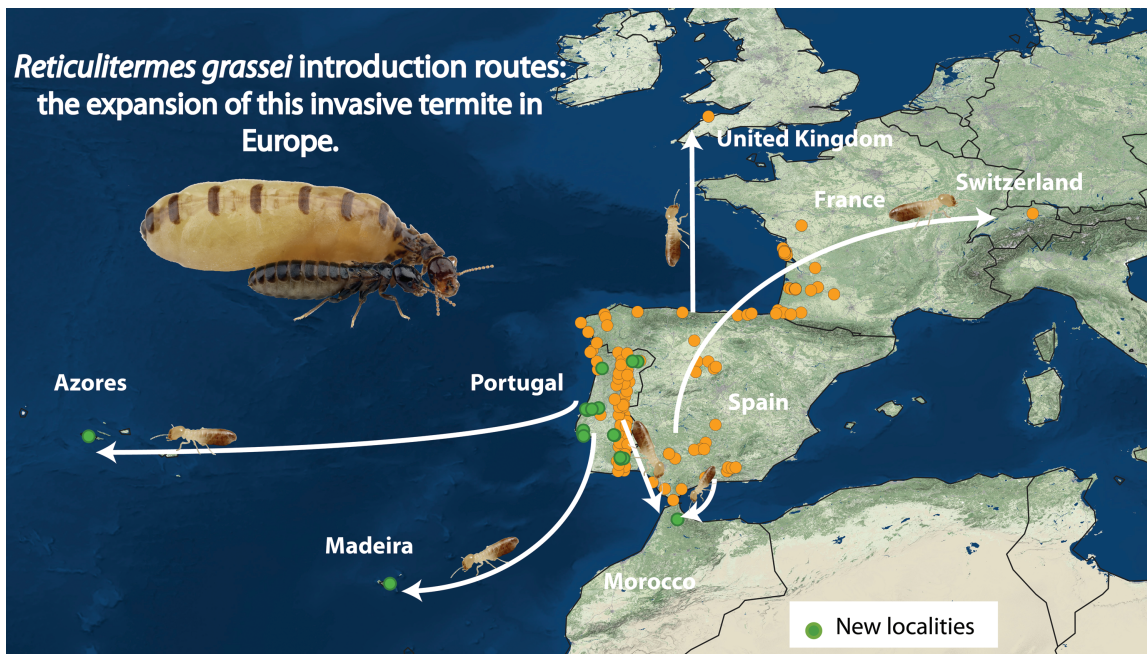
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Despite providing important ecosystem services, termites are also serious pests of wooden structures. Termites are highly adaptive organisms that cause concern as an invasive species. Predictions of the future spread of their distribution range due to factors such as climate change, urban growth, and global trade present new challenges to our capacity to protect our wood and wood-based materials and structures effectively. *Reticulitermes grassei* Clément, 1978 (Blattodea: Rhinotermitidae) is a subterranean termite native to the Iberian Peninsula and France, whose global distribution has widened over recent years. This article updates the distribution range of this species, confirming its identification in the Azores, Madeira, and Morocco through molecular analysis. The origin and consequences of these putative invasive populations are discussed in light of previously available data. The resulting network showed a highly structured base consisting of many haplotypes from the southern and southwestern Iberian Peninsula (Spain and Portugal), including those from Morocco (in natural landscapes) and Switzerland (in infrastructures). The more derived part of the network includes the haplotypes from southwest France, the northwest Iberian Peninsula, the United Kingdom, Azores, and Madeira, the last 3 being linked probably to human-mediated transportation events. The potential impacts of invasive subterranean termite populations expanding into new regions are concerning, especially in urban environments, and remain uncertain in natural areas. The challenges posed by these termites could be especially worrying in island ecosystems. Hence, it is crucial to implement early warning systems and monitoring programs in regions susceptible to subterranean termite invasions.

**Key words:** biological invasion, genetic identification, integrated pest management, invasive species, islands

## Graphical Abstract



## Introduction

Termites are recognized for their roles in a wide range of habitats, as ecosystem engineers, and contributing to carbon turnover or soil fertility. However, within the built environment, some species are considered severe pests of wooden structures and have detrimental effects on agricultural and forestry practices (Jouquet et al. 2011, Govorushko 2019, Chen et al. 2023). One of the implications of global warming is an increase in the number of termite species becoming invaders and an expansion of the distribution range (Buczowski and Bertelsmeier 2017, Evans 2021). There have been projections of climate change scenarios, attempting to predict how species will increase their potential range size in the future. Thus, Buczowski and Bertelsmeier (2017) calculated that *Cryptotermes brevis* (Walker, 1853) (Kalotermitidae) is likely to see an increase of 7.5% in its distribution range, *Reticulitermes flavipes* Kollar, 1837 (Rhinotermitidae) potentially 16.7%, and *Reticulitermes grassei* Clément, 1978 (Rhinotermitidae) 25.0%. Additional factors like global trade and continuous urban growth favor the establishment and expansion of termite populations worldwide. The invasive success of any termite species depends on 3 main characteristics, namely their ability to (i) eat wood, (ii) nest in wood and cultivated plants, and (iii) easily generate secondary reproductives (Evans et al. 2013).

Invasive insects in the suborder Isoptera mainly belong to the subterranean termite family (Evans et al. 2013). Their invasions are particularly challenging to detect due to their cryptic habits (e.g., Nobre et al. 2006, Evans et al. 2013). The *Reticulitermes* Holmgren genus has a Holarctic distribution, with several species occurring naturally in the Mediterranean area. Termite damage in these areas has been increasing, and there has been concern over expansion and new introductions of various termite species and subsequent damage, above all, in areas north of their natural range. These dispersal and regional expansion cases are probably aided by changing climatic conditions.

Some subterranean termites might become successful invasive organisms, as some of them showed the ability to overcome the genetic bottleneck effect after their introduction and establishment in

a new region through admixture among termites from previous colonization events. For example, it has been observed that several new termite population arrivals increased the genetic diversity pool in the new population, enhancing the invasion success of the species *R. flavipes* (Eyer et al. 2021).

There are 3 native termite species present in the Iberian Peninsula: the subterranean termites *R. grassei* and *R. banyulensis*, both having a wide distribution in natural and urban environments and are recognized as pests affecting wooden structures (Kutnik et al. 2004, Nunes 2008, Sequeira et al. 2022); and *Kalotermites flavicollis* (Fabricius, 1793) (Kalotermitidae), a species more commonly associated with their natural habitat than as a pest of applied wood (Nunes 2008). Recently, the invasive species *C. brevis* was identified in continental Spain and Portugal (Nunes et al. 2010). It is considered a major pest in the Azores (Guerreiro et al. 2014) and is globally recognized for attacking wooden structures. On Madeira island, 2 kalotermitid species have been identified: *C. brevis* (Mateus and Goes 1953) and *Postelectrotermes praecox* (Hagen, 1858) (Carvalho 1979). In the Azores archipelago, 4 species of termites were identified (Borges et al. 2014): *C. brevis*, *R. flavipes*, *R. grassei*, and *K. flavicollis*. To date, *K. flavicollis* has only been observed in trees and natural habitats. The other 3 are associated with urban and man-made wooden structures, recognized as voracious pests of wood and wooden products.

*Reticulitermes grassei* originates from southwestern Europe (Iberian Peninsula and France), though there have been cases of infestation in Devon (United Kingdom) and Horta (Faial, Azores) (Jenkins et al. 2001, Ferreira et al. 2013), while there has been a recent case of infestation identified in the canton of Zürich, Switzerland, a country with no native termites (Ghesini et al. 2020). This termite colony's breeding structure ranges from simple family colonies (one reproductive pair) to extended-family colonies (with multiple reproductives) (DeHeer et al. 2005, Nobre et al. 2008, Vargo et al. 2013). *Reticulitermes grassei* is the termite most commonly found in the Iberian Peninsula, in both its natural and urban range. Together with *R. banyulensis* in Spain, it is responsible for most of the insect

damage to wooden structures (Nunes 2008). The damage caused in the town of Horta (Azores) by this termite has already attracted the authorities' attention, resulting in a control program using in-ground termite monitoring stations established across the city (Borges et al. 2016).

The aim of this work was to confirm the presence of *R. grassei* on the island of Faial in Azores, the island of Madeira (both part of Portugal), and Morocco and make a preliminary comparison with all available mtDNA sequences of this species within its known natural or invasive ranges. Fifteen extra samples were included from locations in continental Portugal, 2 samples from Faial Island (Azores), 2 from the island of Madeira, and 3 from Morocco. These 22 samples expand the data available for discussing the introduction routes to new areas and possible implications for pest management strategies.

## Materials and Methods

Subterranean termites were collected in 2 locations on Faial in the Azores, 1 on Madeira, 2 in Morocco, and also 15 in mainland Portugal (Fig. 1; Table 1; see Supplementary Table S1 for information on habitat type) and kept in 96%–100% ethanol. The specimens were previously identified based on physiological and morphological traits at the genus level. The use of morphological traits may pose difficulties in distinguishing between different species of the genus *Reticulitermes* in Europe due to their proximity. Therefore, molecular analysis was needed to confirm the identity of the specimens at the species level. DNA from Azores and Portugal specimens was extracted from 5 termite heads per site using a DNA extraction kit (Qiagen). A fragment (ca. 530 bp) of the mitochondrial cytochrome c oxidase subunit II (COII) gene was amplified according to a previously established protocol with the same forward (5'-TAA GAC TAC AAG ACG GAG CA-3') and reverse primers (5'-AAG TGA TTT GCC GAT ACT CT-3') (Nobre et al. 2006). PCR products were

purified with SureClean (Bioline) and sequenced by MacroGen Inc. (<http://www.macrogen.com>). One amplicon from each location was sequenced on both strands and assembled into sets of overlapping DNA segments that together represent a consensus region of DNA (contigs) using SEQUENCHER 4.2 (GeneCodes, Ann Arbor, MI, USA).

Later, DNA was extracted from Madeira and Morocco specimens (one individual from each) using a Chelex protocol (Casquet et al. 2012). The COII was amplified using TL-J-3037(TED)/TK-N-3785 (EVA) primers (Simon et al. 1994, Gómez-Zurita et al., 2000). The differences in the primers used do not compromise the overlapping of the sequenced regions of the COII of *R. grassei* from different locations, which was of 501 bp. These sequences were MAFFT-aligned with sequences of *R. grassei* from Portugal, Spain, France, the United Kingdom, and Switzerland hosted in GenBank, using the program GENEIOUS 8.0.5. A matrix of 170 *R. grassei* sequences plus a sequence of *R. flavipes* from Genbank, used as an outgroup, was analyzed (see Supplementary Table S2). To unravel the relationships between different populations, a median-joining haplotype network (Bandelt et al. 1999) was calculated with PopART 1.7, keeping parameter  $e = 0$ , starting with minimum spanning trees combined within a single network, and then adding median vectors (consensus sequences) to reduce tree length. The similarity of the haplotypes in the new areas to those in the rest of the previously known distribution was calculated based on the minimum number of DNA sequence changes in the network. In addition, a reduced matrix with 50 *R. grassei* sequences, one for each haplotype, was used for phylogenetic analysis. Maximum likelihood phylogenetic searches were performed through a web server for IQ-TREE (W-IQ-TREE), with an automatic identification of the best-fit substitution model (Trifinopoulos et al. 2016). Finally, a phylogeographic analysis was performed to visualize genetic variation over space. We used PGT software version 1.0.0 (Xia 2019).

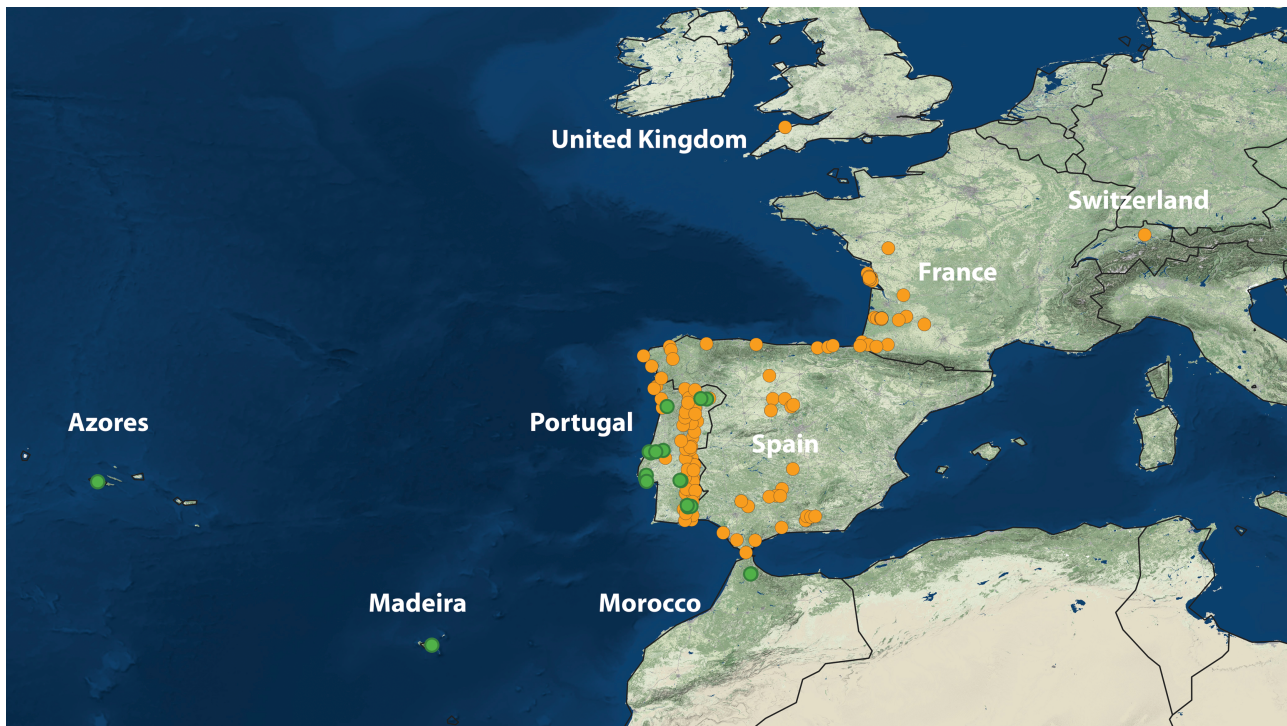


Fig. 1. Geographical distribution of all molecularly identified subterranean termite *Reticulitermes grassei* colonies. Known localities are shown in orange, and new ones, which were contributed by this study, are in green.

**Table 1.** Location of *Reticulitermes grassei* samples of workers collected and sequenced within this study in continental Portugal ( $n = 15$  locations), Azores ( $n = 2$  locations), Madeira ( $n = 1$  location), and Morocco ( $n = 2$  locations)

Country	Region	Location	Latitude	Longitude
Portugal	Azores	Faial island, Horta	38.528506°	-28.627731°
Portugal	Azores	Faial island, Horta	38.528025°	-28.628456°
Portugal	Continental Portugal	Leiria, Alcobaça	39.550336°	-8.978164°
Portugal	Continental Portugal	Setúbal, Almada	38.659253°	-9.168069°
Portugal	Continental Portugal	Porto, Baltar	41.200414°	-8.402031°
Portugal	Continental Portugal	Bragança, Bornes	41.464808°	-6.997286°
Portugal	Continental Portugal	Évora, city	38.567316°	-7.909528°
Portugal	Continental Portugal	Évora, forest	38.578524°	-7.943678°
Portugal	Continental Portugal	Lisbon	38.761094°	-9.141178°
Portugal	Continental Portugal	Beja, Mértola	37.636375°	-7.665686°
Portugal	Continental Portugal	Bragança, Mirandela	41.480161°	-7.201867°
Portugal	Continental Portugal	Beja, Moreanes	37.655944°	-7.553328°
Portugal	Continental Portugal	Leiria, Nazaré	39.591694°	-9.038058°
Portugal	Continental Portugal	Santarém, Ourém	39.640806°	-8.540944°
Portugal	Continental Portugal	Santarém, Porto de Mós	39.592806°	-8.803667°
Portugal	Continental Portugal	Beja, Pulo do Lobo	37.689886°	-7.686953°
Portugal	Continental Portugal	Setúbal, Sesimbra	38.542500°	-9.129250°
Portugal	Madeira	Madeira island, Ponta de S. Lourenço	32.726472°	-16.757979°
Morocco	Morocco	Bouhachem Natural Park, rich forest	35.259444°	-5.421667°
Morocco	Morocco	Bouhachem Natural Park, mountain	35.252395°	-5.435291°

## Results

A 501 bp fragment was obtained from the sequenced specimens and included in a matrix representing 170 individuals of *R. grassei* across all known countries within its distribution (Fig. 1). Molecular identification confirmed that the species present on the islands Faial (Azores) and Madeira, previously identified from morphological characters, is *R. grassei*, and its presence has also been confirmed in Morocco, as Lefebvre et al. (2016) first suggested. Sequences from Madeira were 100% identical to those belonging to *R. grassei* from the central and southern Iberian Peninsula (JQ431004, AY510591, AY954407). Similarly, sequences from the Azores were found to be 99.8% identical to the sequence JQ431014, belonging to the same species from northeast Spain. All sequenced specimens from Madeira and the Azores were genetically identical within each archipelago. However, the specimens from Morocco, specifically the 3 sampled colonies from 2 locations (rich forest and mountain) in the Bouhachem Natural Park, were genetically different from them. Bouhachem 1 (rich forest) was 99.4% identical to sequences from the central and southern Iberian Peninsula (AY954426, AY954407, AY954418, AY510585, JQ431003, AY510591, JQ430999, JQ431004). Bouhachem 2 (rich forest) was 99.6% identical to sequences from southern Spain (AY510585, JQ431003, AY510593, AY510594, JQ430999). Bouhachem 3 (mountain) was 99.2% identical to those from the southern Iberian Peninsula (JQ430999, JQ431003, AY510585, AY954426, AY954418).

The network showed a highly structured basal area with many haplotypes from the southern and southwestern Iberian Peninsula (Spain and Portugal), including those from Morocco and Switzerland. The more derived part of the network included the haplotypes from southwest France, northwest Iberian Peninsula, the United Kingdom, Azores, and Madeira (Fig. 2 and Supplementary Fig. S1).

A similarity analysis of all known haplotypes of this species showed that the populations closest to the new localities were as follows: the westernmost part of the Iberian Peninsula for the Azores populations, the western Iberian Peninsula for the Madeira populations, and the southern Iberian Peninsula in the case of Morocco. In addition, the UK population shares a haplotype with

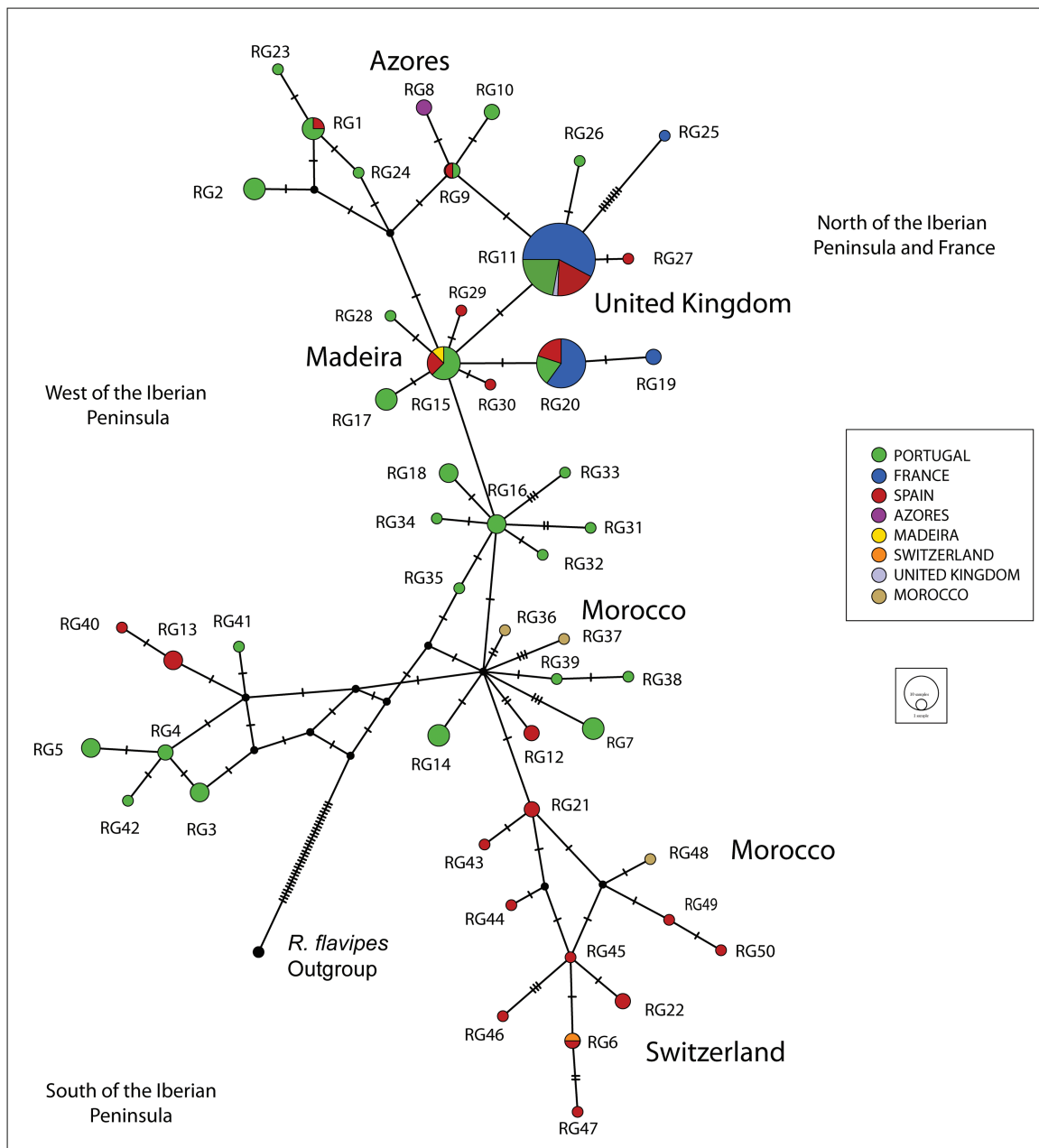
populations from the northern Iberian Peninsula to southwestern France and the Swiss population with the southern Iberian Peninsula (Fig. 3; Supplementary Table S3). In addition, the image obtained from the geophylogenetic analysis shows a similar relationship between the localities (Supplementary Figs. S2 and S3).

## Discussion

The genetic identification of the termite species *R. grassei* herein presented on Faial and Madeira, as well as from Morocco, confirms the distribution of this species in 6 countries: Portugal (continental, Faial, and Madeira), Spain, France, United Kingdom, Switzerland, and Morocco (Fig. 1). In the United Kingdom, no subterranean termite activity has been detected since 2010, despite biannual or annual inspections until 2021, when the eradication program was decommissioned and deemed successful (Verkerk and Suttie 2022). In Switzerland, although eradication has been previously claimed (Ghesini et al. 2020), currently this termite species is still known to be present (G. Müller, personal communication). Within its native area, it occupies more than half the Iberian Peninsula. In Portugal and southern Spain, there is wider haplotype variability but lower variability in the north of Spain and southwestern France (see Fig. 2; Supplementary Figs. S1–S3), similar to results obtained by Lefebvre et al. (2016). This seems to indicate a rapid expansion in the north, perhaps after a postglacial colonization event, as suggested by Kutnik et al. (2004).

In addition, previous studies showed that the southern Iberian Peninsula populations exhibited higher numbers of single-family colonies, while the northern populations were comprised of predominantly extended-family colonies (DeHeer et al. 2005, Vargo and Husseneder 2009, Vargo et al. 2013), which also supports the idea of a northward expansion. Environmental factors like wood availability and soil composition may also influence these patterns (Vargo et al. 2013, Bankhead-Dronnet et al. 2015).

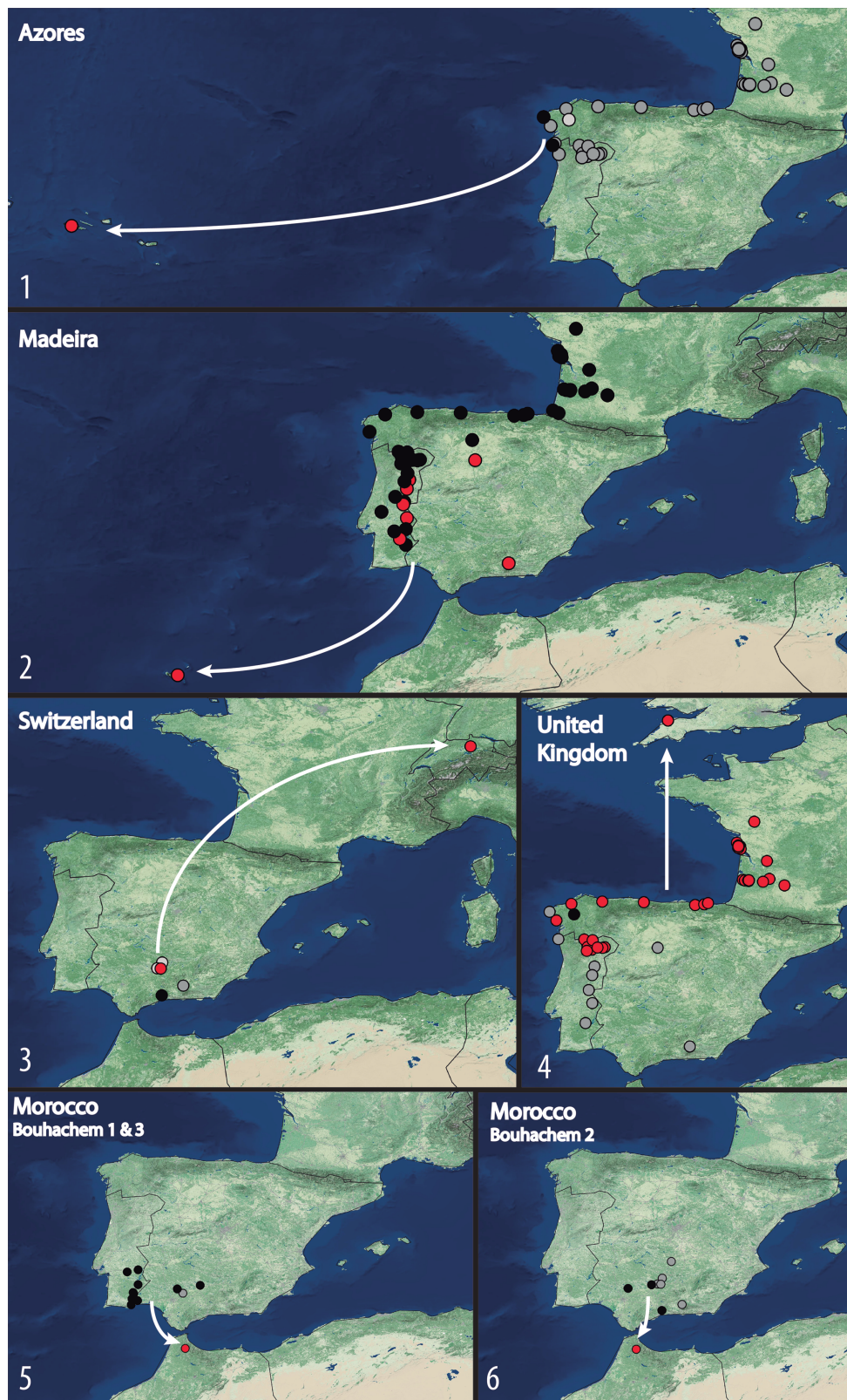
Invasive populations of *R. flavipes* seem to benefit from their propensity to fuse into mixed families, as this will decrease the costs of aggressive behavior between termites from the same species



**Fig. 2.** Median-joining networks of *Reticulitermes grassei* species based on the mitochondrial data set (cox2) from a sample of 155 individuals and 501 bp DNA sequences. Only specimens with different haplotypes and locality were taken into account. Circle size is proportional to the number of samples within a given haplotype, and lines between haplotypes represent mutational steps within alleles. Colors denote the geographic origin of the individuals used in the analysis. *Reticulitermes flavipes* was used as an outgroup in the analysis.

(Perdereau et al. 2015). This is a clear advantage for conquering new habitats. *Reticulitermes grassei* apparently benefitted from the Iberian refugium (in the south of the Peninsula) during the last glacial era and then dispersed to its known distribution range (northern Iberian Peninsula and south of France) (Kutnik et al. 2004). Potentially, it may have also spread south into Morocco, which could help explain the higher genetic variability noted there, although a higher number of samples and genetic markers are needed to confirm this. Three different sequences belonging to 2 basal areas of the network came from 2 close localities within Bouhachem Natural Park (Fig. 2 and Supplementary Fig. S1). The 3 are grouped with samples from different areas of the southern Iberian Peninsula. It appears to be a natural colonization, and therefore, the populations in Morocco are

likely native. If this were the case, the hypothesis put forward by Lefebvre et al. (2016) of an African origin of the Iberian species of *Reticulitermes* could also be possible. However, a more significant sampling effort in Morocco would be needed to test this hypothesis. The Iberian Peninsula and Africa separated in the Miocene period, and termite populations may have become isolated during the geological events that formed the Strait of Gibraltar. It has been shown that the geography of the Strait of Gibraltar has little impact on some species (including arthropods) with a low dispersal capacity, which colonized the Iberian Peninsula after the formation of the sea barrier (Harris et al. 2002, Veith et al. 2004, Planas et al. 2013). Also, some species were able to naturally cross this barrier after its formation (e.g., Nesbit et al. 2009). A mechanism that explains



**Fig. 3.** Map with routes to new areas, showing the geographical distribution of the closest haplotypes. 1: Azores; 2: Madeira; 3: Switzerland; 4: United Kingdom; 5–6: Morocco. Each level of similarity is represented by the colors (Supplementary Table S3): identical haplotype in red; high similarity in black; medium-high in gray; medium in light gray; and low in white. To facilitate visualization of the possible origins of the introductions where necessary, only haplotypes with the highest similarities are represented.

transoceanic termite distributions lies in the natural transport of driftwood. Together with the development of salinity tolerance, this could constitute a possible dispersal route in our case (Chiu et al. 2020). Although less likely, another hypothesis is that their introduction via human migration and commerce routes some centuries ago since the termites now established in natural areas. Further studies are necessary to unveil the origin of Moroccan *R. grassei* and determine whether these populations may be ancestors of the current *R. grassei* populations in the Iberian Peninsula. It is important to note that this is a preliminary comparison, as other DNA regions should be sequenced. Together with an analysis of cuticular hydrocarbons, such advances would provide a more robust assessment. The latter analysis would allow the comparison of the chemical profiles of the *R. grassei* populations as in previous studies, which did not include Moroccan samples (Kutnik et al. 2004, Lefebvre et al. 2016).

*Reticulitermes grassei* populations in a putative invasive range were closely related to those in different parts of the Iberian Peninsula, which may be attributed to human-mediated transportation events. The *R. grassei* identified in Switzerland were in the basal part of the network with haplotypes from the southern Iberian Peninsula (Fig. 2 and Supplementary Fig. S1). This could be due to an introduction event (or more than one) via infested material from this part of the Peninsula, as proposed by Ghesini et al. (2020). Samples from Madeira were grouped with others mainly from the western Iberian Peninsula and Azores samples from the westernmost part of the Iberian Peninsula. Both are included in the most derived part of the network. This finding, given that termites were found only in urban areas, suggests human-mediated transportation events rather than transoceanic dispersal routes through driftwood. However, the samples from the United Kingdom were grouped with those from the Cantabrian cornice of northern Spain across southwestern France within the same haplotype, belonging to the derived part of the network (Fig. 3; Supplementary Figs. 2 and S3). This supports the 2 possible sources previously suggested, namely wine crates from Bordeaux and/or plants from northern Spain (Jenkins et al. 2001). The fact that ongoing gene flow between the 2 *Reticulitermes* species present in the Iberian Peninsula (*R. grassei* and *R. banyulensis*) was suggested in previous studies (Lefebvre et al. 2016) should also be considered regarding the genetic differences among *R. grassei* populations from the northern Iberian Peninsula.

The availability of palatable cellulose sources with low durability against termite attack will facilitate introduction events. In the case of Madeira, weather conditions are different from those in the natural distribution area of *R. grassei*, with more stable temperatures, probably influencing subterranean termite establishment. Its maxima are above 20 °C for more than 40 days per year, and minima reach their lowest at 12 or 13 °C except at its summit, where it can reach below 0 °C (Climate Atlas 2012). Subterranean termite that attacks on wooden structures are a major concern due to the difficulty in controlling their infestation. On oceanic islands, invasion by a termite is even worse (e.g., *R. flavipes* on Tenerife, Hernández-Teixidor et al. 2019). When detection is timely, eradication should be pursued to protect wooden structures, wood-derived buildings, and furniture, crops, and insular ecosystems. On Faial island, a monitoring scheme intended to eradicate invasive *R. grassei* began in 2016 (Borges et al. 2016). However, difficulties arose due to regular floodings, which increased the relative humidity of wood and wooden materials, hampering the treatments. In Madeira, it is essential to establish further steps to monitor and control *R. grassei*. Knowing the extent of its distribution range and whether it has spread into natural habitats are useful clues. Once termites become established, eradication may not be possible, and long-term monitoring programs with

baiting systems become the most widely adopted technique to protect buildings (Oi 2022). Therefore, special attention must be given to preventive measures, especially in areas with native termites and oceanic islands prone to subterranean termite invasions. To protect wooden structures from subterranean termite damage, suitable construction techniques should be addressed, for example, through attention to leakage points causing damp areas in buildings, known to play a major role in termite establishment (Evans et al. 2013, Duarte et al. 2018). Other preventive measures like the application of naturally resistant wood species, treated wood, proper building design, and construction codes formulated to tackle the issue are all crucial and valuable, especially in such susceptible areas (Nobre and Nunes 2007, Forschler 2011, Oi 2022). Termites may transport water into the attacked wood by building mud tubes for foraging, enabling them to establish and feed on wood normally described as dry (e.g., 12–15%). Recently, higher temperatures have been proven to be a key factor that fosters termite dispersal since they can increase wood discovery and consumption by these pests (Zanne et al. 2022). These findings are thus linked with predictions of further global climatic change. Along with being successful invaders and highly adaptive organisms (Evans et al. 2013, Buczkowski and Bertelsmeier 2017), subterranean termites challenge our efforts to protect our wood, wood-based materials, and fruit crops. Furthermore, their ecological impact on invaded natural areas is yet to be evaluated (Evans 2021). Ensuring early detection and promptly implementing eradication and monitoring programs is of utmost importance to prevent establishment in new regions.

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## Author Contributions

David Hernández-Teixidor (Data curation [lead], Formal analysis [lead], Funding acquisition [lead], Investigation [equal], Methodology [equal], Project administration [equal], Resources [equal], Visualization [lead], Writing—review & editing [equal]), Sónia Duarte (Investigation [equal], Methodology [equal], Writing—original draft [lead], Writing—review & editing [equal]), Ahmed Taheri (Investigation [supporting], Methodology [supporting], Project administration [supporting], Resources [supporting], Writing—review & editing [supporting]), Paulo A. V. Borges (Project administration [equal], Resources [equal], Validation [equal], Writing—review & editing [supporting]), and Lina Nunes (Conceptualization [lead], Resources [equal], Supervision [lead], Validation [equal], Writing—review & editing [supporting])

## Supplementary Material

Supplementary material is available at *Journal of Economic Entomology* online.

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