



ORIGINAL ARTICLE

The effect of the Messinian salinity crisis on the early diversification of the *Tettigettalna* cicadas

Gonçalo J. Costa¹  | Vera L. Nunes¹  | Eduardo Marabuto¹ | Raquel Mendes¹ | Diogo N. Silva¹ | Pere Pons² | Josep M. Bas² | Thomas Hertach^{3,4} | Octávio S. Paulo¹ | Paula C. Simões¹

¹Centre for Ecology, Evolution and Environmental Changes & CHANGE - Global Change and Sustainability Institute, Faculdade de Ciências, Universidade de Lisboa, Lisbon, Portugal

²Departament de Ciències Ambientals, Universitat de Girona, Girona, Catalonia, Spain

³Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland

⁴Natural History Museum of Bern, Bern, Switzerland

Correspondence

Vera L. Nunes, Centre for Ecology, Evolution and Environmental Changes & CHANGE - Global Change and Sustainability Institute, Faculdade de Ciências, Universidade de Lisboa, Lisbon 1749-016, Portugal.
Email: vlunes@fc.ul.pt

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Abstract

The current distribution patterns of many Mediterranean species are often a consequence of large and impactful past geoclimatic events, such as the Messinian Salinity Crisis (MSC) and the Quaternary glacial cycles. Cicadas are flying insects with poor dispersal ability, which have experienced intense local differentiation in the Mediterranean, where the genus *Tettigettalna* has surfaced as a biogeographic model. The genus includes 10 species with species-specific calling songs but identical morphology. All *Tettigettalna* species are restricted to Southern Iberia, with the exception of *T. estrellae* (northwest Iberia), the widespread *T. argentata* (mainly Iberia, France and Italy), and *T. afroamissa* (Morocco). With an expanded genetic dataset involving nuclear (*EF1α*) and mitochondrial (5' and 3' *COI* and *ATP*) loci, we reconstructed the phylogeny of the genus and estimated divergence dates for *Tettigettalna* species under a Bayesian framework. Phylogeny with the new mitochondrial dataset was in agreement with previous studies, whereas the nuclear *EF1α* supported *T. josei* and *T. afroamissa* as monophyletic clades but lacked resolution to resolve the remaining taxa. Some sister taxa share mitochondrial haplotypes, hinting for incomplete lineage sorting. Estimates of divergence time settled *T. josei* as the earliest diverging lineage, likely as a pre- or early-MSC event. As for the origin of *T. afroamissa* in Morocco, though time estimates could not entirely rule out post-MSC dispersal, the most likely scenario points to isolation of African *Tettigettalna* after the reopening of the strait of Gibraltar. The Pleistocene glaciations that followed likely impacted on the diversification of the remaining species of the genus in southern Iberia refugia.

KEYWORDS

cicada, Messinian salinity crisis, molecular dating, phylogeny, *Tettigettalna*, Western Mediterranean

1 | INTRODUCTION

The complex historical biogeography of Western Mediterranean has been extensively studied for many

plant and animal taxa, where two major geo-climatic events have been often evoked to explain current patterns of distribution and molecular diversity: the Pleistocene Ice Ages (<2.6 Ma) and the Messinian Salinity Crisis

(MSC) at the end of the Miocene, 5.97–5.33 Ma (Gómez & Lunt, 2007; Hewitt, 1999, 2000; Marabuto et al., 2020; Médail & Diadema, 2009; Schmitt, 2007; Trájer et al., 2021).

The MSC was triggered by tectonic movements that isolated the Mediterranean Sea from the Atlantic Ocean and accompanied by climatic changes that led to several series of evaporation cycles, progressively lowering the sea level to its nearly complete desiccation (Krijgsman et al., 1999, 2018; Manzi et al., 2013). Extensive land connections were formed between Europe and North Africa during this process (see Figure 1), functioning as land bridges for fauna and flora exchange among the two continents (Gibert et al., 2013; Husemann et al., 2014). These connections were suddenly interrupted when the Mediterranean

basin was refilled through the Gibraltar Strait corridor, at 5.33 Ma (Blanc, 2002). This event marked the beginning of the Pliocene and created a sea barrier for poorly dispersive biota and triggered differentiation on either side of the strait, contributing to the high endemism observed today in this biodiversity hotspot (Lavergne et al., 2013; Médail & Diadema, 2009; Puissant & Sueur, 2010).

By late Pliocene, the Iberian Peninsula had already acquired its modern coastal configuration (Elez et al., 2016; Jolivet et al., 2006), with the closest distance between Europe and Africa found at the Gibraltar Strait (14 km, Figure 1C). In the following period, the Pleistocene, the Northern Hemisphere was subject to several glacial cycles, which strongly affected the

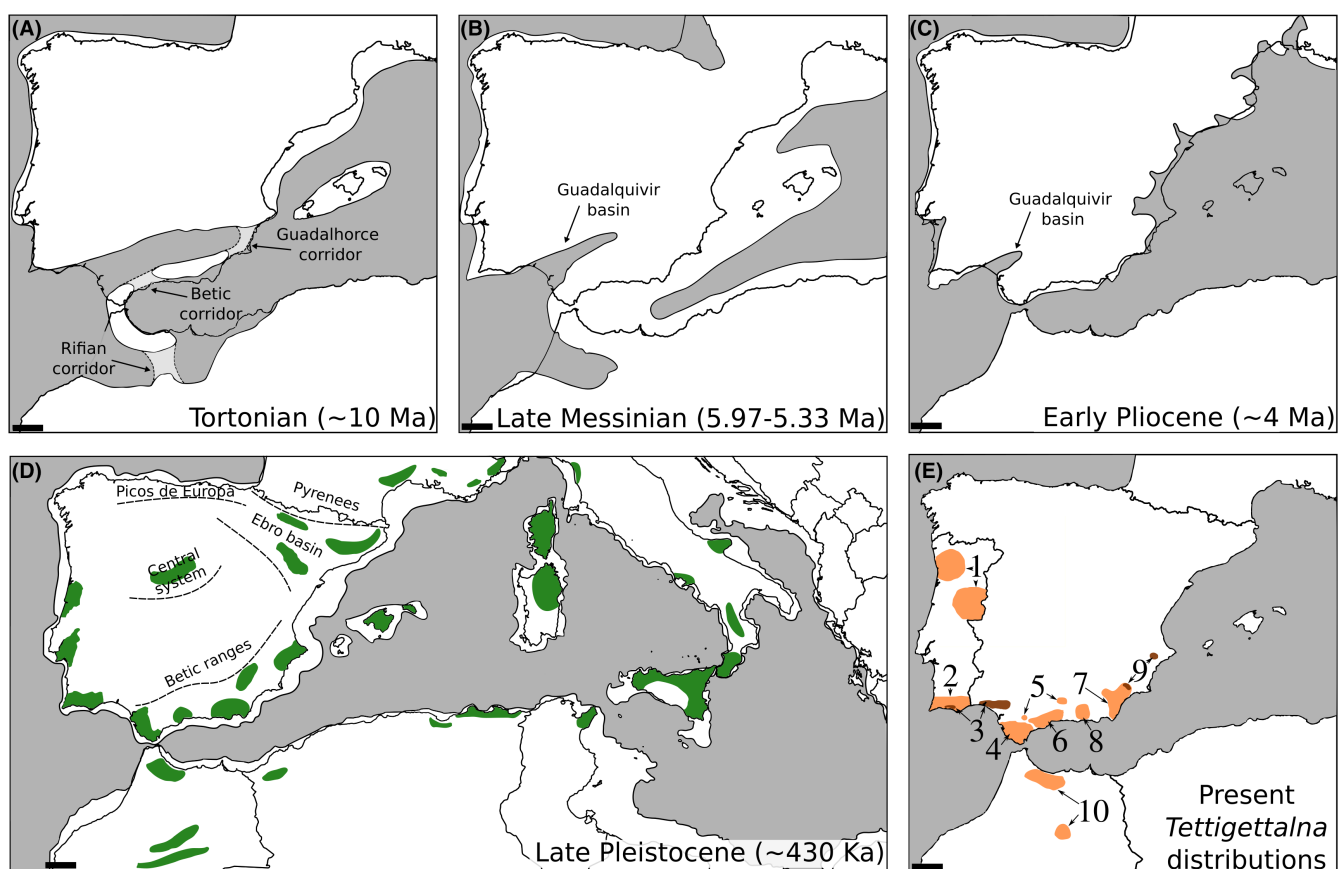


FIGURE 1 Major geological events of the Western Mediterranean, Pleistocenic glacial refugia, and *Tettigettalna* spp. distributions. Panels (a–d) show a schematic of the evolution of the West Mediterranean region from the Tortonian to the late Pleistocene. (a) Mid Tortonian, depicting the three Eurafrican corridors that later closed, between 7.8 and 6.0 ma. (b) Late Messinian, during the salinity crisis an extensive land bridge formed between Iberia and North Africa. Arrow points to the Guadalquivir basin, a large saltwater basin. (c) Early Pliocene, land bridge is now disrupted, and the Guadalquivir basin has almost retreated. (d) Late Pleistocene, during the period when sea level was the lowest, according to Rohling et al. (2014), approx. 150 m lower. No land bridges are present during this period. Putative Pleistocenic glacial refugia of the Western Mediterranean inferred for flora (Médail & Diadema, 2009) are represented in green, and terrestrial fauna and flora (Gómez & Lunt, 2007) shown with broken lines. (e) Present day *Tettigettalna* spp. distributions are shown in orange, according to Puissant and Sueur (2010), Simões et al. (2014), Nunes, Mendes, Quartau, et al. (2014) and Costa et al. (2017). Legend: 1—*T. estrellae*; 2—*T. josei*; 3—*T. mariae*; 4—*T. armandi*; 5—*T. aneabi*; 6—*T. defauti*; 7—*T. helianthemi helianthemi*; 8—*T. h. Galantei*; 9—*T. boulardi*; 10—*T. afroamissa*. Species' distributions shown in brown overlap with those of other species. The distribution of *T. argentata* is not shown as it is widespread across several European countries and the Iberian Peninsula with exception of the Baetic ranges in southeastern Iberia. Scale bar = 100 km

distribution and survival of biota (Hewitt, 1999, 2000). The heterogeneity in topography and habitats within the Iberian Peninsula and Maghreb enabled the survival of previously widespread lineages into a plethora of local refugia (Tzedakis, 2009), supporting the concept of “refugia within refugia” (Figure 1D, Feliner, 2011; Gómez & Lunt, 2007; Martínez-Freiría et al., 2020; Miraldo et al., 2011; Petit, 2003). Nevertheless, overseas dispersal could have been facilitated under glacial maxima by a significant drop in sea levels, which were estimated to have reached over 100 m lower than today (Dumitru et al., 2021; Rohling et al., 2014). Post-MSL overseas dispersal to either side of Gibraltar has been suggested for several taxa, such as bell flowers (García-Aloy et al., 2017), lizards (Mendes et al., 2017), newts (Veith et al., 2004), butterflies (Habel et al., 2011; Marabuto et al., 2020), beetles (Mas-Peinado et al., 2021), and spittlebugs (Rodrigues et al., 2014). In some cases, however, divergence time estimates place lineage split events between Europe and Africa much before the reopening of the Strait of Gibraltar (see for e.g. Mendes et al., 2017; Paulo et al., 2008).

Cicadas (Hemiptera: Cicadidae) are a worldwide varied group of insects with over 3000 species (Sanborn, 2014), characterized by low dispersal and long egg-to-adult life cycles (see Table S2 in Simon et al., 2022), with 500 species belonging to the largest tribe Cicadellini (Marshall et al., 2016). The Cicadellini are thought to have an Australasian origin, with two smaller centers of diversity in the Mediterranean and South Africa (Marshall et al., 2016). Cicadas spend multiple years underground as nymphs, contrasting with their ephemeral adult winged stage above ground, generally lasting up to a few weeks in which they seek to mate and lay their eggs. The male uses a unique calling mechanism to attract receptive females. These calls are usually species-specific and recognized as effective for species delimitation (Boulard, 2006). Although several aspects of their biology are still largely unknown, cicadas make interesting models for biogeography, as exemplified by studies from New Zealand, where vicariance appears to have played an important role in their diversification and speciation (Arensburger, Simon, et al., 2004; Bator et al., 2022; Buckley & Simon, 2007; Marshall et al., 2008).

In the western Mediterranean, cicadas of tribe Cicadellini have recently received some attention regarding their diversity and biogeography (Hertach et al., 2015, 2016; Mendes et al., 2014; Nunes, Mendes, Marabuto, et al., 2014; Puissant & Sueur, 2010). The genus *Tettigettalna* was created in 2010 to accommodate nine morphologically similar species of Cicadellini with distinct calling songs (Puissant & Sueur, 2010), with all but one restricted

to the Iberian Peninsula (see Figure 1E). The only species extending its range beyond the Pyrenees is *T. argentata*, reaching southern France, Italy, western Slovenia, and southern Switzerland (Gogala & Gogala, 1999; Hertach & Nagel, 2013; Puissant & Sueur, 2010). The biogeographic setting of the genus changed with the recent discovery of *T. afroamissa*, a new species found in northern Morocco and the first *Tettigettalna* to be reported out of Europe (Costa et al., 2017). Ecologically and morphologically analogous to the widespread *T. argentata*, this new species is allopatric to all other *Tettigettalna*, has a distinctive calling song and is genetically well differentiated (Costa et al., 2017). The discovery of this species led to an important question: How and when did *T. afroamissa* get into North Africa?

Sequence data available thus far to infer the phylogeny of *Tettigettalna* spp. are based on a single mitochondrial gene, the cytochrome c oxidase I (Costa et al., 2017; Nunes, Mendes, Marabuto, et al., 2014). Both *Tettigettalna josei* and *T. afroamissa* stand out as the earliest diverging species of the genus, but single-gene analysis proved as insufficient to resolve which *Tettigettalna* species diverged in the first place (Costa et al., 2017). Mitochondrial genes evolve faster than nuclear ones and often tell a biased story based on a maternally inheritance only (Ballard & Whitlock, 2004; Rubinoff & Holland, 2005; Shaw, 2002). Therefore, we use an extended dataset in this study, with mitochondrial and nuclear gene coverage, and add molecular age dating calibration to unfold the evolutionary history of early *Tettigettalna* diversification and find the best explanation for current distribution of *T. afroamissa* in North Africa.

Three biogeographical scenarios can be hypothesized: The first is an overseas dispersal in either direction, resulting in the splitting of the European and Moroccan lineages before the onset of the MSL at 5.9 Ma. The second scenario, that of vicariance, postulates that a large population existed across the land connection during the MSL, which was divided with the opening of the Gibraltar Strait at the end of the MSL (5.33 Ma). The third scenario places overseas dispersal of *Tettigettalna* from Europe to Africa as a post-MSL event, after 5.3 Ma, which should have happened most likely during the Pleistocene glacial maxima, when sea level was remarkably lower. To determine which of these scenarios is the most likely, we reconstructed the phylogenetic relationships of *Tettigettalna* using two methods for species-tree reconstruction within a Bayesian framework: gene concatenation and multispecies coalescence (Heled & Drummond, 2010; Lambert et al., 2015; Tonini et al., 2015). The results of both methods are discussed under these biogeographical hypotheses to determine the most parsimonious scenario explaining *Tettigettalna* spp. distribution.

2 | MATERIALS AND METHODS

2.1 | Sampling, DNA extraction and sequencing

Sampling from previous *Tettigettalna* phylogenies by Nunes, Mendes, Marabuto, et al. (2014) and Costa et al. (2017) was extended with the collection of 41 new specimens in the Iberian Peninsula. Cicadas were collected by hand or sweeping net and assigned to species according to their male calling song. GPS data was recorded at each capture site and a front leg was removed and preserved in 100% ethanol for genetic analysis (Figure 2; Table S1). Dry specimens are stored at the Department of Animal Biology of the Faculty of Sciences, University of Lisbon, Portugal. Legs from the four Italian specimens of *T. argentata* included in this dataset were provided by Thomas Hertach from his own collection.

Genomic DNA was isolated with the DNeasy Blood & Tissue Kit (Qiagen). Four gene fragments were sequenced, with a total of 2504 base pairs (Table 1): (i) COI-Lep: 5' region of the cytochrome C oxidase I (COI) mitochondrial gene; (ii) COI-CTL: 3' region of the cytochrome C oxidase I (COI) mitochondrial gene; (iii) ATP: mitochondrial locus comprising tRNA-Asp gene (complete sequence), ATPase subunit 8 gene (complete coding sequence) and ATPase subunit 6 gene (partial sequence); and (iv) EF-1 α : nuclear locus of Elongation Factor 1 α comprising exon2 (partial coding sequence), intron2 (complete sequence), exon3 (complete sequence), intron3 (complete sequence) and exon4 (partial coding sequence). Amplification of each locus by polymerase

chain reaction (PCR) was performed in a total volume of 20 μ l containing 1 \times PCR buffer (Promega), 0.6 U Taq polymerase (Promega), 2.8 mM MgCl₂, 0.10 mM dNTPs, and 0.4 μ M of each primer (see Table S2 for primer sequences and sources). The standard cycling conditions used were 94°C for 3 min, 35 \times (30 s at 94°C, 30 s at the specific annealing temperature as in Table S2 and 30 s at 72°C) followed by a final elongation step at 72°C for 10 min. PCR products were purified with Sureclean (Bioline) following the manufacturer instructions. Purified fragments were sequenced with Big Dye Terminator v.3.1 (Applied Biosystems) on Macrogen or Beckman Coulter Genomics facilities.

Sequences were edited in Sequencher v4.0.5 (Gene Codes Co.) to correct noisy and ambiguous base calling. Mitochondrial sequences were checked for stop codons in DnaSP v5.10 (Librado & Rozas, 2009). Sequences of nuclear EF-1 α found to be heterozygous in length were reconstructed as in Flot et al. (2006). Haplotype phase was inferred with PHASE v2.1.1 (Stephens et al., 2001) with default settings and ran for 1000 iterations (ambiguities were assigned as N if phase probability was <0.70). A total of 262 new sequences were generated by this study and deposited in GenBank (Table S1). This dataset was combined with 107 sequences of COI-Lep from *Tettigettalna* generated by previous works (see Table S1; Costa et al., 2017; Nunes, Mendes, Marabuto, et al., 2014; Simões et al., 2014). All the 148 *Tettigettalna* spp specimens of the dataset were represented by COI-Lep sequences, whilst 61 were successfully sequenced for nuclear EF-1 α and 45 were sequenced for all four DNA fragments (Table S1).

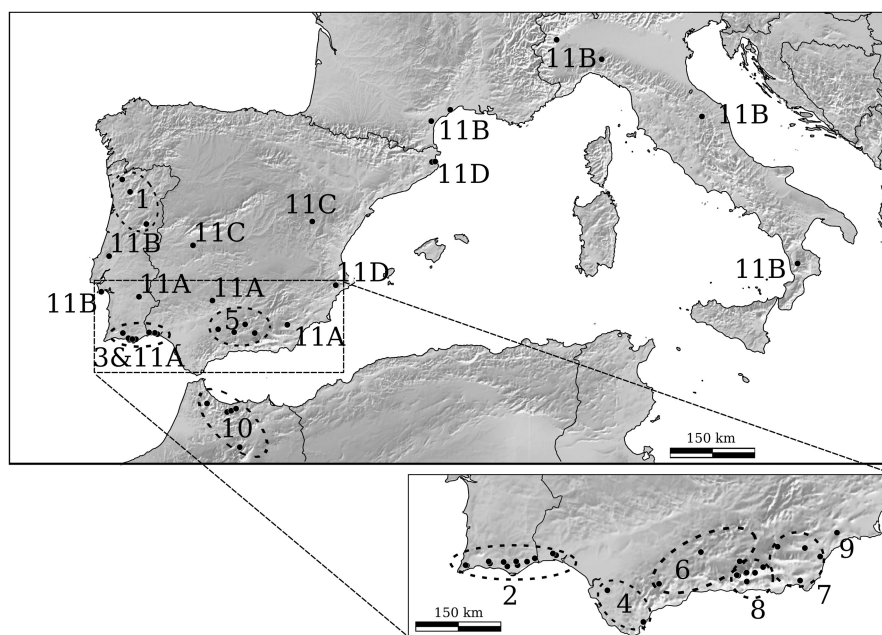


FIGURE 2 Sampling of *Tettigettalna* spp. circles indicate same-species collection points. Due to the volume of sampling from the southern Iberian Peninsula, the smaller box below shows additional sampling points for other species annotated for that area. Legend: 1—*T. estrellae*; 2—*T. josei*; 3—*T. mariae*; 4—*T. armandi*; 5—*T. aneabi*; 6—*T. defauti*; 7—*T. helianthemii helianthemii*; 8—*T. h. Galantei*; 9—*T. bouvardi*; 10—*T. afroamissa*; 11A—*T. argentata* south clade; 11B—*T. argentata* north clade; 11C—*T. argentata* central clade; 11D—*T. argentata* Catalonia clade

TABLE 1 Loci sequenced for *Tettigettalna*, with information about sequence length, number of individuals sequenced (N), number of haplotypes, number of variable sites (V), and number of parsimony-informative sites (P)

Locus name	Locus size (bp)	N	Haplotypes	V	P
COI-Lep mtDNA	581	148	83	208	175
COI-CTL mtDNA	683	59	41	106	76
ATP mtDNA	668	55	42	211	162
EF-1 α nuDNA	561	63	59 ^a	82 ^a	30

^aIncluding indels.

2.2 | Phylogenetic analysis

Sequence alignments were carried out with MAFFT v7.273 (Kato & Standley, 2013). Site substitution saturation was tested in DAMBE (Xia et al., 2003; Xia & Xie, 2001) for each codon position at coding sequences and found to be non-significant (p -value > 0.05) for all mitochondrial loci. Conversion of files from FASTA to NEXUS or PHYLIP format as well as gene concatenation were performed with TriFusion (available at <https://github.com/ODiogoSilva/TriFusion>). Maximum likelihood (ML) trees were obtained by assigning each separate locus dataset a GTRCAT model, 1000 replicates and a rapid bootstrap analysis ($-x$) on RAxML-HPC v.8 (Stamatakis, 2014). For the Bayesian inference (BI), each dataset was partitioned into loci subsets and coding sequences were further partitioned into codon positions. These partitions were subsequently tested and assigned an evolution model on PartitionFinder v2 (Lanfear et al., 2016) under the corrected Akaike information criterion (AICc), with a greedy search parameter. Bayesian inference trees were generated on MrBayes v3.2.6 (Ronquist et al., 2012). Each dataset was assigned with two independent runs with four chains, 5×10^7 generations with burn-in set to the initial 25% trees and the evolution models previously selected with PartitionFinder2. Parameter's convergence was checked in TRACER and if confluence was not attained, runs were assigned additional 5×10^7 generations and checked again for confluence. *Hilaphura varipes*, *Cicada orni*, and *Cicada barbara* were chosen as outgroups (Table S1). All analyses were run as implemented on the CIPRES Science Gateway (Miller et al., 2010). Output trees were visualized in FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) and imaged in Inkscape.

2.3 | General approach for BEAST model optimization

BEAST v.1.8.4 (Drummond et al., 2012) was used to estimate divergence times as implemented in the CIPRES Science Gateway. Input files for BEAST analysis were initially assembled on BEAUti (a package of BEAST) and then visually inspected or adjusted manually for

substitution site models not implemented on BEAUti. Initial substitution site models were the same used for the BI tree analysis, i.e. selected under an AICc criterion on PartitionFinder v2. Site models with low ESS values would be discarded and the next best site model, ranked under AICc, would be selected. After optimization, the selected site models were: TIM1e for the COI-CTL, COI-LEP, and ATP unlinked partitions; HKY+G for EF-1 α exon and HKY for EF-1 α intron. Tree models were linked, with a Yule process prior, for the three mitochondrial partitions and for the EF-1 α partitions. Clock models were linked for the COI partitions (COI-LEP + COI-CTL). Because the parameter “uclid.stdev” of the EF-1 α exon partition abutted 0 on preliminary runs, the clock model was changed from “uncorrelated relaxed” to a “strict clock with a lognormal distribution”. The remaining partitions were assigned an uncorrelated relaxed clock, with a lognormal distribution with “mean in real space” checked.

Fossil records of Cicadidae are scarce worldwide (reviewed by Moulds, 2018), and none is adequate for clock calibration for *Tettigettalna* cicadas. Clock rate estimates for COI and EF-1 α follow the ones of Marshall et al. (2016) set for phylogenetic analysis of Cicadettini with a relaxed clock (analysis K – subclades), in which *Tettigettalna josei* and *T. argentata* were included (see Table S3 (COI) and Table S4 (EF-1 α subclade I) in Marshall et al. 2016). The values used were $M = 0.01172$ with $S = 0.288$ for COI partition, $M = 0.001965$ with $S = 2.0$ for EF-1 α exon and $M = 0.0075$ with $S = 2.0$ for EF-1 α intron.

As no calibrations were available for ATP, we assigned an uniform clock prior bound between 1.0×10^{-4} and 100 to this gene during preliminary runs and afterward inferred with a lognormal clock prior assigned with $M = 0.0149$ and $S = 2.0$. The *Tettigettalna* clade was constrained to be monophyletic, regardless of the outgroup being *Cicada orni*, *Cicada barbara*, or *Hilaphura varipes*. The parameter “uclid.stdev” was set to an exponential distribution bound between 0 and 3.33. MCMC chain length was set for 2.5×10^8 iterations logging every 25,000th iteration and ran 6 to 7 times to check for repeatability.

Tracer v1.4 was employed to assess convergence and correct mixing of all parameters by visually inspecting the .log files and securing the Effective Sample Size (ESS) of each informative parameter to be over 200. Logcombiner

was used to combine the replicate runs with a 10% burning of each run for the log and tree files.

2.3.1 | Time estimates with concatenation model

The gene concatenation model relies on a super-matrix of multiple concatenated loci to retrieve a species-tree. One of the shortcomings of this method is that mitochondrial loci generally have more variable positions which often mask the phylogenetic signal provided by nuclear loci, thus losing much of its resolution, especially on deeper-level phylogenies (Rubinoff & Holland, 2005).

To infer divergence time, we selected a subset of 38 *Tettigetta* samples that represented all relevant branches of the phylogeny: two samples per species/lineage were selected, each corresponding to the most ancestral and most recently derived haplotypes. All samples in this subset were sequenced for all four loci (except for *T. afroamissa*, which could not be successfully sequenced for COI-CTL). To implement the concatenated model in BEAST, each locus was defined as a separate, unlinked site model, and clock models were assigned as previously stated in the general approach. Tree models were linked across nuclear and mitochondrial loci, on BEAUti. The input file for this analysis can be accessed in the Appendix S1.

2.3.2 | Time estimates with multispecies coalescence model

Multispecies coalescence models are often considered as superior to concatenation by differently weight – and unlink – nuclear and mitochondrial loci, thus accounting for sources of gene and species-tree mismatch, such as incomplete lineage sorting (Text S3 in Bouckaert et al., 2014; Lanier & Knowles, 2015; McCormack et al., 2010). For time estimates with the multispecies coalescent model, *BEAST (Heled & Drummond, 2010) was used instead, which is an extension package of BEAST v.1.8.4 (Drummond et al., 2012). *BEAST enables the user to provide information on the ploidy of the locus – i.e. differentially weighing the mutations that occur in the slower-evolving nuclear loci and the faster-evolving mitochondrial loci. Preliminary runs with the full mitochondrial and nuclear datasets (148 individuals) mixed poorly, and Tracer showed low convergence among most parameters, resulting in very low effective sample sizes, probably due to the lack of resolution of the only nuclear gene included. Since nuclear EF-1 α only resolves *T. josei* and *T. afroamissa* as monophyletic clades, leaving the remainder of the *Tettigetta* as a large polytomy, we

decided it was best to place these under a single monophyletic entity, which was named “Core *Tettigetta*” (i.e. *T. argentata*, *T. estrellae*, *T. mariae*, *T. aneabi*, *T. defaulti*, *T. armandi*, *T. helianthemi* and *T. bouldardi*). Model optimization was rapidly obtained with this reduced trait set (*T. josei*, *T. afroamissa* and Core *Tettigetta*). The final dataset for the *BEAST analysis included 5 partitions: COI-CTL, COI-LEP, ATP, EF-1 α intron, and EF-1 α exon, and the input file can be accessed in the Appendix S2.

Densitree was used to represent the distribution and topology of the large number of generated trees, and to retrieve clade support. Time to the most recent common ancestor (tMRCA) boxplots were generated in R Studio.

3 | RESULTS

3.1 | Single and concatenated gene phylogenies

Phylogenetic trees constructed for each mitochondrial locus with BI and ML are broadly concordant and successfully retrieve most song-delimited species (Figures S1 and S2). While mitochondrial loci efficiently reconstruct more recent relationships, all fail individually to reconstruct the deep nodes among *Tettigetta*, having low support values, particularly the ones involving *T. josei* and *T. afroamissa*. Their relationship remains unclear and even conflicting in single-gene analyses.

When concatenating all three mitochondrial loci, most clades defined with COI-Lep in previous studies were here retrieved with both BI (Figure 3A) and ML (Figure S3) analyses. The concatenated tree provides good support for considering *T. josei* as sister to all other *Tettigetta*, in both BI (1 pp, Figure 3A) and ML (99% BS, Figure S3), followed by *T. afroamissa* and the remainder of the *Tettigetta* (0.98 pp, <70% BS).

As in Nunes, Mendes, Marabuto, et al. (2014), an unresolved clade in the concatenated tree joins *T. argentata*, *T. aneabi*, and *T. mariae* (BI 1 pp; ML 99% BS). Regarding *T. helianthemi*, the apparent polyphyly of this species was confirmed in this study, where samples of subspecies *T. h. galantei* from Western Sierra Nevada, defined as type II by Nunes, Mendes, Marabuto, et al. (2014), group with *T. bouldardi* (BI 1 pp). The remainder of *T. h. galantei*, referred as type I by Nunes, Mendes, Marabuto, et al. (2014), form a well-supported sister clade to *T. h. helianthemi*, as it would be expected from their song differences (BI 1 pp, ML 99% BS).

The enlarged sampling enabled the resolution of a standing *T. defaulti* and *T. armandi* polytomy (1 pp, Figure 3A; 92% BS, Figure S3). The new dataset also revealed additional lineages with marked geographical

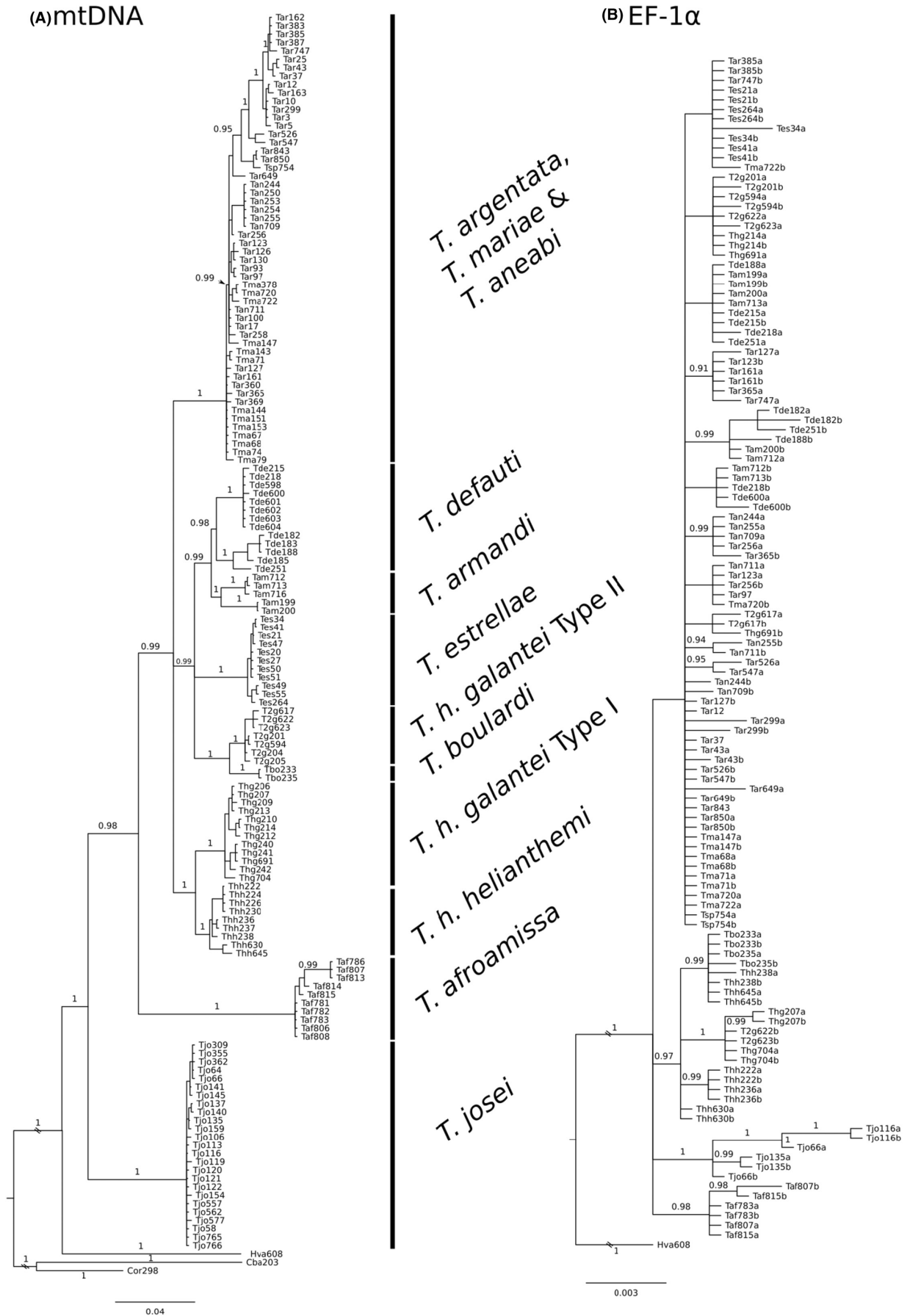


FIGURE 3 Bayesian phylogenetic trees for the concatenated mitochondrial loci (a) and nuclear EF-1α (b). Posterior probabilities >0.90 are shown next to branch nodes. Scale bar represents the number of estimated changes per branch length. *Hilaphura varipes* (Hva608), *Cicada barbara* (Cba203), and *Cicada orni* (Cor298) were set as outgroup. Root length was truncated for imaging purposes.

structure: Sierra Nevada and Ronda & Zagra populations of *T. defaulti* (0.89 pp, Figure 3A; 77% BS, Figure S3) and Jerez and Gibraltar populations of *T. armandi* (1 pp, Figure 3A; 94% BS, Figure S3).

Sequence data from the nuclear gene EF-1 α have about five times fewer parsimony-informative sites than mitochondrial loci (Table 1) and 39 out of 63 sequenced individuals were heterozygous for indel length (length of indels varied between 1 and 4 bases). Indels were coded as N for phylogenetic analyses. Their distribution among taxa was random, without diagnostic value. Both BI and ML phylogenetic reconstruction with nuclear gene EF-1 α have low resolution (Figure 3B and Figure S4). This locus fully retrieved *T. afroamissa* as well as *T. josei* as monophyletic taxa, whereas the remainder of the specimens form a large and weakly supported polytomy. Nevertheless, it is noteworthy that among *T. helianthemi*, while mitochondrial DNA clearly defines two lineages corresponding to the two acoustically defined subspecies (*T. h. helianthemi* and *T. h. galantei* Type I) and a third diverging lineage of *T. h. galantei* (Type II) clustering with specimens of *T. bouhardi* (Figure 3A), such genetic structure is not supported by EF-1 α , where *T. bouhardi* groups with some specimens of the subspecies *T. h. helianthemi* instead (Figure 3B). Samples Thh645 and Thh238 of *T. h. helianthemi* actually share the same EF-1 α haplotype with *T. bouhardi* samples, signalling incomplete lineage sorting among these parapatric species in southern Spain.

3.2 | Divergence time with concatenation method

Divergence time estimates for *Tettigettna* nodes using BEAST are summarized in Table 2 and Figure 4. The concatenated species-tree is congruent with both the BI and ML trees of mitochondrial datasets, apart from the basal relationships recovered within the core *Tettigettna*. BEAST concatenated species-tree confirms *T. josei* (from southwestern Iberia) as the earliest diverging taxon (node A), between 9.6 and 2.8 Ma, with a mean age estimate of 5.9 Ma. The divergence of Moroccan *T. afroamissa* (node B) was estimated between 7.8 and 2.3 Ma, with a mean age estimate of 4.8, after the reopening of the Strait of Gibraltar. The remaining taxa were arranged in two main subclades (node C) with a mean age estimate of 3.4 Ma, during late Pliocene. One subclade comprises *T. estrellae*, *T. bouhardi*, *T. helianthemi galantei* Type II, *T. defaulti*, and *T. armandi* (BS = 1). *Tettigettna estrellae* remains *incertae-sedis* within this subclade. Relationships between *T. defaulti* and *T. armandi*, and between *T. bouhardi* and *T. h. galantei* Type II remain well supported (both BS = 1). Estimates place divergence of *T. defaulti* and *T. armandi*

TABLE 2 Mean age estimates in million years ago (ma) and 95% highest posterior density (HPD) intervals for nodes A to J, as in Figure 4, according to BEAST analysis (concatenation model)

Node	Mean \pm SE	95% HPD interval
A	5.9191 \pm 9.18 $\times 10^{-3}$	2.8488–9.6307
B	4.8203 \pm 7.48 $\times 10^{-3}$	2.2826–7.8374
C	3.4142 \pm 5.26 $\times 10^{-3}$	1.6048–5.5004
D	2.7216 \pm 4.21 $\times 10^{-3}$	1.3082–4.4336
E	2.5574 \pm 4.00 $\times 10^{-3}$	1.2458–4.1976
F	1.8258 \pm 2.84 $\times 10^{-3}$	0.8167–2.9201
G	1.2287 \pm 2.15 $\times 10^{-3}$	0.4944–2.1052
H	2.9220 \pm 4.60 $\times 10^{-3}$	1.3616–4.7695
I	1.5040 \pm 2.43 $\times 10^{-3}$	0.6722–2.5244
J	1.2858 \pm 2.05 $\times 10^{-3}$	0.5653–2.0989

(node F) between 2.9 and 0.8 Ma and divergence of *T. bouhardi* from *T. h. galantei* Type II (node G) between 2.1 and 0.5 Ma, both during the Pleistocene.

The second subclade (node H, BS = 0.92) comprises the *T. argentata* species complex (*T. argentata*, *T. mariae*, *T. aneabi*) together with its putative sister taxon *T. helianthemi*, including its two recognized subspecies (*T. h. helianthemi* and *T. h. galantei* Type I). Both groups within this subclade are well resolved (BS = 1), but *T. argentata*, *T. mariae*, or *T. aneabi* remain polyphyletic. Divergence time estimates within subclade H (nodes I and J) are recent, during the Pleistocene, between 2.5 and 0.6 Ma.

3.3 | Divergence time with coalescent method

To make a bona fide estimation of the chain of events of the diversification of the *Tettigettna*, we estimated tMRCA of the clades definable by the nuclear dataset: *T. josei*, *T. afroamissa*, and Core *Tettigettna*. Age estimates are summarized in Table 3. Because we were working with a reduced number of ingroup taxa, we could ponder all the three possible phylogenetic relationship scenarios within the *Tettigettna* clade (see introduction). The probabilities of such sub-clades are presented in Table 3 and Figure 5. Of the three likely phylogenetic relationship scenarios (Figure 5), the one with *T. josei* as sister to all other species in the genus has the highest bootstrap support (81.59%, Table 3), against the remaining which have a combined reduced probability (<20%). The mean age estimate for the divergence of *T. josei* is 6.9 Ma, before the MSC, although the 95% highest probability density intervals are wide (Table 3). The tMRCA for the *T. afroamissa* – Core *Tettigettna* split was estimated between 9.5 and 2.0 Ma, with a mean age estimate of 5.3 Ma, coinciding

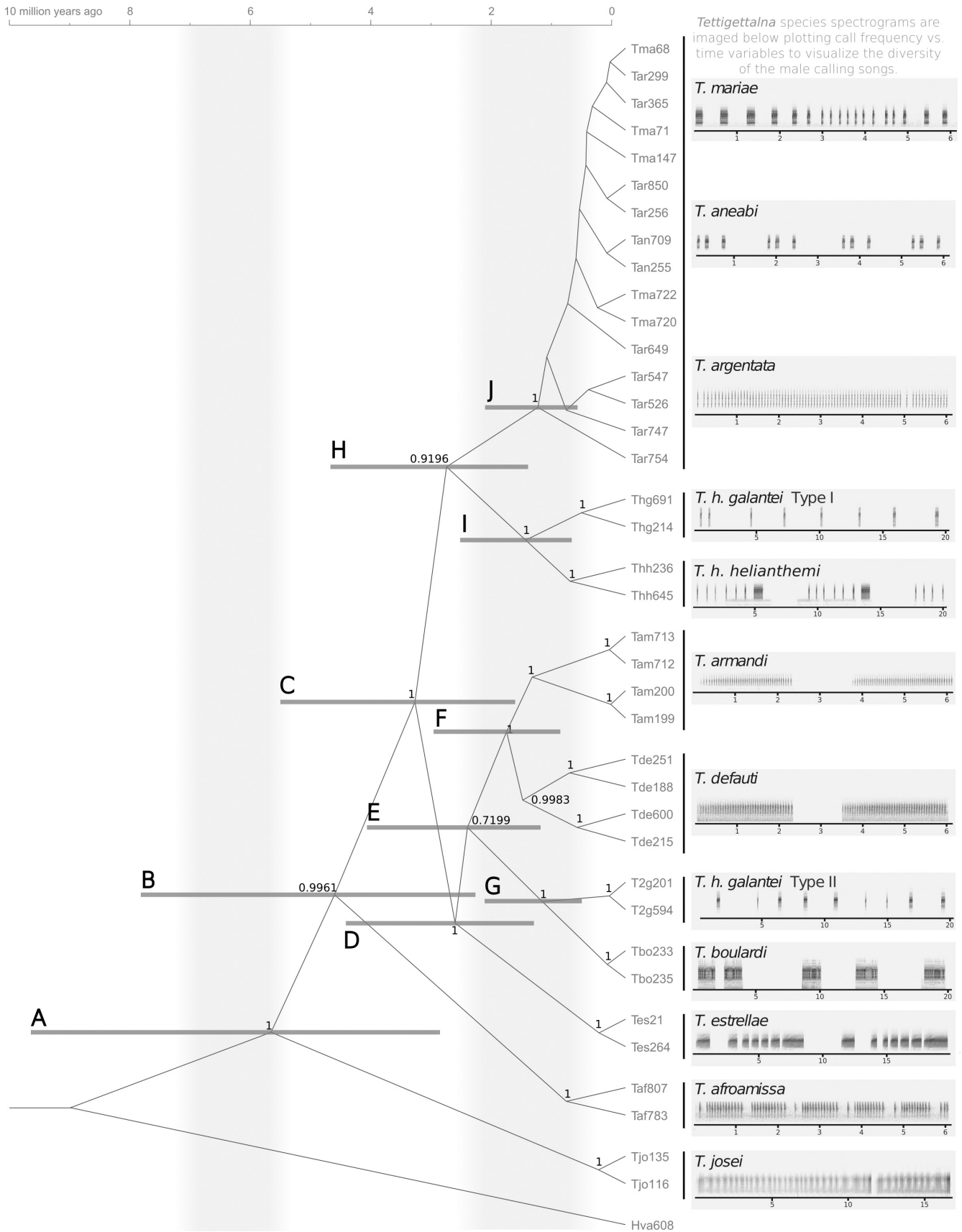


FIGURE 4 *Tettigettalna* species tree with concatenation model as output of BEAST. Posterior probabilities >0.9 are shown next to each node. Node bars (A-J) illustrate the 95% HPD interval (age estimates for each node are listed in Table 2). Vertical grey shading under the timescale bar refers to two past geological events: The Messinian salinity crisis and the Pleistocene ice ages. To illustrate song diversity within the target genus, oscillograms are shown next to each taxon.

Split	Mean \pm SE	95% HPD interval	Support (%)
<i>Tettigettalna</i> spp	7.039 \pm 0.080	2.638–12.274	95.12
<i>T. afroamissa</i> / <i>T. josei</i>	6.952 \pm 0.080	2.429 $\times 10^{-3}$ –12.41	9.00
<i>T. afroamissa</i> /"Core" <i>Tettigettalna</i>	5.308 \pm 0.054	2.047–9.565	81.59
<i>T. josei</i> /"Core" <i>Tettigettalna</i>	6.965 \pm 0.080	2.548–12.297	8.40

TABLE 3 Mean age estimates in million years ago (ma) and 95% highest probability density intervals of tMRCA as in Figure 5 according to *BEAST analysis (multispecies coalescent model). Clade support is given in percentage of trees post-burnin that support that topology

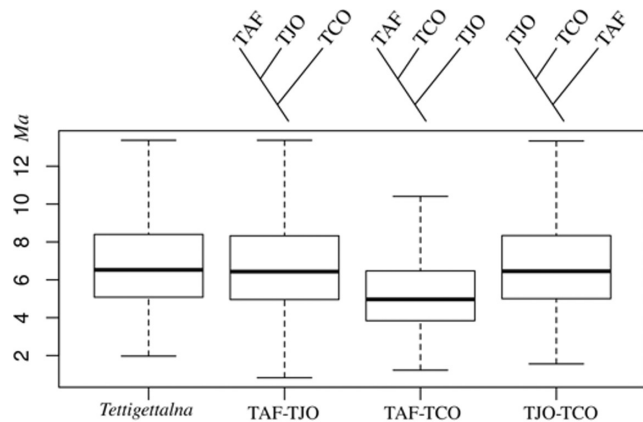


FIGURE 5 Age estimate boxplots of the possible nodes by the multispecies coalescent species-tree with *BEAST. The first boxplot plots the age estimates of the basal node of *Tettigettalna*, with the remainder plotting a different topology (TAF, *T. afroamissa*, TJO, *T. josei*, TCO, "core" *Tettigettalna*).

with the reopening of the Strait of Gibraltar and the end of the MSC (Figure 6).

4 | DISCUSSION

4.1 | Phylogeny of *Tettigettalna*

With this study, we used an extended set of mitochondrial sequences and obtained the first nuclear data to investigate the evolution of the Mediterranean genus *Tettigettalna*. These small-sized and colour-cryptic cicadas occur at fairly low density, being very difficult to sample without the use of acoustic location. Hence, and because females are mute and seldom seen, the dataset is composed of males only. The inclusion of nuclear sequences is particularly relevant to corroborate patterns found with mitochondrial data and discard potential bias in male dispersal. As expected, nuclear EF-1 α had much lower diversity than mitochondrial genes and lacked resolution in *Tettigettalna*, though without conflicting with mitochondrial phylogenies.

Regarding the order of lineage splitting among *Tettigettalna*, our study gives support to *T. josei* as sister

to the remaining extant members of the genus, consistent with morphological and acoustic differentiation observed for this species (Mendes et al., 2014). Nonetheless, the other two phylogenetic scenarios in the species coalescent tree (i.e. subclades *T. josei* – Core *Tettigettalna* and *T. afroamissa* – *T. josei*) have some degree of support (8%–9%). Species *T. afroamissa* and *T. argentata* are among the most genetically distant taxa in the phylogeny, even though they share a similar calling song pattern. *Tettigettalna* has no parallel with any other cicada genus occurring in Iberia, being the most species rich, with species defined mainly by song characterization (Puissant & Sueur, 2010). Such diversity of songs within *Tettigettalna* has evolved without significant morphological divergence, not even at genitalia morphology, often a key trait to distinguish between closely related species, as it may prevent successful mating of heterospecific couples (Knowles et al., 2016; Langerhans et al., 2016). A few song operational taxonomic units within *Tettigettalna* remain polyphyletic with the new sequence data as in Nunes, Mendes, Marabuto, et al. (2014) and Costa et al. (2017), namely the widespread *T. argentata* with cryptic *T. mariae* and *T. aneabi*, which present partially overlapping distribution ranges (Nunes, Mendes, Quartau, et al., 2014). Insect songs are genetically inherited but the genes ruling them remain poorly known, in particular for cicadas (Fujisawa et al., 2018; Sueur, 2006; Xu & Shaw, 2019, 2021). Neutral genetic markers fail to differentiate among such close species, either because divergence among their songs is too recent for complete lineage sorting or episodes of introgression on secondary contact have eroded such divergence, or very likely both. Similar cases of polyphyly among cryptic species of Cicadettinni have been detected in *Cicadetta* of Italy and Greece (Hertach et al., 2015, 2016; Wade et al., 2015), and in *Kikihia* and *Maoricicada* of New Zealand (Buckley et al., 2006; Marshall et al., 2008), where molecular phylogeny failed to recover some acoustically defined taxa.

Only a few nuclear genes have been sequenced thus far to investigate phylogenetic relationships among cicadas worldwide, being EF-1 α the most extensively used and informative (Arensburger, Buckley, et al., 2004; Banker et al., 2017; Buckley & Simon, 2007; Hill et al., 2021; Lee & Hill, 2010; Marshall et al., 2008, 2016, 2018; Owen

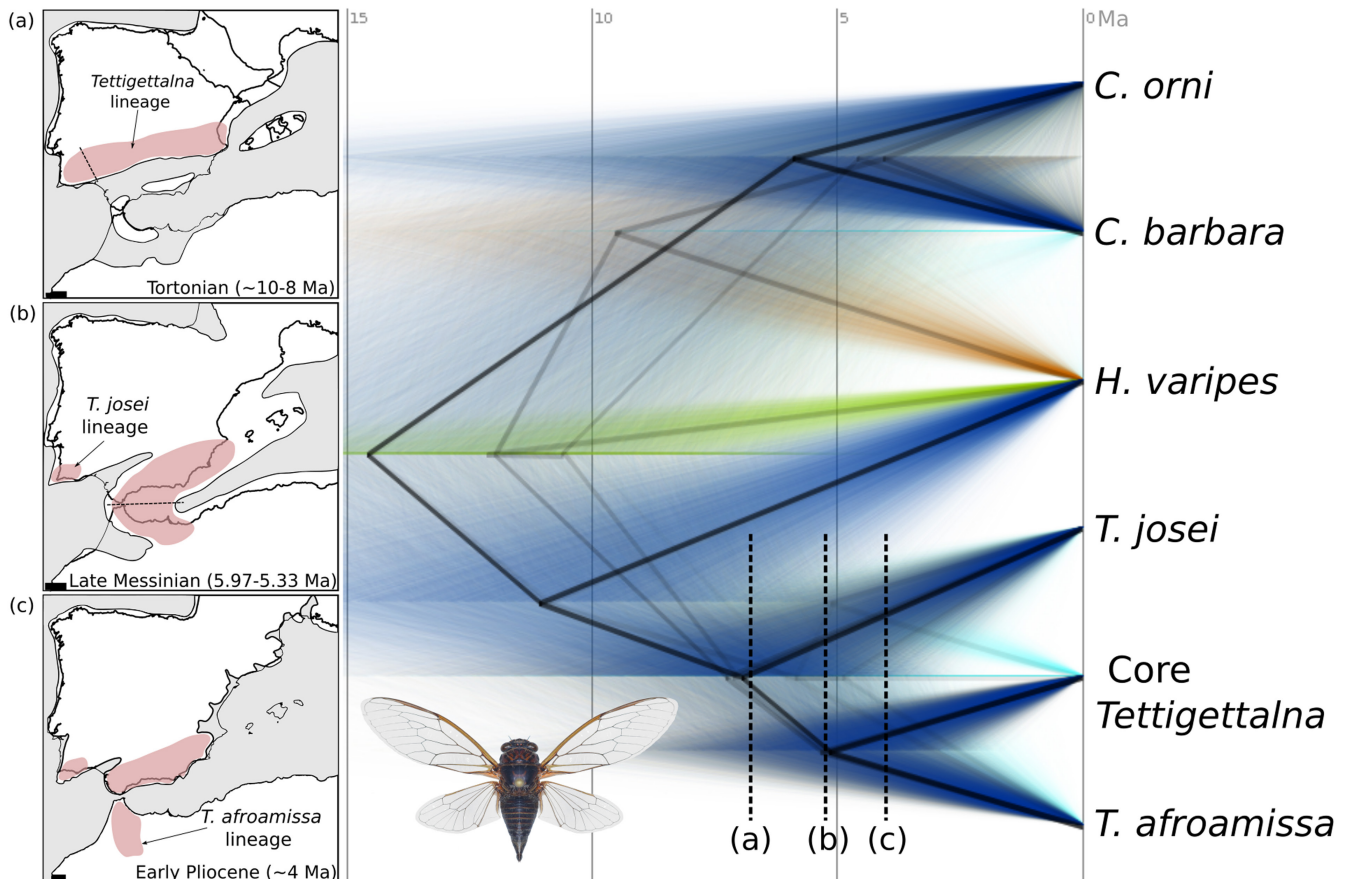


FIGURE 6 DensiTree output of the Bayesian inference species tree of *Tettigetta* with the partitioned unlinked mtCOI and nuEF-1 α dataset. The consensus trees are shown by the bold blue line. Uncertainty of node heights and topology is shown by the transparent green, purple and red lines. Core *Tettigetta* refers to the clade composed of the remainder of the *Tettigetta* (see methods for explanation). Scale bar indicates ma. The broken lines (A-C) refer to key moments in time illustrated in the left panes. (A) Mid-Tortonian (~10–8 ma) when the ancestral population of the *Tettigetta* occurred in the southern Iberian Peninsula; the broken line marks the separation of the *T. josei* lineage from the main ancestral population. (B) Late Messinian, during the salinity crisis, when the main population disperses to North Africa, via the formed land bridge; the broken line indicates the rupture caused by the opening of the Gibraltar Strait by end of the Messinian (5.33 ma). (C) Early Pliocene (~4 ma), showing the three lineages: *T. josei* in southwestern Iberia; *T. afroamissa* in Morocco and the remainder of the European *Tettigetta* lineage, which would later diverge into all other species. In the lower left corner, a female of the Moroccan species *T. afroamissa* is shown

et al., 2017; Price et al., 2019; Simon et al., 2019). Nuclear genes have been shown as insufficient to resolve the phylogeny of cryptic cicada species where hybridization is suspected, even when multiple genes were used (Banker et al., 2017; Buckley & Simon, 2007; Wade et al., 2015). A genomic approach and a fine-scale sampling at contact zones would be preferable to overcome single-gene shortcomings and will certainly help to disentangle introgression events and incomplete lineage sorting among *T. argentata*, *T. mariae* and *T. aneabi*.

Another incongruence between acoustics and genetics found within the *T. helianthemis* ssp. remains unexplained, as *T. h. galantei* Type II, though sharing the same calling song with *T. h. galantei* Type I, is

remarkably different at mitochondrial data. *T. h. galantei* Type II has a parapatric distribution with Type I (they were not overlapping) with no obvious breaks in habitat features to justify such level of genetic divergence. Amplification of nuclear mitochondrial DNA sequences (NUMTs) could be a reason for this pattern of divergence i.e., mutations at the primer binding sites that would bias amplification towards nuclear copies of COI at this particular taxon (Song et al., 2008). This seems unlikely since the same phylogenetic pattern was recovered for all three independently amplified fragments of mitochondrial DNA (COI-LEP, COI-CTL and ATP). Further studies with phylogenomic data should bring some light to the roots to this mismatch.

4.2 | Biogeographic scenarios

Time estimates inferred with the concatenated model and the multispecies coalescence model for the oldest splits within *Tettigettalna* have both very wide confidence intervals (12–2 Ma), with which we cannot confidently exclude any of the three scenarios. However, if considering mean ages, both methods place the divergence of *T. josei* as a likely pre-MSA or early MSA event, probably before the land-bridge was fully established (mean ages of 5.9 and 6.9 Ma, respectively). As for the divergence of the Moroccan *T. afroamissa* from the remainder of the European *Tettigettalna*, mean age estimates place this event as a likely post-MSA event (4.8 and 5.3 Ma, respectively). Divergence time estimates are sensitive to assumptions used in clock calibration, hence a considerable degree of uncertainty is inevitably associated to inferred dates (Carruthers & Scotland, 2020), particularly among cicadas, where no accurate fossil calibration can be used. When dating divergence events among Cicadellini, Marshall et al. (2016) tested many assumptions and attempted to correct inflation when using literature based values from other arthropods. Even though relaxed clock models accommodate more uncertainty about the substitution rate, configuring analyses such that they adequately account for uncertainty remains a challenge (Carruthers & Scotland, 2020).

When coupling divergence-time estimates with the tree topology, our most parsimonious biogeographic reconstruction has a chain of events scenario involving three phases (Figure 6). First, an ancestral *Tettigettalna* population was likely present across the southern Iberian Massif before or during early Messinian (Figure 6A). Then, in the second phase, the ancestor of *T. josei* became geographically or ecologically disconnected from the remainder of the *Tettigettalna* in the southwest, where today is southern Portugal (Figure 6B). This divergence is concurrent with the closing of the Baetic and Guadalhorca corridors, 7.3 and 6.8 Ma, respectively, and the formation of the Guadalquivir Basin (Martin et al., 2001; Martín et al., 2009). Presently, there is little recorded evidence for the role of the Guadalquivir Sea basin as a biogeographical barrier within the Iberian Peninsula, but it has been implied in the divergence of two Iberian subspecies of fire salamander, though more recently, during the early Pliocene (Antunes et al., 2018; García-París et al., 1998). The progressive uplift of the Baetic basement basin led to the formation of the Eurafrikan land connection (Schoorl & Veldkamp, 2003), enabling the main *Tettigettalna* ancestral population to migrate southwards, into Africa (Figure 6B). Finally, in the third phase, the reopening of the Strait of Gibraltar at 5.33 Ma likely worked as an effective barrier to gene flow between the ancestor of

T. afroamissa in Africa and “core” *Tettigettalna* in south-eastern Iberia (Figure 6C). Examples of rupture of genetic and biological intercontinental continuity with the end of MSA are well known among western Mediterranean lineages occurring in Iberia and/or the Maghreb. To cite only some of the most remarkable, the re-opening of the Strait of Gibraltar isolated midwife toads *Alytes maurus* from *Alytes dickhilleni* and *Alytes muletensis* on opposite sides of the Mediterranean (Martínez-Solano et al., 2004), and the same happened to trapdoor spiders *Ummidia* sp. ‘Tarifa’ and *U. aedificatoria* (Opatova et al., 2016) and to Anthocharidini butterflies (Marabuto et al., 2020).

The lower 95% HPD for the divergence of *T. afroamissa* points to less than 2.5 Ma, although a founder event resulting from post-MSA long dispersal to Africa seems very unlikely. Adult cicadas are short-lived (1–6 weeks) and need to feed daily on plant sap, turning long travelling overseas without feeding sources on the way into a risky endeavour. Dispersal in cicadas seems to occur mostly in a step-wise fashion, with travel distances reported as not exceeding 1 km for large-body *Quesada gigas* (Andrade et al., 2020), 500 m for *Cicada orni* (Simões & Quartau, 2007), or 150 m for *Magicicada* spp. (Karban, 1981). Even in a scenario where dispersal across Gibraltar was mediated by extreme winds or other stochastic events (Mas-Peinado et al., 2021; Osozawa et al., 2021), the likelihood of survival and successful settling afterward is low. Some accidental human-mediated introductions of cicada nymphs have been occasionally reported, likely via potted plants trading (Hertach & Nagel, 2013; Hill et al., 2005; Osozawa et al., 2021; Pons et al., 2021), but this hypothesis can be safely discarded for *T. afroamissa*, as it was found in remote and nearly untouched habitats of Morocco (Costa et al., 2017). Adding to this, the deep level of divergence of *T. afroamissa* from Iberian *Tettigettalna* is not compatible with an anthropogenic founder event.

4.3 | The effect of Pleistocene climate changes

After the MSA, with *T. afroamissa* isolated in the Maghreb, and *T. josei* in SW Iberia, the chain of events therein is however less well supported. Nevertheless, it seems to be closely tied to the Pleistocene climate changes, since age estimates inferred with the concatenated model for species within “core” *Tettigettalna* resulted in upper 95% HPD that do not predate 2.9 Ma, well after the reopening of the Strait of Gibraltar. Splits are estimated to have occurred in the early to mid-Pleistocene (Figure 4: nodes F, G, I and J), during the Gelasian and Calabrian periods (2.58–0.781 Ma), coinciding with the acceleration of glacial conditions and the retreat of populations into more

favourable areas, especially the thermophilic ones (Gómez & Lunt, 2007). Given the heightened species richness of this area, complex geography, and climatic stability over long periods of time (Manzano et al., 2017), it is conceivable that the most recent common ancestor for most modern *Tettigetta* could have inhabited the southern portion of the Iberian Peninsula, and especially the Baetic area. Nowadays, *Tettigetta* spp. in southern Iberia have parapatric or partially overlapping distributions, and it is likely that finer-scale sampling would detect hybrid zones between sister taxa, as detected for Cicadettini cicadas of the genus *Kikihia* (Banker et al., 2017; Marshall et al., 2008, 2011). The genus *Kikihia* provides a remarkable example of species-rich lineages that experienced rapid evolutionary radiation of song-defined cryptic species during the Pleistocene, as mountain-building isolated populations and affected habitat structure in New Zealand (Marshall et al., 2008, 2011). This process might be analogous to what could have happened with *Tettigetta* in southern Iberia, at a smaller geographical scale.

Among extant Iberian “core” *Tettigetta*, only *T. estrellae* seems not to be affiliated with the Baetic southern hotspot of diversity. Instead, the NW Iberian distribution of *T. estrellae* seems to gravitate around a putative glacial refugium represented by the Douro valley or mountains around it, as seen for other ectotherms such as newts (Martínez-Solano et al., 2006), lizards (Miraldo et al., 2011; Paulo et al., 2001), and vipers (Martínez-Freiria et al., 2020). Likewise, the northern clade of widespread *T. argentata* probably expanded from some uncertain refugia, up and beyond the Iberian Peninsula. Haplotypes found in France and Italy seem to be the most derived, but further sampling and genomic data is necessary to investigate the phylogeography of *T. argentata* in its full range. We found no consistent differences in ecology or morphology compared with other *Tettigetta* spp. that could explain *T. argentata*'s success in expanding northwards (Mendes et al., 2014; Nunes, Mendes, Quartau, et al., 2014; Puissant & Sueur, 2010). Perhaps tolerance to temperate climate, adaptation to a wider range of plant hosts or less competition with other *Tettigetta* while expanding from southern refugia could be hypotheses to explore in the future.

Several other cicada species share a similar trans-Mediterranean distribution as *Tettigetta*. Genera such as *Cicada*, *Tibicina*, *Euryphara*, or *Pseudotettigetta* have species on both sides of the Strait of Gibraltar, but the North-African counterparts remain poorly studied (Pinto-Juma et al., 2009; Puissant & Sueur, 2010; Sueur et al., 2007). We hope that future studies may lessen the knowledge gap between the two continents and investigate the underlying causes for their current patterns of distribution.

AUTHOR CONTRIBUTIONS

Study design: GJC, VLN, OSP, PCS; Sampling: VLN, EM, RM, PP, JMB, TH; DNA isolation and sequencing: VLN; Data analyses: GJC, VLN, DNS; Writing: GJC, VLN. All authors contributed to review the final manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ORCID

Gonçalo J. Costa  <https://orcid.org/0000-0002-9972-6244>

Vera L. Nunes  <https://orcid.org/0000-0003-2689-2409>

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