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A review of cross-backed grasshoppers of the genus *Dociostaurus* Fieber (Orthoptera: Acrididae) from the western Mediterranean: insights from phylogenetic analyses and DNA-based species delimitation

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Abstract. Phylogenetic analyses and species delimitation methods are powerful tools for understanding patterns of species diversity. Given the current biodiversity crisis, such approaches are invaluable for urgent assessment and delimitation of truthful species, particularly of endangered and morphologically cryptic taxa from vulnerable areas submitted to strong climate change and progressive human intervention such as the Mediterranean region. In this study, we applied two DNA-based species delimitation methods and performed a Bayesian phylogenetic reconstruction using three mitochondrial gene fragments (*12S*, *16S* and *COI)* to solve several taxonomic uncertainties among species of cross-backed grasshoppers (genus *Dociostaurus* Fieber) from the western Mediterranean. Phylogenetic analyses demonstrate the polyphyletic character of subgenera *Dociostaurus*, *Kazakia* Bey-Bienko and *Stauronotulus* Tarbinsky and, thus, the necessity of revising the currently accepted taxonomic subgenera within the genus *Dociostaurus*. We propose the split of closely related taxa with allopatric distributions such as *D. (S.) kraussi* and *D. (S.) crassiusculus*, considering the later a distinct species limited to the Iberian Peninsula and excluding the name *crassiusculus* from other forms of *D. (S.) kraussi* from East Europe and Asia. Estimates of divergence times indicate that diversification of *Dociostaurus* probably happened during the Miocene–Pliocene (3–7 Ma), and the split of the studied pairs of sister taxa took place during the middle and late Pleistocene (1–2 Ma). This study highlights the need for more molecular studies on the genus and their different species for a better understanding of their evolution, genetic variation and population dynamics in order to prioritize strategies for their adequate conservation and management.

Introduction

Understanding the origin and diversity of the living world requires a revision of traditional taxonomic practices and the advent of molecular tools has greatly assisted this process (Tautz *et al.*, 2003; Pons *et al.*, 2006; Vogler & Monaghan, 2006). Phylogenetic analyses, coupled with molecular-based species delimitation methods, are nowadays considered fundamental in comprehending current patterns of biological diversity (e.g. Fujisawa & Barraclough, 2013; Huang *et al.*, 2013; Zhang *et al.*, 2013; Solis-Lemus *et al.*, 2015; Yang, 2015). Mitochondrial DNA has proven to be very useful for phylogenetic inference and species delimitation due to its universally amplifiable loci, small genome size, fast rates of molecular evolution, low or absent sequence recombination, and evolutionarily conserved gene products (Pons *et al.*, 2006; Zhang *et al.*, 2013; Amaral

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et al., 2016). Protein-coding genes are suitable to resolve phylogenetic relationships among related species, whereas the most conserved regions of ribosomal RNA genes are useful to establish deep levels of divergence (Simon *et al.*, 1994). In recent years, the employment of DNA markers in taxonomic delimitation has steadily increased and contributed to unravelling cryptic patterns of species diversity that could not be resolved by classical morphological studies (e.g. Allegrucci *et al.*, 2009; Grzywacz *et al.*, 2013; Bocek & Bocak, 2016). This is particularly relevant in groups of species inhabiting geographical regions severely impacted by abrupt climate change and human activities, as these processes may lead rapidly to environmental degradation and the stochastic decline of natural populations (Myers *et al.*, 2000).

Although the Mediterranean region is one of the areas of the world historically most altered by humans (Blondel & Aronson, 1999; Ortego *et al.*, 2015), it constitutes an important biodiversity hotspot (Médail & Quézel, 1999; Myers *et al.*, 2000; Brooks *et al.*, 2006). The main reason for the great species richness and endemism of this region is believed to be associated with its historically high climatic stability in comparison with northern Temperate areas (Blondel & Aronson, 1999; Hewitt, 2000). It is also widely accepted that northern Mediterranean Peninsulas have served both as glacial refugia and as important diversification hotspots (Hewitt, 1999; Petit *et al.*, 2003). Accordingly, most European thermophilous taxa present deep patterns of phylogeographical divergence driven by their retraction into five main refugia during the Pleistocene glacial cycles: the Iberian Peninsula, the Apennine Peninsula, the Balkans, Anatolia and North Africa (Hewitt, 1999). The Mediterranean region is predicted to experience the highest proportion of biodiversity loss among all terrestrial biomes due to its particular sensitivity to a wide range of threats, including land-use alterations and global climate change, and their negative interactions (Giorgi & Lionello, 2008; Klausmeyer & Shaw, 2009). For these reasons, understanding the biological diversity from the Mediterranean region is necessary in order to establish priorities for conservation and inform management practices aimed to preserve its unique diversity (Blondel & Aronson, 1999).

In this study, we adopt an integrative taxonomic approach by examining the phylogenetic relationships and taxonomic status of western Mediterranean *Dociostaurini* (Mistshenko, 1974), a tribe of Orthoptera comprising several species of either great conservation concern or important economic interest (e.g. Latchininsky, 1998, 2013; Hochkirch *et al.*, 2016). This tribe comprises eight different genera, with three of them (genera *Dociostaurus*, *Notostaurus* and *Xerohippus*) being represented in the Western Mediterranean region. *Dociostaurus* are grasshoppers with an 'X'-shaped pattern on their pronotum, three well-developed transverse grooves and obliterated lateral ridges in the posterior half of the prozona, convex vertex and closed foveolae with distinct sharp margins visible (Fieber, 1853). Species within this genus are distributed mainly in South Europe, North Africa, Angola, the Canary Islands, the Madeira archipelago, Central Asia, and the Hawaiian Islands (Cigliano *et al.*, 2017). They constitute one of the most common grasshoppers living in desert and semi-desert landscapes of the Palaearctic region (e.g. Sirin & Mol, 2013). The genus comprises 30 described species and three subgenera separated by some morphological traits: 16 species in the subgenus *Dociostaurus* Fieber; 6 species in the subgenus *Kazakia* Bey-Bienko; 7 species in the subgenus *Stauronotulus* Tarbinsky (Cigliano *et al.*, 2017), and a new controversial species, *Dociostaurus biskrensis* Moussi & Petit, that has not been yet assigned to any subgenus (Moussi *et al.*, 2014). The description of new species in *Dociostaurus* is relatively recent as more than half of the taxa within the genus were described in the 20th Century. However, most taxonomic efforts on the group have been focused on the identification of morphological diagnostic traits and bioacoustic signals (e.g. Harz, 1975; Soltani, 1978; García *et al.*, 2005), an approach presenting certain limitations when dealing with some closely related and phenotypically similar species (e.g. sibling and/or cryptic species).

The employment of DNA markers can help to define species boundaries and resolve several taxonomic ambiguities within *Dociostaurus*, which is of particular interest given that different species of this genus are of great conservation concern or constitute important agricultural pests. According to the International Union for Conservation of Nature (IUCN), there are several species of *Dociostaurus* included in the European Red List of Orthoptera (Hochkirch *et al.*, 2016). One of them is *Dociostaurus* (*Dociostaurus*) *minutus* La Greca, a brachypterous narrow endemic species restricted to some coastal dunes in south-east Sicily (Massa, 2011; Massa *et al.*, 2012). The extremely small distribution range of this species, together with the considerable degradation of coastal habitats, has motivated its inclusion in the European Red List of Orthoptera with the category 'endangered' (Bushell, 2013; Hochkirch *et al.*, 2016). The situation is very different for the Moroccan locust *Dociostaurus* (*Dociostaurus*) *maroccanus* (Thumberg), a pest species of many crops with considerable economic impacts, although currently scarce in many areas (reviewed in Latchininsky, 1998, 2013). Among Mediterranean species, there are also a couple of interesting cases of sibling/cryptic species of conservation concern that present disjunctive distributions in vast areas between the Western Mediterranean, on the one side, and Eastern Europe and Central Asia, on the other side: *D. (Stauronotulus) crassiusculus crassiusculus* (Pantel) for *D. (S.) kraussi* (Ingenitskii), and *D. (D.) hispanicus* Bolívar for *D. (K.) brevicollis* (Eversmann). *Dociostaurus (S.) c. crassiusculus* and *D.* (*D.*) *hispanicus* are Iberian endemics that have been recently assigned to the categories 'endangered' and 'near threatened', respectively, due to the high fragmentation of their small populations (Cordero *et al.*, 2010; Hochkirch *et al.*, 2016). The taxonomic relationship of *D. (S.) crassiusculus* and *D. (S.) kraussi* is controversial and has been modified by different authors according to morphological criteria (e.g. Harz, 1975; Soltani, 1978; Hodjat, 2016). Resolving the taxonomic status of these two putative species is particularly interesting because they involve one endangered Iberian endemic and a relatively abundant species presenting a disjunctive distribution in Eastern Europe and Asia. The same controversial case occurs with the pair *D. (D.) hispanicus* and *D. (K.) brevicollis*, also with disjunctive distributions and the last one being a widely distributed common species in Eastern Europe (Cigliano *et al.*, 2017). Taxonomic problems also involve common and widely distributed species like *Dociostaurus* (*Kazakia*) *jagoi* Soltani, with populations showing subtle morphological differences at different sides of the Mediterranean Sea: *D. (K.) j. occidentalis* in South Europe and *D. (K.) j. jagoi* in North Africa.

The systematics of *Dociostaurus* from the western arc of the Mediterranean region is reviewed. In particular, this study: (i) analyses the phylogenetic relationships for most taxa of the genus; (ii) evaluates the validity of current supraspecific classification (i.e. genera/subgenera); and (iii) employs species-delimitation methods to resolve the taxonomic status of controversial sibling species with a disjunctive Palaearctic distribution.

Material and methods

Taxon sampling

Between 2007 and 2015, samples from different species belonging to the *Dociostaurini* tribe (Mistshenko, 1974) were collected: seven species of the genus *Dociostaurus* and one species of the genus *Notostaurus* (Table S1; Fig. 1). For each specimen, collection date, locality, geographical coordinates and elevation were recorded. Fresh whole adult specimens were stored in 2000 μL ethanol 96% at −20°C until needed for DNA extraction. The identification of doubtful specimens was checked against the entomological collection of the National Museum of Natural History (MNCN) in Madrid. Nine specimens of *D. (K.) brevicollis* collected in different localities from Turkey and Russia, and sequences of *D. (S.) kraussi* and *D. (S.) crassiusculus nigrogeniculatus* deposited in the GenBank (accession numbers KR005944, KR014937 and KM816675) were used for genetic comparison with *D. (D.) hispanicus* and *D. (S.) c. crassiusculus*, their respective putative sibling species from the Western Mediterranean region.

DNA extraction, amplification and sequencing

Nucleo Spin Tissue kits (Macherey-Nagel, Durën, Germany) were used to extract and purify total DNA (mitochondrial DNA + genomic DNA) from a hind leg of each specimen. Segments of three mitochondrial genes: 12S rRNA (*12S*), 16S rRNA (*16S*) and cytochrome oxidase subunit I (*COI*) were amplified for each sample by PCR (Table S2). Previous studies in Orthoptera have demonstrated that these molecular markers are informative and useful for comparison within and among species (e.g. Ortego *et al.*, 2009; Vedenina & Mugue, 2011; Çiplak *et al.*, 2014). A nuclear gene fragment, the internal transcribed spacer 2 (*ITS2*), was also amplified but it could not be used for subsequent analyses due to the high frequency of indels and unambiguous peaks in most sequences.

PCR amplifications were performed in 15 μL reaction volumes including 1× reaction buffer, 2 mM $MgCl_2$, 10 mM of each dNTPs, 10 μM of each primer and 5 U/μL of Immolase DNA Polymerase (Bioline Reagents, London, U.K.). Amplifications were carried out on a Mastercycler EpgradientS (Eppendorf, Hamburg, Germany) thermal cycler under the following program: 9 min denaturing at 95°C followed by 40 cycles of 30 s at 94°C, 45 s at the annealing temperature (*12S*: 50°C; *16S*: 60°C; *COI*: 48°C) and 45 s at 72°C, ending with a 10 min final elongation stage at 72°C. PCR products were visualized on 2% agarose gels stained with Orange G (10 mM Tris–HCl pH 7.6, 0.15% Orange G, 60% glycerol, 60 mM EDTA). Amplified products were commercially purified and sequenced (Macrogen, Seoul, South Korea).

Bayesian phylogenetic reconstruction

All sequences were visually inspected, edited and trimmed to the same length to remove ambiguous ends using the software SEQUENCHER v5.2.4 (Gene Codes Corporation, Ann Arbor, MI). Sequences were submitted to GenBank with accession numbers KX954639-KX954810 (Table S1). The genetic dataset was complemented with three sequences of *COI* from GenBank: two of *D. (S.) kraussi* (accession numbers KR005944 and KR014937) and one of *D. (S.) c. nigrogeniculatus* (accession number KM816675), all of them obtained from specimens collected in Xinjiang (China). Sequences were aligned using CLUSTALW on the Web Server of Kyoto University Bioinformatics Center with opening gap = 10 and extension gap penalty = 0.10 (e.g. Allegrucci *et al.*, 2014). The number of haplotypes, haplotype diversity and the number of polymorphic sites were calculated in DNASP v5 (Librado & Rozas, 2009). Neutrality tests (Fu and Li′s *D*-statistic tests and Tajima's *D*-tests) were performed as implemented in DNASP v5. Genetic differentiation among sequences was estimated using Kimura 2-parameter genetic distances in MEGA v5.0 (Tamura *et al.*, 2011).

The three mitochondrial gene fragments (*12S*, *16S* and *COI*) were treated as separate data partitions in phylogenetic analyses. JMODELTEST v2.1.7 was used to find the best-fitting model of nucleotide evolution for each gene fragment in a hierarchal hypothesis testing framework based on the Bayesian Information Criterion (BIC) (Darriba *et al.*, 2012). For phylogenetic analyses, the three mitochondrial gene fragments were concatenated in a data matrix of 1507 bp using MEGA v5.0 (Tamura *et al.*, 2011).

We inferred an ultrametric tree and estimated divergence times for mtDNA sequences using BEAST 1.8.3 (Drummond *et al.*, 2012). Analyses on BEAST were performed using concatenated data from the three mitochondrial gene fragments and only using the *COI* gene. For both, different clocks and demographic models were considered. Each analysis was run with two independent Markov chains for 100 million generations sampled every 10 000 generations (i.e. 10 000 retained genealogies). Posterior probabilities were calculated from post-burn trees. Fossil evidence or adequate events of geological variance to calibrate the molecular clock are not available. Thus, to approximate absolute ages of divergence among *Dociostaurus* species, molecular clocks were calibrated

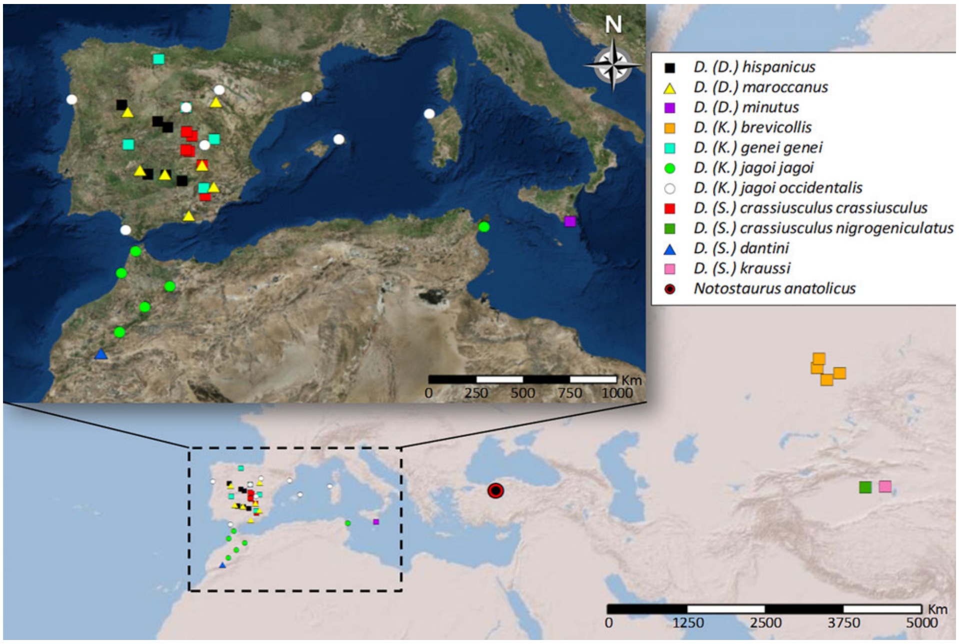

Fig. 1. Geographical location of samples of different species/subspecies of the genus *Dociostaurus* used for phylogenetic analyses. *Dociostaurus (S.) kraussi* and *D. (S.) c. nigrogeniculatus* were retrieved from GenBank and their location is approximate. *Notostaurus anatolicus* was used as outgroup. [Colour figure can be viewed at wileyonlinelibrary.com].

using mutation rates reported in the literature for insects (mean ± S.D.; *16S* = 0.0049 ± 0.0008 substitutions/site/Ma; *COI* = 0.0169 ± 0.0019 substitutions/site/Ma; Papadopoulou *et al.*, 2010) and used default values from BEAST for the *12S* gene. Each run was inspected in TRACER v1.6 (Rambaut *et al.*, 2014) in order to check the convergence to stationary of model parameters and that Effective Sample Sizes (ESS) were always much higher than 200. Afterwards, the two replicate independent runs for each analysis were combined using LOGCOMBINER v1.8.3 (Drummond *et al.*, 2012). The first 10 million generations were discarded as burn-in period [burn-in of 10% of Markov Chain Monte Carlo (MCMC)]. The best-fitting clock and demographic model were determined using Akaike's information criterion through Markov Chain Monte Carlo (AICM; Baele *et al.*, 2012) with 100 bootstraps as implemented in TRACER 1.6. Finally, TREEANNOTATOR v1.8.3 (Drummond *et al.*, 2012) and FIGTREE v1.4.2 (Rambaut, 2014) were used to draw Bayesian consensus trees and obtain 95% highest posterior density (HPD) intervals.

DNA-based species delimitation

We used two independent species delimitation methods that do not require a priori taxonomic information: the General Mixed Yule Coalescent (GMYC) model (Fujisawa & Barraclough, 2013) and the Bayesian Poisson Tree Processes model (bPTP) (Zhang *et al.*, 2013). The GMYC model requires an ultrametric tree as input, so the Bayesian tree generated by BEAST as described above was used, with and after outgroup removal to provide the most robust diversity estimates (Montagna *et al.*, 2017). The Bayesian tree generated by BEAST was converted into a Newick file with FIGTREE and used to run GMYC with a single-threshold method in the species delimitation web server (http://species.h-its.org/gmyc/).

In the bPTP species delimitation model, branch lengths represent the number of substitutions, not time, eliminating the problems associated with requiring a calibrated tree when *a priori* information on divergence time is not available (Zhang *et al.*, 2013). bPTP tends to overestimate the number of species when using multiple sequences per population (Zhang *et al.*, 2013). Thus, the number of sequences in the data matrix was reduced to one individual per population ($n = 50$) for this species delimitation analysis. RAXML v8.2.9 (Stamatakis, 2014) was used to build a non-ultrametric phylogenetic tree in the CIPRES Science Gateway (Miller *et al.*, 2010). The output from RAXML with RAXML-HPC BLACK BOX model was used as input for bPTP analyses in the species delimitation web server (http://species.h-its.org/ptp/), specifying outgroup, considering 100 000 MCMC generations, a thinning value of 100, and a burn-in of 10%.

Results

mtDNA sequence data and polymorphism

Fifty-eight individual sequences of *COI*, *16S* and *12S* gene fragments were obtained, and three more *COI* sequences were retrieved from GenBank (KR005944, KR014937 and KM816675).

Table 1. Descriptive statistics for the three mtDNA genes used in this study (*12S*, *16S* and *COI*): number of analysed individuals (*N*), number of haplotypes (*H*), number of polymorphic sites (*S*), haplotype diversity (*Hd*), nucleotide diversity (π) and Theta per sequence from S (*Theta-W*).

	12S	*16S*	*COI*
N	58	56	61
H	21	22	39
S	51	57	177
Hd	0.940	0.949	0.978
π	0.032	0.024	0.100
Theta-W	11.017	12.409	37.822

No sequence presented nucleotide double peaks that could prompt the existence of nuclear mitochondrial DNA sequences (NUMTs). In the case of the *COI*, internal stop codons that could suggest the amplification of pseudogenes were also absent. Results of the genetic variability analyses obtained from the three different mtDNA genes used (*12S*, *16S* and *COI*) and calculated without considering gaps and missing data are shown in Table 1. In particular, *COI* revealed the highest nucleotide and haplotype diversity values (Table 1). For the concatenated dataset of mitochondrial DNA fragments, Fu and Li′s *D*-statistic was 2.21 and significantly higher than zero ($P < 0.02$). However, Tajima's test of selective neutrality was not significant ($P > 0.1$). The average number of nucleotide differences for the concatenated dataset was 23.33 between sequences of *D. (S.) crassiusculus* and *D. (S.) kraussi* and 49.67 for the comparison involving *D. (D.) hispanicus* and *D. (K.) brevicollis*. Kimura two-parameter genetic distances among species for *COI* spanned between 4.0 and 16.0%. Interspecific genetic distances for *COI* between pairs of closely related species ranged between 4.0% for *D. (S.) crassiusculus* and *D. (S.) kraussi*, and 7.6% for *D. (D.) hispanicus* and *D. (K.) brevicollis*. Between the two putative subspecies of *D. (S.) crassiusculus* [*D. (S.) c. crassiusculus* and *D. (S.) c. nigrogeniculatus*] pairwise genetic distance was 2.8%.

Bayesian phylogenetic reconstruction

For phylogenetic analyses in BEAST, the Hasegawa–Kishino–Yano model with invariable sites (HKY+I) was used for *12S* and *16S*, and the General Time-Reversible nucleotide substitution model with gamma-distributed rate heterogeneity (GTR+G) for *COI*. The clock and demographic model best fitting (i.e. with the lowest AICM values) the concatenated dataset (*12S*, *16S* and *COI*) was a 'strict' molecular clock model with coalescent exponential growth. All BEAST runs converged and ESS values obtained were always above 200. Bayesian and maximum-likelihood (ML) phylogenetic analyses produced a similar consensus topology and, in general, most clades were well supported by both Bayesian posterior probabilities and bootstrap values (Fig. 2). The obtained phylogenetic tree grouped most species/subspecies in good agreement with traditional classification based on phenotypic data, but the analyses also revealed that the current taxonomic nomenclature within the genus *Dociostaurus* present certain incongruences that require revision (Fig. 2): (i) Phylogenetic analyses indicated that species within the genus *Dociostaurus* from the Western Mediterranean constitute a polyphyletic group; (ii) *D. (S.) dantini* Bolívar, 1914 from Morocco resulted the most distant taxon and grouped with the outgroup (genus *Notostaurus*); (iii) the two species from the subgenus *Stauronotulus*, the Iberian *D. (S.) c. crassiusculus* and the Asian *D. (S.) kraussi*, grouped together as sister species in a monophyletic clade that was quite distant from the rest of species; (iv) in this clade, *D. (S.) c. nigrogeniculatus* grouped with *D. (S.) kraussi* (sequences from GenBank) but not with *D. (S.) c. crassiusculus* contrary to expectations from current taxonomic classification; (v) the Iberian *D. (D.) hispanicus* clustered as sister taxon of *D. (K.) brevicollis* specimens from Russia and Turkey; (vi) the two subspecies of *D. (K.) jagoi*, *D. (K.) j. occidentalis* corresponding to specimens from southern Europe and *D. (K.) j. jagoi* from North Africa, grouped as sister taxa; (vii) finally, the analyses showed that the subgenera *Kazakia, Dociostaurus* and *Stauronotulus* are polyphyletic, indicating that current hypotheses of subgeneric delimitation require revision (Table 2; Fig. 2).

Divergence time estimates based on the three mitochondrial gene fragments (*12S*, *16S* and *COI*) indicated that the split into major clades occurred during the Miocene (~6.36 Ma; HPD: 4.82–8.16). Sister taxa splits such as *D. (S.) c. crassiusculus – D. (S.) kraussi* and *D. (D.) hispanicus – D. (K.) brevicollis* occurred around 1.01 Ma (HPD: 0.6–1.52) and 1.88 Ma (HPD: 1.30–2.56), respectively. Subspecies of *D. (S.) c. nigrogeniculatus* diverged from *D. (S.) kraussi* 0.35 Ma (HPD: 0.13–0.63), more recently than the well-established subspecies of *D. (K.) j. jagoi* and *D. (K.) j. occidentalis* (0.71 Ma; HPD: 0.46–1.03) (Fig. 3).

DNA-based species delimitation

The GMYC model of species delimitation yielded 10 ML entities, including outgroup, with a confidence interval of 8–12. In agreement with the current number of recognized species, the results of GMYC showed that each entity corresponded to a described morphological taxon. Accordingly, sequences corresponding to different subspecies clustered together in a unique species (Fig. 2). The bPTP model retrieved 12 species, including outgroup, with a confidence interval of 10–21 estimated species. The most conservative number of species (10) yielded by the bPTP model matched exactly with the taxa established by current taxonomic classification. The bPTP model set boundaries of species delimitation at a lower taxonomic level than GMYC, identifying subspecies as species. However, the lowest posterior delimitation probabilities corresponded to those assigned to the two subspecies of *D. (K.) jagoi* and to the separation of *D. (S.) kraussi* and *D. (S.) c. nigrogeniculatus* (Fig. 2).

Discussion

The GMYC and bPTP models yielded 10–12 taxonomic entities that correspond well with the current number of accepted species

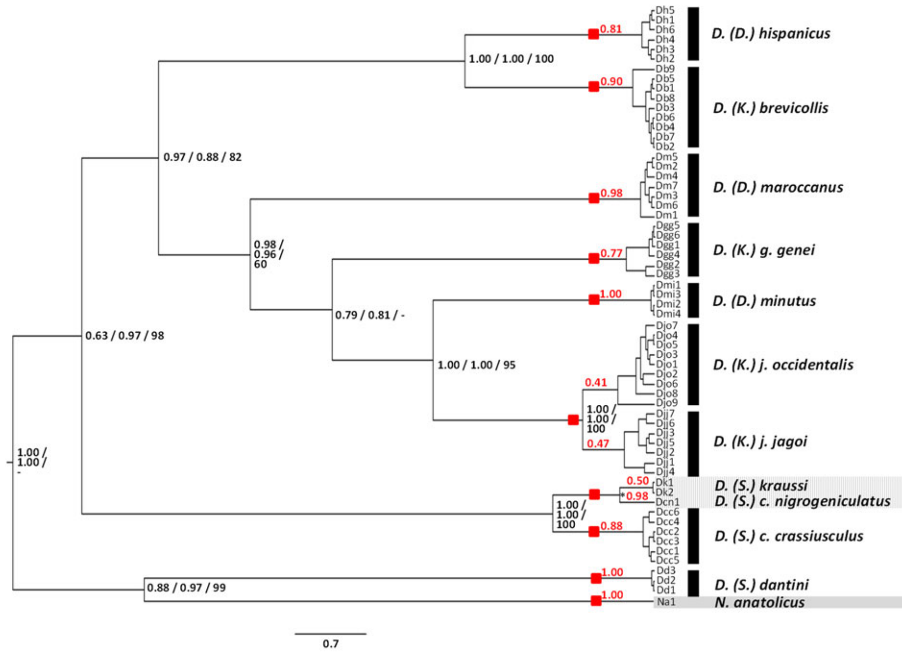

Fig. 2. Phylogenetic reconstruction of the genus *Dociostaurus* using Bayesian analyses in BEAST 1.8.3 and maximum-likelihood analyses in RAXML. Numbers in nodes indicate posterior probabilities from BEAST analyses performed on concatenated data for three mtDNA genes (*12S*, *16S* and *COI*), posterior probabilities from BEAST analyses based on *COI* gene only, and bootstrap support values (over 50%) from RAXML analyses based on the three mtDNA genes. Species delimited using the General Mixed Yule Coalescent (GMYC) model are indicated with small red filled squares and posterior delimitation probabilities from the Bayesian Poisson Tree Processes (bPTP) model are shown with red numbers. *Notostaurus anatolicus* was used as outgroup (shaded in grey). Sequences of *Dociostaurus (S.) kraussi* and *D. (S.) c. nigrogeniculatus* (only available for *COI* gene) were retrieved from GenBank (striped in grey). Species names follow Cigliano *et al.* (2017). *1.00/1.00/85. [Colour figure can be viewed at wileyonlinelibrary.com].

(Fig. 2). However, the general agreement between molecular and classical taxonomy at the species/subspecies level contrasts with a remarkable number of incongruences at a higher taxonomic level (genus/subgenus) that require thorough revision (Cigliano *et al.*, 2017). These analyses point out that increasing the number of molecular markers or adding different data to attempt higher success in species delimitation does not necessary improve the performance of the analysis, although it could improve its statistical power (Blaimer, 2012). Phylogenetic analyses revealed that, at the subgeneric level, *Dociostaurus*, *Kazakia* and *Stauronotulus* do not cluster according to the expectations of current proposed taxonomic classification (Cigliano *et al.*, 2017). Soltani (1978) completely reordered the genus *Dociostaurus*, synonymizing the subgenera *Dociostaurus* and *Stauronotulus* (see Table 2) and including the subgenus *Notostaurus* within the genus *Dociostaurus*. He also included the species *D. (D.) minutus* into the subgenus *Kazakia*, with *D. (K.) brevicollis*, *D. (K.) jagoi* and *D. (K.) genei*, instead of in its current subgenus *Dociostaurus*. This author also proposed inclusion of *D. (D.) hispanicus* into the subgenus *Kazakia* by its proximity with *D. (K.) brevicollis*, which is congruent with the results of this study (Figs 2, 3). The phylogenetic analyses placed *D. (S.) dantini* together with *Notostaurus anatolicus* (Krauss) (outgroup) (Figs 2, 3). This result is in agreement with the study by Soltani (1978) suggesting that the genus *Notostaurus* should be considered a subgenus within the genus *Dociostaurus*, a nomenclature also followed by other more recent studies (e.g. Sirin & Mol, 2013; Mol *et al.*, 2014). Considering different ways of assigning species, the results of the present study suggest a classification more akin to Soltani's proposal (Table 2). Given that taxonomic changes have occurred very often within the genus *Dociostaurus* on the basis of phenotypic traits that may vary among populations (e.g. Mistshenko, 1974; Soltani, 1978; Hodjat, 2016), we conclude that the status of subgenera within *Dociostaurus* is not satisfactory. A possible alternative is using the results of the present phylogenetic study, which are in good agreement with the particular morphological traits previously described to separate the subgenus *Dociostaurus* (Bey-Bienko, 1933; Bey-Bienko & Mistshenko, 1951; Mistshenko, 1974). Thus, both morphological and genetic data support that *D. (K.) brevicollis*, *D. (K.) genei* and *D. (K.) jagoi* should be assigned to subgenus *Dociostaurus*. Further phylogenetic analyses including a wider range of species from the tribe *Dociostaurini* would be of great help to re-evaluate these incongruences and determine the taxonomic value of currently accepted supraspecific classification (Cigliano *et al.*, 2017).

Intraspecific polymorphism, different kinds of morphological crypsis and hybridization are the main limitations on establishing species boundaries (Evangelista *et al.*, 2014). The analyses presented here resolve some taxonomic ambiguities involving closely related taxa with allopatric distributions. This is the case of the controversial taxonomic classification of *D. (S.) kraussi*,

Table 2. Summary of the different taxonomic classifications and synonyms for *Dociostaurus crassiusculus* and *D. kraussi*.

Past taxonomic classification (**1–6**)		Soltani taxonomic classification (**7**)		Current taxonomic classification (**8–9**)		Proposed taxonomic classification (**10**)	
Species	Subspecies	Species	Subspecies	Species	Subspecies	Species	Subspecies
Dociostaurus (=Stauronotus) crassiusculus (**1**) (**2**) *Dociostaurus (S.) crassiusculus* (**6**)	–	*Dociostaurus (D.) crassiusculus*	*D. (D.) c. crassiusculus*	*Dociostaurus (S.) crassiusculus*	*D. (S.) c. crassiusculus* (**8, 9**)	*Dociostaurus crassiusculus*	–
Dociostaurus (=Stauronotus) kraussi (**2**)	*D. k. kraussi* (**3, 4**) *D. (S.) k. kraussi* (**5, 6**)	*Dociostaurus (D.) crassiusculus*	*D. (D.) c. kraussi*	*Dociostaurus (S.) kraussi* (**8, 9**)	–	*Dociostaurus kraussi*	*D. k. kraussi*
Dociostaurus (=Stauronotus) kraussi (**2**)	*D. k. nigrogeniculatus* (**3, 4**) *D. (S.) k. nigrogeniculatus* (**5, 6**)	*Dociostaurus (D.) crassiusculus*	*D. (D.) c. nigrogeniculatus*	*Dociostaurus (S.) crassiusculus*	*D. (S.) c. nigrogeniculatus* (**8, 9**)	*Dociostaurus kraussi*	*D. k. nigrogenicu-latus*
Dociostaurus (=Stauronotus) kraussi (**2**)	*D. k. aurantipes* (**4**) *D. (S.) k. aurantipes* (**5, 6**)	*Dociostaurus (D.) crassiusculus*	*D. (D.) c. kraussi*	*Dociostaurus (S.) crassiusculus*	*D. (S.) c. aurantipes* (**9**)	*Dociostaurus kraussi*	*D. k. aurantipes*
Dociostaurus (=Stauronotus) kraussi (**2**)	*D. (S.) k. claripes* (**5, 6**)	*Dociostaurus (D.) crassiusculus*	*D. (D.) c. kraussi*	*Dociostaurus (S.) kraussi* (**8, 9**)	–	*Dociostaurus kraussi*	*D. k. kraussi*
Dociostaurus (=Stauronotus) kraussi (**2**)	*D. (S.) k. ornatus* (**5, 6**)	*Dociostaurus (D.) crassiusculus*	*D. (D.) c. kraussi*	*Dociostaurus (S.) kraussi* (**8, 9**)	–	*Dociostuarus kraussi*	*D. k. kraussi*

(*S.*), subgenus *Stauronotulus*; (*D.*), subgenus *Dociostaurus*. Each row corresponds to a single taxon and its synonymous names are indicated in different columns. References: **1**, Pantel (1886); **2**, Ingenitskii (1897); **3**, Tarbinsky (1928); **4**, Bey-Bienko (1933); **5**, Bey-Bienko & Mistshenko (1951); **6**, Mistshenko (1974); **7**, Soltani (1978); **8**, Hodjat (2016); **9**, Cigliano *et al.* (2017); **10**, this study.

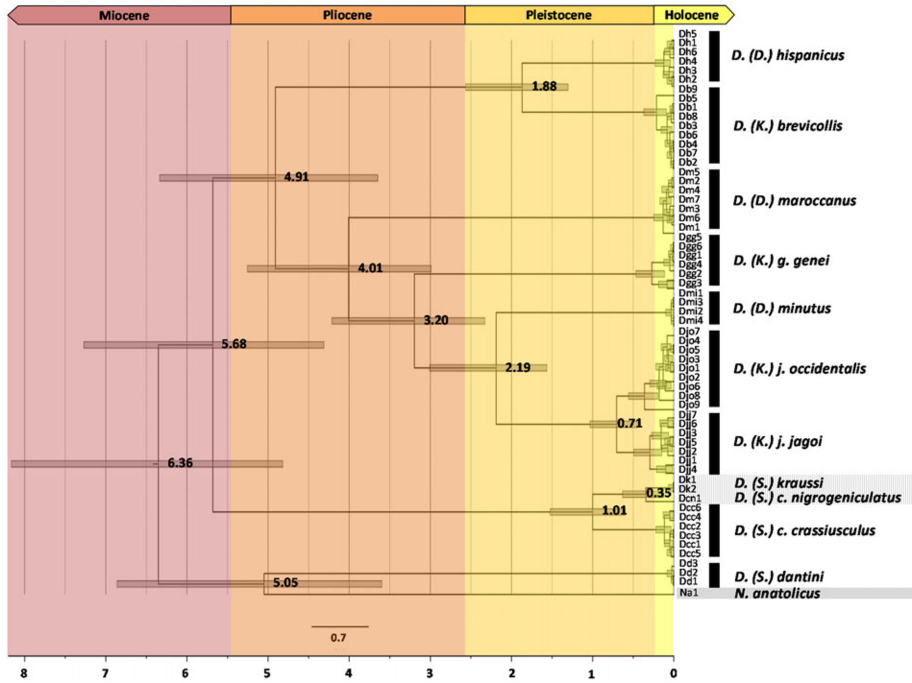

Fig. 3. Phylogenetic tree showing the relationship between species of the genus *Dociostaurus* from the Western Mediterranean. Analyses were performed in BEAST 1.8.3 using sequence information from three mtDNA genes (*12S*, *16S* and *COI*) and considering a strict clock and a coalescent exponential growth model. Mean ages (Ma) of each node are given and horizontal shadow bars indicate the 95% highest posterior density (HPD) intervals. *Notostaurus anatolicus* was used as outgroup (shaded in grey). Sequences of *Dociostaurus (S.) kraussi* and *D. (S.) c. nigrogeniculatus* (only available for *COI* gene) were retrieved from GenBank (striped in grey). Species names follow Cigliano *et al.* (2017). [Colour figure can be viewed at wileyonlinelibrary.com].

D. (S.) crassiusculus and their respective subspecies. A summary of historical nomenclatural changes of these two species and our own proposal is shown in Table 2. The results from the present study indicate that the first historical taxonomic classification is probably more accurate than the present one. Based on our results, we propose that: (i) *D. crassiusculus* is a young species endemic to the Iberian Peninsula. Thus, this taxon should be considered a conservation priority given the high fragmentation of its few and declining populations (Cordero *et al.*, 2010); (ii) phylogenetic analyses indicate that *D. c. nigrogeniculatus* is much more closely related to *D. kraussi* than to *D. crassiusculus*. Thus, *D. c. nigrogeniculatus* should be considered a subspecies of *D. kraussi* (e.g. Tarbinsky, 1928; Bey-Bienko, 1933; Bey-Bienko & Mistshenko, 1951; Mistshenko, 1974); (iii) similarly, *D. c. aurantipes* from Republic of Tajikistan in Asia should probably be considered as a subspecies of *D. kraussi* (e.g. Bey-Bienko & Mistshenko, 1951; Mistshenko, 1974) instead of a subspecies of *D. crassiusculus* until more analyses can be performed (Soltani, 1978; Hodjat, 2016; Cigliano *et al.*, 2017) (Figs 2, 3; Table 2). The fact that *D. kraussi* has a wide distribution in Eastern Europe and Central and South Asia could explain the description of many different subspecies that may simply reflect phenotypic plasticity among populations (Mal'kotskii, 1963). Accordingly, most subspecies of *D. kraussi*, such as *D. k. claripes* and *D. k. ornatus* (Bey-Bienko & Mistshenko, 1951), recently have been suggested as synonyms of *D. kraussi* (Cigliano *et al.*, 2017). Information on acoustic signals obtained in previous studies agrees with the obtained phylogenetic relationships here (Blondheim, 1990; García *et al.*, 1994, 2005; Ragge & Reynolds, 1998; Savitsky, 2000; Vedenina & Mugue, 2011; Sirin & Mol, 2013). The calling song of *D. c. crassiusculus* (García *et al.*, 2005) is almost identical to *D. kraussi* (Savitsky, 2000), which supports the close relationship between these species (Fig. 2). By contrast, the songs of *D. brevicollis* (Savitsky, 2000; Vedenina & Mugue, 2011; Sirin & Mol, 2013) and *D. hispanicus* (Ragge & Reynolds, 1998; García *et al.*, 2005) are more different, which is concordant with the estimation of an earlier split between these two sister taxa (Fig. 2).

Phylogenetic analyses and molecular dating in BEAST revealed that the main diversification of the genus *Dociostaurus* probably took place during the late Miocene and the Pliocene (Fig. 3). This may be explained by the progressively drier and cooler climate during these epochs, which is known to have promoted the expansion of typical habitats for *Dociostaurus* such as steppes, savannahs and grasslands (Dowsett *et al.*, 1994; Thompson & Fleming, 1996). Thus, the opening of a new niche space in many areas and founder events after the colonization of suitable habitats might have favoured the diversification of the genus since the late Miocene (Song *et al.*, 2015). The split of sister taxa (1.88 Ma: *D. hispanicus* – *D. brevicollis*; 1.01 Ma: *D. c. crassiusculus* – *D. kraussi*) and subspecies (0.71 Ma:

D. j. jagoi – D. j. occidentalis; 0.35 Ma: *D. kraussi – D. c. nigrogeniculatus*) took place more recently, during the middle and late Pleistocene (Fig. 3). These findings are in agreement with previous studies suggesting that speciation in Orthoptera can happen in as little as 1–2 Ma (e.g. Hemp *et al.*, 2015). As shown in many other Mediterranean taxa, these recent or incipient speciation events have probably resulted from long-term population isolation mediated by geographical barriers (e.g. *D. j. jagoi – D. j. occidentalis*) or as a consequence of distributional shifts driven by Pleistocene climatic oscillations (e.g. *D. hispanicus – D. brevicollis*) (Knowles & Richards, 2005; Noguerales *et al.*, 2016).

The phylogenetic relationships and timing of divergence of *D. minutus* and *D. jagoi* provide some insights about the biogeographical origin of the former. The Sicilian *D. minutus* is a species of great conservation concern that has been included in the European Red List of Orthoptera with the category 'endangered' (Bushell, 2013; Hochkirch *et al.*, 2016). The distribution of this brachypterous species is limited to a very restricted area in southern Sicily and our results indicate that it diverged from *D. jagoi* around the early Pleistocene (~2.19 Ma; Fig. 3). The colonization of Sicily by the ancestor of the current *D. minutus* may have occurred through the Siculo-Tunisian Strait, an area that has been hypothesized to facilitate the exchange of terrestrial fauna between European and African continents when the lower sea level characterizing Pleistocene glacial periods resulted in the emergence of 'stepping stone islands' and reduced the distance between the North African palaeocoast and the Sicilian landmass (Stöck *et al.*, 2008). This is in accordance with the presence in Sicily of other grasshopper taxa also distributed in North Africa such as *Euchorthippus albolineatus* (Lucas, 1849), *Acinipe calabra* (Costa, 1836) and *Ocneridia nigropunctata* (Lucas, 1849) (Massa, 2011; Massa *et al.*, 2012). The ancestor of *D. minutus* would have evolved to a short-winged form due to isolation and genetic drift in Sicily where *D. jagoi* is absent (Massa *et al.*, 2012).

Overall, this study reveals the importance of considering genetic information to disentangle the intricate taxonomy of *Dociostaurus* and establishes background knowledge for future studies aimed to reconsider the value of the currently accepted supraspecific classification into genera and subgenera (Cigliano *et al.*, 2017). Future studies should consider employing nuclear markers for phylogenetic reconstructions (e.g. Song *et al.*, 2015), a wider number of taxa, and sequencing more specimens from more localities for taxa with large distribution ranges (e.g. *D. brevicollis, D. kraussi* and *D. maroccanus*). Detailed phylogeographical and population genetic analyses of some species of particular interest would also greatly contribute to increase knowledge about their biogeography and evolutionary history, establish evolutionary significant units for conservation (Vogler & Desalle, 1994), identify corridors for gene flow (e.g. Ortego *et al.*, 2009, 2015) and, in the case of pest species (i.e. *D. maroccanus*), reach a better understanding of their demographic dynamics (e.g. Chapuis *et al.*, 2008, 2009).

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference:
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Table S1.List of species names according to Cigliano *et al.* (2017) included in this study with their individual reference (ID), number of samples per locality (*n*), geographical location and GenBank accession numbers (corresponding to the *12S* and *16S* ribosomal RNA genes, and the COI gene; in this order, respectively).

Table S2. mtDNA genes, PCR amplicon sizes, primers used for amplification and their respective sequences (Simon *et al.*, 1994).

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