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A molecular phylogenetic and phylogeographic study of two forms of *Calliptamus barbarus* (Costa 1836) (Orthoptera: Acrididae, Calliptaminae) from two regions of Algeria

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Summary. *Calliptamus barbarus* (Orthoptera: Acrididae) is the most polymorphic species within the genus *Calliptamus*. It shows a morphological polymorphism (three hind femoral spots, or only one hind femoral spot). Several studies have been made in order to distinguish the two forms: morphometry, number of ovarioles, sound production, protein and enzyme system. The aim of our work is to assess whether the two forms can be considered as different taxa and to perform a molecular phylogenetic study of two populations of *C. barbarus* collected from two different Algerian localities. No clear genetic differentiation was found between the samples with different morphologies. Additionally, the samples from Algeria do not form a monophyletic sister clade compared to the one formed by the sequences from GenBank from other geographical regions. Despite the morphological differences shown between the two populations, our molecular study indicates that there are no differences at a molecular level using the two mitochondrial genes COI and 16S.

Résumé. Étude phylogénétique et phylogéographique de deux formes de *Calliptamus barbarus* (Costa 1836) (Orthoptera : Acrididae, Calliptaminae) de deux régions d'Algérie. *Calliptamus barbarus* est l'espèce la plus polymorphe au sein du genre *Calliptamus*. Elle montre un polymorphisme morphologique (une ou trois taches au niveau des fémurs postérieurs). Plusieurs études ont été réalisées dans le but de distinguer les deux formes : morphométrie, nombre d'ovarioles, production sonore, protéines et système enzymatique. Le but de notre travail est d'évaluer si les deux formes peuvent être considérées comme des taxons différents et de réaliser une étude moléculaire phylogénétique de deux populations de *C. barbarus* recueillies à partir de deux localités différentes d'Algérie. Aucune différence génétique claire n'a été observée entre les échantillons morphologiquement différents. En outre, les échantillons provenant d'Algérie ne forment pas un groupe monophylétique par rapport à celui formé par les séquences tirées de GenBank et provenant d'individus d'autres régions géographiques. En dépit des différences morphologiques observées entre les deux populations, notre étude montre qu'il n'y a pas de différence au niveau moléculaire en utilisant les deux gènes mitochondriaux COI et 16S.

http://www.zoobank.org/urn:lsid:zoobank.org:pub:1BD2D778-C800-43C7-8EAE-F032AF75FFB7

Keywords: *Calliptamus barbarus*; form; femoral spot; phylogeography; COI

Mots-clés: *Calliptamus barbarus*; forme; tache fémorale; phylogéographie; COI

Calliptamus barbarus (Costa 1836), also called "the Caloptene ochrace" or "prickly locust ", belongs to the subfamily Calliptaminae. It is included in a group of four closely related species whose identification often proves difficult, including the Italian Caloptene, *Calliptamus italicus* (L. 1758), the Provenzal Caloptene, *C. siciliae* (Ramme 1927) and the Occitan Caloptene, *C. wattenwylianus* (Pantel 1896).

Many identification keys are available to identify species of the genus *Calliptamus*, all based on morphology (tegmina, femoral and phallic complex), e.g. Chopard (1943) for North Africa, Chopard (1951), Harz (1975) and Defaut (1988) for the western Palearctic region, Llorente (1982) for Spain, Bellman and Luquet (1995) for Europe, Fontana et al. (2002) for Italy and Olmo-Vidal (2006) for Catalonia. However, the revision of the genus *Calliptamus* by Jago (1963) remains the best at present time. In *Calliptamus barbarus*, the identification of male specimens is often easier than that of females and juveniles (Bellman & Luquet 1995; Blanchet 2009; Blanchet et al. 2012a).

Among *Calliptamus*, *C. barbarus* (Figure 1) is the most polymorphic. It presents a chromatic polymorphism in the hind femora (ruby color with three bold and separate femoral spots, or pale orange, with only one large femoral spot) that corresponds to its ecological distribution and habitat: the form with one femoral spot can be found almost exclusively in the semiarid environments

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Figure 1. *Calliptamus barbarus* female with 3 spots (left) and 1 spot (right) (photo: Rouibah, 2015).

whereas the form with three spots is encountered in less arid places. Its distribution area stretches from Portugal in the west (Larrosa et al. 2007) to Afganistan and China to the east (Fabry et al. 1987; Larrosa et al. 2007), and from north of Russia (Stolyarov 2000) to Pakistan in the south, through Europe, Mediterranean sea and North Africa and Middle East (COPR 1982) (Figure 2).

On the bio-ecological plan, *C. barbarus* is a thermophilic and xerophytic species (Monard 1986) with a preference for arid land, sparse vegetation, wasteland and open scrubland surrounded by fallow. In Algeria, this species can usually be found near the sea to 1100 m. According to Louveaux et al. (1996), it can exceed this altitude in some cases, e.g. in Morocco. This Calliptaminae usually overwinter as eggs but rarely as the adult stage (Tumbrinck 2006).

In Algeria (Figure 3), two different populations can be found: one living near the coast (e.g. Jijel, Boumerdes and Tizi Ouzou), corresponding to the form with three femoral spots, and the other living in the steppe area near the desert (e.g. Medea and Djelfa) with individuals having only one femoral spot.

Under the most recent edition of the International Code of Zoological Nomenclature (ICZN, 1999), "form" is a term that is deemed to denote infrasubspecific ranks that are published after 1960. Jago (1963) was the first to propose the term of "form" for the *Calliptamus barbarus* variants. Several authors have attempted to compare the two forms on the basis of morphology. The form with one femoral spot is larger than the form with three spots, for both males and females (Clemente et al. 1987; Louveaux

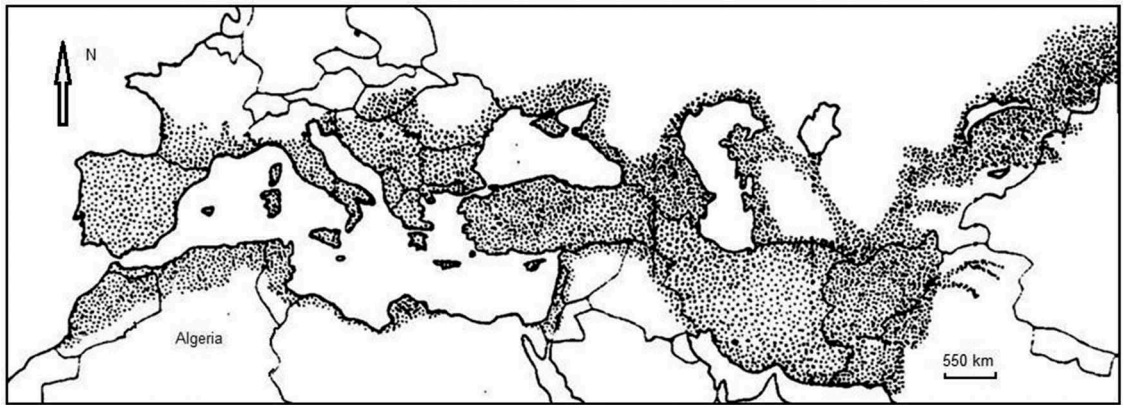

Figure 2. Geographic distribution of *Calliptamus barbarus* in the world (modified, according to Jago 1963).

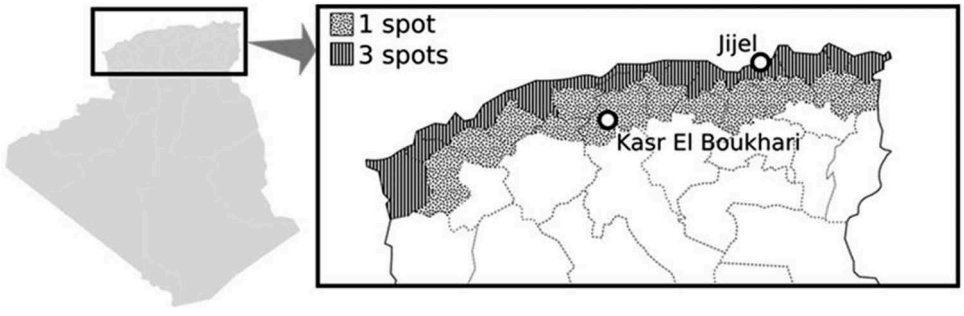

Figure 3. Geographic distribution of the two forms of *Calliptamus barbarus* in Algeria and sampling localities (in clear: 1S form, in dark: 3S form).

1991; Benzara 2004; Larrosa et al. 2004). On the other hand, according to Larrosa et al. (2008), the time domain features of the acoustic emissions of the males, and to a lesser degree for females, of both forms showed significant differences: the syllable length and the number of emitted pulses are greater in the one-spot form than in the three-spot form. Larrosa et al. (2007) reported that, regarding sexual behavior, there are some differences between the two forms in the inter and intrasexual relationships (convulsive and alternative movement of hind femora, walk up and down hind femora, advance and jump) and proposed that the two forms appear to be following a speciation process (Larrosa et al. 2007). Furthermore, females with one femoral spot have more ovarioles (an average of 62) than females with three femoral spots (only 51) (Benzara 2004). The same author reported the presence of some differences between the two forms concerning total proteins and enzyme systems of hemolymph and wing muscle such as tetrazolium oxidase and alpha-glycerophosphate; however, the phosphatase acid indicates a close relationship between the two populations.

Molecular studies have already shown their effectiveness to characterize the populations of grasshoppers, for example: Selkoe and Toonen (2006) and Sword et al. (2007) for *Hesperotettix viridis* (Thomas 1872); Chapuis (2006) for *Locusta migratoria* L. 1758, Huo et al. (2007) for Arcypteridae; Berthier et al. (2008) for *Oedaleus decorus* (Germar 1825); Chapuis et al. (2008) for *Chortoicetes*; Chapuis et al. (2011) for Orthoptera, Blanchet (2009); Blanchet et al. (2010); Blanchet et al. (2012b); for *Calliptamus*; Berthier et al. (2011) for *Chortoicetes terminifera* (Walker 1870); Umbers et al. (2012) for *Kosciuscola tristis* Sjöstedt 1934; and Saglam et al. (2013) for *Phonochorion*.

Currently, no molecular studies of *C. barbarus* have been performed in order to compare the two forms of this species. If they actually correspond to separate taxa, this difference should be revealed by means of a phylogenetic and phylogeographic study. The mitochondrial DNA fragment cytochrome oxidase subunit 1 (COI) is one of the most popular molecular markers used in phylogenetic studies, not only in Orthoptera (Bensasson et al. 2000; Burgov et al. 2006; Blanchet et al. 2010) but also in other insect groups (Jermiin & Crozier 1994; Zhang & Hewitt 1996; Guryev et al. 2001). The 16S ribosomal RNA (16S) has already been successfully tested by Lu and Huang (2006) for the phylogeny of Oedipodinae and López-López and Galian (2010, 2012), and López-López et al. (2015) for Cicindelinae.

The purpose of this work is to examine the systematic position of both forms of *C. barbarus* based on the sequence analysis of these two mitochondrial genes: cytochrome oxidase subunit 1 (COI) and the 16S RNA isolated from samples collected from the two populations of this species (with one and three femoral spots) taken from two geographically different regions of Algeria. Additionally, this analysis will confirm or refute the speciation process proposed by Benzara (2004) and Larrosa et al. (2008).

Material and methods

Samples of *Calliptamus barbarus* were collected during August 2014, 38 samples with one femoral spot (25 males and 13 females) in Kasr El Boukhari [35°86′07″N 2°76′07″E], and 30 with three femoral spots (11 males and 19 females) in Texenna (Jijel) [36°41′41″N 5°46′34″E] (Table 1, Figure 3).

After sampling, all collected specimens were brought to the laboratory, preserved in individual tubes filled with 100% alcohol and stored in a refrigerator at 4°C before DNA extraction.

DNA isolation from the hind femora was performed using the Invisorb® Spin Tissue Mini Kit (Invitek, Berlin, Germany) following the manufacturer's indications. Two fragments of the mitochondrial DNA were amplified using the Kapa® Taq DNA polymerase with the primers mtd6 (5′-GGAGGATTTGGAAATTGATTAGTTCC-3′) and mtd11 (5′-ACTGTAAATATATGATGAGCTCA-3′) (Contreras & Chapco 2006) for the cytochrome oxidase 1 (COI) and the primers 16S-F (5′-CCGAGTATTTTGACTGTGC-3′) and 16S-R (5′-TAATCCAACATCGAGGTCGCAA-3′) (Zerm et al. 2007) for the 16S ribosomal RNA (16S).

PCR reaction was performed under the following conditions: 5 min of denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C (primer annealing), 1 min at 72°C and then at 72°C for 10 min (final elongation).

PCR amplification was checked in a 1.5% agarose gel and sequenced in Macrogen (Amsterdam, the Netherlands). The

Table 1. Collection data for *C. barbarus* samples used for the molecular analysis.

Sample	Sex	Locality	Date	Form	GenBank accession n°	
					16S	COI
A01–A12, B01–B12, C01	M	Kasr Elboukhari	17.VIII.2014	One spot	KT158469- KT158493	KT158532- KT158545
C02–C12, D01–D02	F	Kasr Elboukhari	17.VIII.2014	Three spot	KT158494- KT158505	KT158546- KT158556
D03–D06, D08, D10–D12, E01–E03	M	Texenna	20.VIII.2014	Three spots	KT158506- KT158514	KT158557- KT158566
E04–E12, F02, F03, F05–F12	F	Texenna	20.VIII.2014	Three spots	KT158515- KT158531	KT158567- KT158581

sequences were edited in GENEIOUS 5.4 (Drummond et al. 2011) and aligned in the same program using the MUSCLE algorithm. Some *C. barbarus* sequences available in the GenBank database were added to the alignment, and sequences from other species of *Calliptamus* were added as outgroups (Table 2).

The most appropriate nucleotide substitution model was determined using jMODELTEST v2.1.7 (Darriba et al. 2012). A phylogenetic analysis of a concatenated matrix composed of the two fragments (COI and 16S) was carried out in BEAST 1.8.2 (Drummond et al. 2012), with a coalescent tree model with constant population size. The analysis ran for 10 million generations, sampling each 1000 steps. The first 1000 trees were discarded and the consensus tree was built with TREEANNOTATOR 1.8.1 (available at http://beast.bio.ed.ac.uk/). A phylogeographic network was built in the program POPART (available at http://popart.otago.ac.nz) using a modified version of the COI matrix, in which only the portion that was not

Table 2. List of outgroups and additional *C. barbarus* sequences obtained from the GenBank database.

Accession number	Species	Locality	Marker
DQ366833	*C. barbarus*	China	16S
FJ555215	*C. barbarus*	China	16S
FJ555220	*C. barbarus*	China	16S
FJ555221	*C. barbarus*	China	16S
FJ555222	*C. barbarus*	China	16S
FJ555223	*C. barbarus*	China	16S
JX033916	*C. barbarus*	NA	COI
KC139829	*C. barbarus*	China	COI
KC139830	*C. barbarus*	China	COI
KC261372	*C. barbarus*	NA	COI
AY379752	*C. abbreviatus*	NA	16S
DQ366836	*C. abbreviatus*	China	16S
KC139803	*C. abbreviatus*	China	COI
KC139804	*C. abbreviatus*	China	COI
KC139805	*C. abbreviatus*	China	COI
KC139806	*C. abbreviatus*	China	COI
KC139807	*C. abbreviatus*	China	COI
KC139808	*C. abbreviatus*	China	COI
KC139809	*C. abbreviatus*	China	COI
KC139810	*C. abbreviatus*	China	COI
KC139811	*C. abbreviatus*	China	COI
KC139812	*C. abbreviatus*	China	COI
KC139813	*C. abbreviatus*	China	COI
KC139814	*C. abbreviatus*	China	COI
KC139815	*C. abbreviatus*	China	COI
KC139816	*C. abbreviatus*	China	COI
KC139817	*C. abbreviatus*	China	COI
KC139818	*C. abbreviatus*	China	COI
KC139819	*C. abbreviatus*	China	COI
KC139820	*C. abbreviatus*	China	COI
KC139821	*C. abbreviatus*	China	COI
KC139822	*C. abbreviatus*	China	COI
KC139823	*C. abbreviatus*	China	COI
KC139824	*C. abbreviatus*	China	COI
KC139825	*C. abbreviatus*	China	COI
KC139826	*C. abbreviatus*	China	COI
KC139827	*C. abbreviatus*	China	COI
KC139828	*C. abbreviatus*	China	COI
EU589054	*C. italicus*	NA	COI
EU589059	*C. italicus*	NA	COI
EU589086	*C. italicus*	NA	COI
EU589087	*C. italicus*	NA	COI
EU589088	*C. italicus*	NA	COI
EU589089	*C. italicus*	NA	COI
EU589090	*C. italicus*	NA	COI
EU589091	*C. italicus*	NA	COI
EU589092	*C. italicus*	NA	COI
EU589093	*C. italicus*	NA	COI

(*continued*)

Table 2. (*Continued*).

Accession number	Species	Locality	Marker
EU589094	*C. italicus*	NA	COI
FJ555212	*C. italicus*	China	16S
FJ555213	*C. italicus*	China	16S
FJ555214	*C. italicus*	China	16S
FJ555216	*C. italicus*	China	16S
FJ555217	*C. italicus*	China	16S
FJ555218	*C. italicus*	China	16S
FJ555219	*C. italicus*	China	16S
KC139831	*C. italicus*	China	COI
KC139832	*C. italicus*	China	COI
KC139833	*C. italicus*	China	COI
KC139834	*C. italicus*	China	COI
KC261373	*C. italicus*	NA	COI
KR005871	*C. italicus*	China	COI
GQ355954	*C. siciliae*	France	COI
GQ355950	*C. wattenwylianus*	Spain	COI

Note: NA, locality not available.

missing from any sequence was conserved. The algorithm used for building the network was median joining, as it usually correctly resolves the relationships among haplotypes and has been successfully used in similar cases (Cassens et al. 2005).

Results and discussion

The obtained COI fragment had a length of 525 bp (GenBank accession codes KT158532-KT158581) and the 16S fragment was of 323 bp (GenBank accession codes KT158469-KT158531). The COI matrix used for the phylogeographic analysis had a length of 322 bp. The selected nucleotide substitution model for both fragments was the GTR + I + Γ.

The node support of the trees obtained from the concatenate matrix (Figure 4) and the 16S (Figure 6) was generally low, except for the most basal nodes. This can be explained by the low variability of the 16S fragment, which makes it impossible to accurately resolve the relationships among the different clades. The different topology of the 16S and COI trees (Figures 6 and 7), mainly due to homoplasy, is the main reason for the low support of the tree obtained from the concatenated matrix (Figure 4), as the phylogenetic analysis is unable to create a tree that correctly depicts the history of both fragments.

The possibility of that one of the two fragments could be actually a numt (a pseudogene originated by a transposition of a mitochondrial fragment to the nuclear genome) was ruled out. The COI sequences could be translated into the correct amino acid sequences, and the 16S fragment had the same nucleotide composition and structure as the sequences obtained from GenBank.

In all the phylogenetic trees, no clear differentiation can be found between the samples with different morphologies (Figure 4). These traits seem to be randomly distributed across all the branches of the tree. Additionally, the samples from Algeria do not form a monophyletic group, the sequences from GenBank from other geographical regions being included within them.

In the phylogeographic network (Figure 5), a central haplotype with high frequency can be observed, surrounded by several less frequent haplotypes only separated by one or two mutational steps. Other haplotypes can be found in the outer parts of the network, at the end of long branches.

Despite the differences shown between the two forms concerning different aspects, our molecular study indicates that there are no differences at molecular level using the two genes COI and 16S (Figures 4 and 5). The samples from both localities (one or three femoral spots) are not shown to be phylogenetically separated from each other; instead they are mixed, forming a polyphyletic group. This pattern is also observed in the phylogeographic network. The fact that the sequences obtained from GenBank, coming from other localities, are included within the Algerian samples, both in the phylogenetic tree and in the phylogeographic network, indicates a lack of geographic structure. From these results we can infer that the phylogenetic grouping does not correspond with neither the morphology nor the geographic origin of each sample. The morphology (big with one spot, small with three spots) does not seem to be related with the mitochondrial lineage. In fact, there is no genetic differentiation between both kinds of samples as far as we can infer from our data.

The lack of genetic structure inferred from the phylogenetic and phylogeographic analyses can be explained by the great dispersal power of this species, which is able to perform long flights. Our data hints that the individuals of this species could be continuously moving and transporting their genes

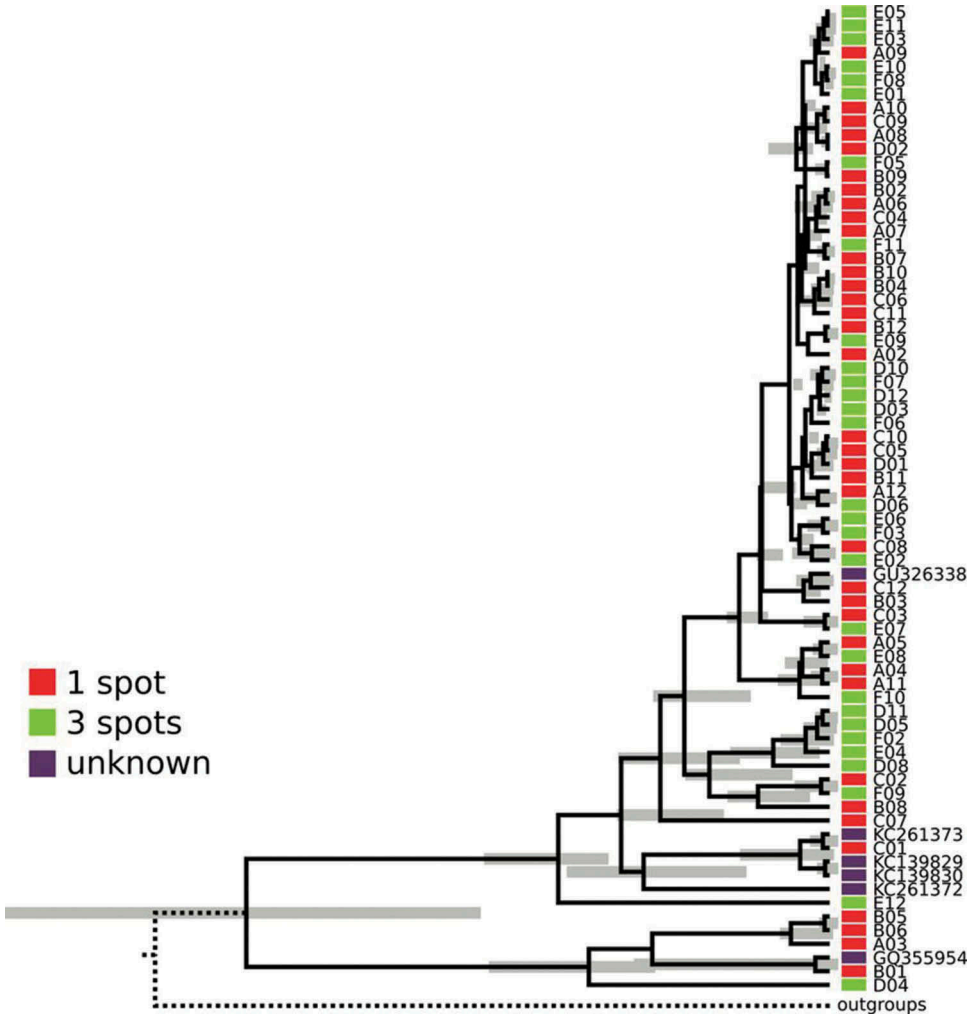

Figure 4. Bayesian inference tree obtained for the concatenated matrix of *Calliptamus barbarus* from the concatenated matrix including COI and 16S data. The coloration pattern in the inner side of the femora of the grasshopper sampled is marked either in red (one spot) or green (three spots). The node bars represent the node height with a 95% confidence interval. No support value is given because all the ingroup nodes had a posterior probability < 0.5.

throughout the Palearctic region, but this needs to be asserted using more data from all the distribution range.

In their study on the genus *Calliptamus*, Blanchet et al. (2012a) reported lower genetic diversity levels in *C. barbarus* populations when compared with populations of two related species (*C. wattenwylianus* and *C. italicus*) using microsatellites. This result has also been found in a recent phylogenetic analysis of several *Calliptamus* species (Sofrane et al. 2015). Our results confirm this low genetic diversity, and highlight a lack of separation between the sequences of our populations and the sequences of other localities (China and Morocco) obtained from GenBank (Figures 4 and 5). Furthermore, no significant correlation could be found between the genetic variability and geographic or morphological parameters. During many years of study in the field, no one-spot individual was found in the region of Jijel (at an altitude of 503 m), and no three-spot individual was found in Ksar El Boukhari region (at an altitude of 792 m). The two regions are separated by the Tell Atlas range. These geographic barriers did not affect the genetic diversity of the two forms of *C. barbarus*. According to Benzara (2004), this mountain chain is not a high enough barrier to block the dispersion of individuals, but the bioclimatic gradient is very strong between littoral and desert. On the other hand, Blanchet et al. (2012b), reported that *C. barbarus* did not show any genetic differentiation when comparing populations of different sites. Furthermore, the genetic diversity was slight in *C. barbarus* locality samples. They inferred that gene flow is not limited by distance or discrete geographic barriers in males of *Calliptamus* species.

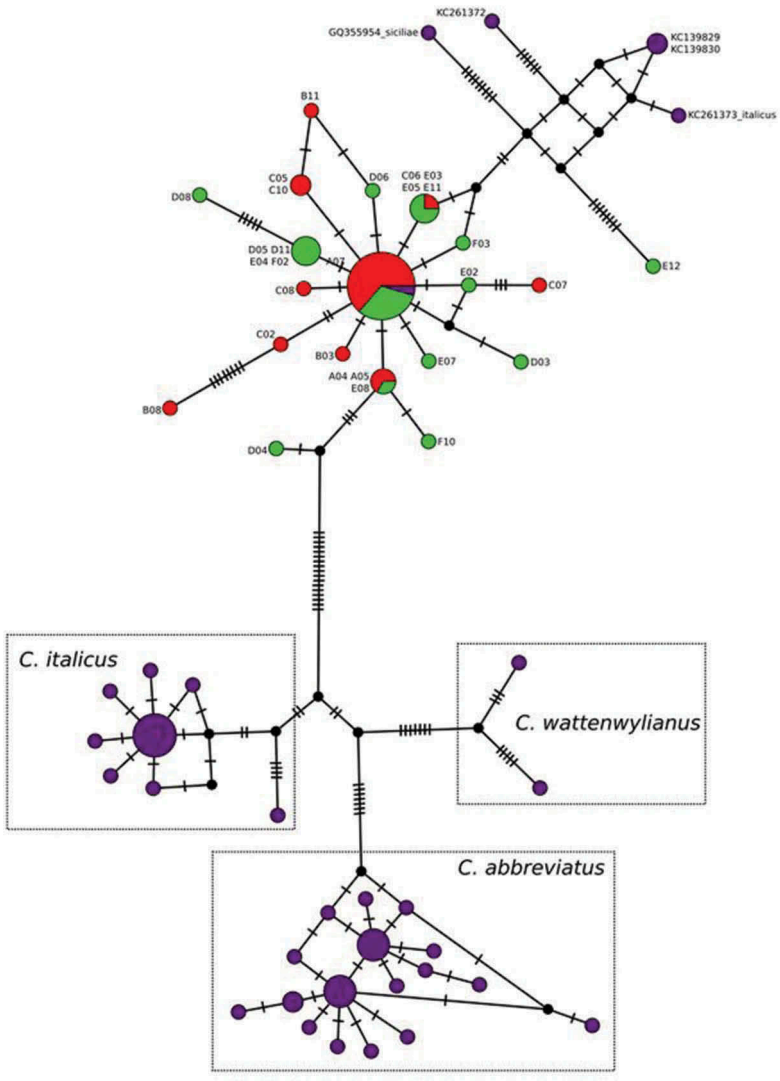

Figure 5. Haplotype network for *Calliptamus barbarus* obtained from COI data, including outgroups. The size of each haplotype is proportional to the number of samples that share it. The number of hatch marks in each link represents the number of mutational steps that separate the haplotypes. Black dots represent haplotypes not sampled (either extinct or not found in our sampling) but necessary to connect sampled haplotypes. Codes of the samples in each haplotype are indicated. The coloration pattern in the inner side of the femora of the grasshopper sampled is marked either in red (one spot) or green (three spots).

In the phylogeographic network, a central and very frequent haplotype can be distinguished, surrounded by multiple haplotypes only separated by one or two mutational steps. This star-shaped part of the network indicates a recent population expansion, that occurred after a population bottle-neck in the past. The most distant haplotypes could represent remains (older lineages) of an ancestral polymorphism.

Considering these results together, we can infer an interpretation of the history of these populations as follows: in the past, this species would have had a high diversity of haplotypes, distributed along a widespread population. Then, extinctions of haplotypes caused a drastic reduction of the genetic diversity, so only several genetically distant haplotypes remained (the central haplotype in the network and the far ones). More recently, one of the surviving haplotypes (the central one) started to experience an expansion, increasing the number of individuals that share it and generating a high number of haplotypes separated from it by one or two mutations.

Conclusion

Several species and subspecies concepts were proposed by different authors according to different properties based on morphology, biology, recognition, reproductive isolation, and phylogenetics. A unified species concept was proposed by De Queiroz (2007). In this concept, this author

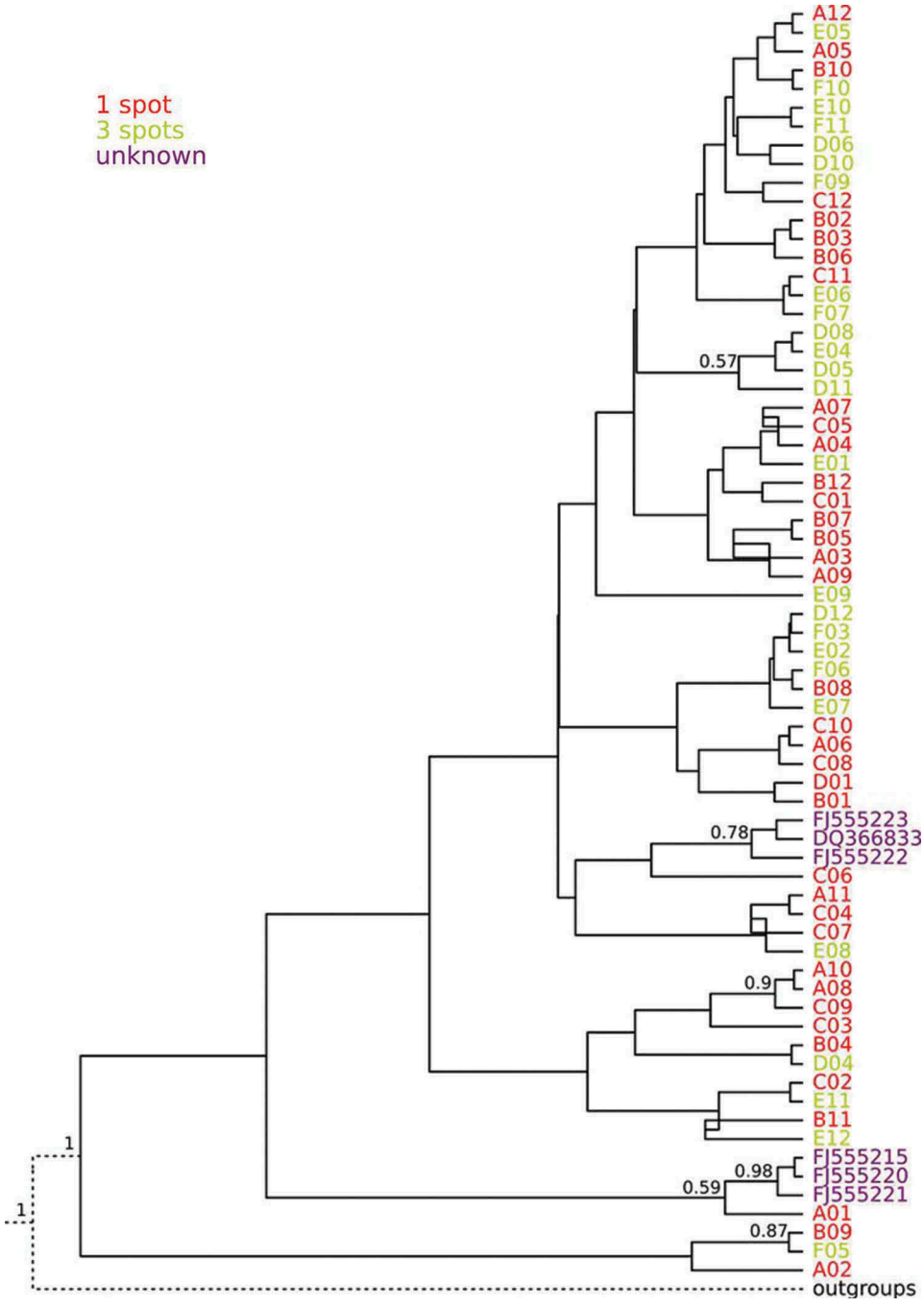

Figure 6. Bayesian inference tree obtained for the 16S fragment. The coloration pattern in the inner side of the femora of the grasshopper sampled is marked either in red (one spot) or green (three spots). Support values are given where the posterior probability value is > 0.5.

determined that a separately evolving metapopulation (inclusive population made up of connected subpopulations) lineage (ancestor-descendant series) is the only necessary property of a species. For Larrosa et al. (2008), the application of a broad biological species concept leads to the recognition of more species than the traditional purely morphological approach. They consider other differences, like sound production among others, to represent the mechanisms of isolation between the two forms of *C. barbarus* that appear to be following a speciation process.

In our study, we adopt the phylogenetics species concept proposed by Hennig (1966). This concept is based on reciprocal monophyly, as in the work by Lecocq et al.

Figure 7. Bayesian inference tree obtained for the COI fragment. The coloration pattern in the inner side of the femora of the grasshopper sampled is marked either in red (one spot) or green (three spots). Support values are given where the posterior probability value is > 0.5.

(2015) about *Bombus pascuorum* (Scopoli 1763) bumblebees. As *C. barbarus*, this species displays a considerable coat color variation, a morphological differentiation and a slight genetic differentiation. It appears as a single species with a high geographic phenotypic differentiation and with a low genetic differentiation. Those authors assess the traditional taxa classification using the groups defined by an integrative taxonomy approach based on genetic markers and ecochemical divergences. They finally considered that a taxon deserved a species status with a high degree of certainty if the taxon was genetically differentiated in all genetic markers and constituted a monophyletic group (Lecocq et al. 2015).

On the other hand, according to Mayr (1942), a subspecies is an aggregation of phenotypically similar populations of a species occurring in a geographical subdivision within the overall range and differing from other conspecific population groups. It might be also considered as one type of ESU (evolutionarily significant unit): a partially isolated lineage that has not quite separated as a result of recent gene flow, with a neutral divergence and genetic differentiation without the necessary reciprocal monophyly, in nuclear and mitochondrial markers and a divergence in characters, shaped by selective pressure (Braby et al. 2012).

The two forms of *C. barbarus* have been found to have differences concerning size, chromatic and geographic polymorphism, ovariole number, sound production, inter and intrasexual relationships, and slight differences in terms of total protein and enzymatic systems. Moreover, no hybrids between sympatric populations of both forms have been recorded. In our case, despite these differences, considered significant enough for their recognition as distinct species (Berrebi et al. 1986), the obtained molecular data do not allow us to corroborate that the two forms are separate taxa or are experiencing a speciation (segregation) process, as was suggested by Benzara (2004) and Larrosa et al. (2008).

Further studies based on larger sampling and including more genetic markers are needed to confirm the results obtained in this work, and will show whether the two forms are an ecological adaptive mechanism (Biron et al. 2002) in which the genetic system controlling the expression of the two phenotypes may be a protective mechanism of genetic variability within a population, conferring certain ecological benefits.

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