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Multiplex PCR assay to discriminate four neighbouring species of the *Calliptamus* genus (Orthoptera: Acrididae) from France

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Abstract

Definition of the genus *Calliptamus* (Orthoptera: Acrididae) has generated many taxonomic debates. Even now, the existence of different geographical morphs hinders species determination, particularly as concerns females and larvae. Some of these species are observed in southern France and are recognized as potential pests. To circumvent problems of species identification in ecological surveys, we developed a single multiplex PCR method based on mitochondrial Cytochrome Oxydase I diagnostic polymorphisms to differentiate between the four species, *Calliptamus italicus*, *C. wattenwylianus, C. siciliae* and *C. barbarus*, in southern regions of France.

Keywords: *Calliptamus*, species identification, molecular diagnostics, mitochondrial Cytochrome Oxydase I

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Introduction

Identification methods based on molecular markers can provide an efficient means of distinguishing species for which morphological traits are not informative or may even be error-prone. Various tools have been developed in this aim, ranging from PCR-based allelic discrimination assays to the use of highly polymorphic markers, such as microsatellites (Blaxter, 2004). In the last decade, some of these molecular tools have been developed towards identifying species of insect which are of economical importance or currently endangered (Phuc *et al*., 2003; Contreras-Diaz *et al*., 2006; Nolan *et al*., 2007). For such species, the lack of accurate morphological keys for adults and juveniles often greatly limits our knowledge regarding various ecological parameters (Behura, 2006).

Morphological identification problems arise more particularly for some species of the Acrididae family belonging to the *Calliptamus* genus, such as *C. siciliae* (Ramme, 1927), or *C. italicus* (Linneaus, 1758) and *C. wattenwylianus* (Pantel, 1896) – both recognized as potential pests – as well as for *C. barbarus* (Costa, 1836). *C. italicus* and *C. barbarus* are observed in an area extending from the Mediterranean Basin to the southern part of Siberia. *Calliptamus barbarus* has a more restricted distribution area than *C. italicus* in Siberia but is observed slightly more to the south, in Italy, Spain and North Africa. *Calliptamus wattenwylianus* is only present along the Mediterranean Coast, in France, Spain and North Africa (COPR, 1982). *Calliptamus siciliae* has the most limited distribution area, which extends from the south-east of France through Italy and Sicily (Harz, 1975). Because these four species are observed in similar habitats in the south of France and are under the focus of ecological surveys, there is a crucial need for accurate

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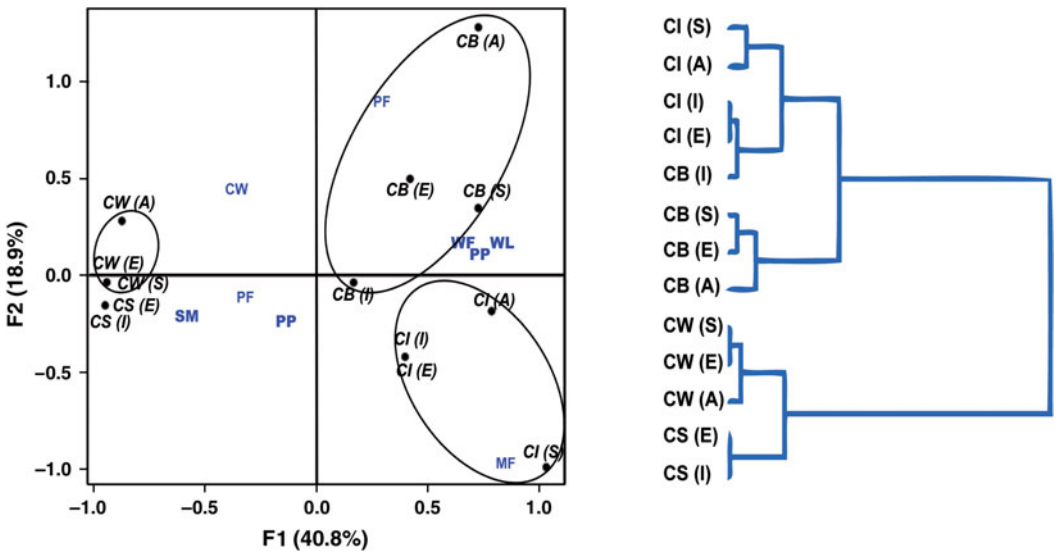

Fig. 1. Discrimination of the four *Calliptamus* species using morphological characteristics taken from guides published for various localities.

On the left, factorial correspondence analysis of table 2. In bold and italic, species associated with different localities: *CW*, *C. wattenwylianus*; *CB*, *C. barbarus*; *CI*, *C. italicus*; and *CS*, *C. siciliae*. In brackets, species guide definitions: *(A)* means North Africa and use of Chopard (1943) to determinate the species; *(E)*, Europe and use of Chopard (1951); *(S)*, Spain and use of Olmo Vidal (2006); and *(I)*, Italy and use of Fontana *et al*. (2002). In standard format, different characteristics taken into account: WF, tegmen form; WL, tegmen length; PF, pallium form; PP, pallium position; CW, colour of posterior wings; MF and SM, number and size of marks on the inner side of posterior femur. Bold characters explain significantly (high contribution) the distribution over axes 1 and 2 of the different species definitions.

On the right, a dendrogram derived from a species classification (ascending hierarchical classification based on the species' coordinates in the first four axes of FCA).

determination tools based on morphological or molecular diagnostics.

Distinction among species of the *Calliptamus* genus relies mainly on males' traits and is based on wings and genitalia characteristics. It is more difficult to identify females and larvae, which have few or no morphological diagnostics enabling determination keys to be designed (Jago, 1963; Harz, 1975). Moreover, depending on the localities, some morphological characteristics used for identification are subject to variation (see fig. 1: Chopard, 1943, 1951; Fontana *et al*., 2002; Olmo Vidal, 2006) and morphological taxonomy still remains unconfirmed by molecular studies. Only a few studies have been performed on the *Calliptamus* genus, focusing mainly on their mitochondrial genome (Bensasson *et al*., 2000). More recently, the sequencing of the mitochondrial genome has revealed the presence of nuclear mitochondrial copies (numts) in *C. italicus* (Fenn *et al*., 2007; Song *et al*., 2008). However, none of these studies have proposed any identification protocol based on unambiguous genetic characteristics. In this paper, we present a quick and inexpensive molecular method to efficiently identify the four species present in southern France using a multiplex PCR assay.

Material and method

Congruence of morphological characteristics

To verify the congruence of different determination keys, we first performed a factorial component analysis (FCA) based on seven different characteristics used for *Calliptamus* determination in various geographical localities (table 1). We used an appropriate guide for each locality: Olmo Vidal (2006) for Spain, Chopard (1943) for North Africa, Chopard (1951) for Europe, and Fontana *et al*. (2002) for Italy. All characteristics were taken as factors and were broken down into different modalities. Some characteristics were not used in all guides (colour of posterior wings, number and size of marks on the inner side of posterior femur). However, these were not considered as non-available data but assigned particular and separate modalities to enable FCA calculation.

Two modalities were considered for the form of anterior wings (WF), tegmen narrowed from the beginning of the second third or tegmen parallel from the beginning of the second third. Two modalities were used for the wing's length (WL), tegmen longer or shorter than posterior knees. For the form of the pallium (membrane covering penis valves, PF) seen in profile, we separated long and curved or rounded. Two modalities were also distinguished for pallium position (PP), proximal or near the apex of subgenital plates. We used four modalities for the colour of posterior wings (CW): colourless, pink, pale pink and 'NA' when this characteristic was not used in a guide. Number (MF) and size (SM) of marks on the inner side of posterior femur were described by three modalities (MF: number of marks is one, two or more, or not used in a guide; SM: marks are small, wide or characteristic not used).

Wing length and form and pallium position appeared to supply the best explanation for the distribution of species over the first two axes (fig. 1). *Calliptamus italicus* and *C. barbarus* were clearly distinguished from *C. siciliae*

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Table 1. Morphological characteristics used by various authors for the identification of *Calliptamus* species.

Species according to guides	WF	WL	PF	PP	CW	MF	SM
C. italicus – (CI(E))	+	+	+	+			
C. italicus – (CI(I))	+	+	+	+			
C. italicus – (CI(A))	+	+	+	+	+	+	
C. italicus – (CI(S))	+	+	+	+		+	+
C. barbarus – (CB(E))	+	+	+	+		+	
C. barbarus – (CB(I))	+	+	+	+			
C. barbarus – (CB(A))	+	+	+	+	+	+	+
C. barbarus – (CB(S))	+	+	+	+		+	+
C. wattenwylianus – (CW(E))	+	+	+	+			
C. wattenwylianus – (CW(A))	+	+	+	+	+		
C. wattenwylianus – (CW(S))	+	+	+	+			
C. siciliae – (CS(I))	+	+	+	+			
C. siciliae – (CS(E))	+	+	+	+			

The first column indicates the species by to geographical zone (E, Europe; A, North Africa; I, Italy; S, Spain) and the determination keys used (Chopard (1951) for Europe; Chopard (1943), for North Africa; Fontana *et al*. (2002) for Italy; Olmo Vidal (2006) for Spain). Following columns show the different characteristics used by these authors to identify the *Calliptamus* species: WF, form of anterior wings; WL, wing length; PF, pallium form; PP, pallium position; CW, colour of posterior wings; MF, number of marks on the inner side of posterior femur; SM, mark size on the inner side of Femur. Crosses indicate that the characteristics are used in a particular guide to determine the respective species.

and *C. wattenwylianus* on the basis of these three combined characteristics. Differentiation between *C. italicus* and *C. barbarus* was more obvious in terms of the size of marks on the inner side of posterior femur and the form of pallium, whereas *C. siciliae* and *C. wattenwylianus* were differentiated on the third axes according to pallium position (PP) and wing colour (CA).

The dendrogram on the right side of fig. 1 shows species classification, on the basis of different guides (ascending hierarchical classification based on the species' coordinates in the first four axes of FCA). *Calliptamus siciliae* and *C. wattenwylianus* stay close to each other but remain clearly distinct, as do *C. barbarus* and *C. italicus*. Only *CB* (I) was not correctly classified within the branch characterizing the *C. barbarus* species. This error of assignment suggests that key characteristics used in different guides could be subject to range overlap and shows their failure to discriminate between species from different localities. In such a context, the development of accurate molecular tools could be especially useful.

Insect sampling

Each *Calliptamus* species observed in France was represented in 123 samples, which were taken from different locations from France, as well as from neighbouring locations such as Spain, Italy, and North Africa (see table 2). Females were tested and came from localities where only one species was present, in order to be certain of their species identity, and were verified through morphological identification proposed by Jago (1963) and Harz (1975). No female of *C. siciliae* could be tested because such a sample was not available.

In order to ensure high specificity to the *Calliptamus* species for the primer design step, we also took into account two additional samples of a species belonging to a genus close to *Calliptamus*. We selected *Paracaloptenus bolivari* (Uvarov, 1942), since the morphology of this species during the first development stages is similar to that of *Calliptamus* species and because it is sympatrically distributed with *Calliptamus* species in some French localities.

Molecular method design and application

DNA was extracted using a CTAB protocol (Doyle & Doyle, 1987) from the hind femur in each of the five species: *C. italicus* (CI), *C. barbarus* (CB), *C. wattenwylianus* (CW), *C. siciliae* (CS) and *Paracaloptenus bolivari* (PB). Using aligned mitochondrial sequences of Cytochrome Oxydase I (COI) from two Acrididae species, *Locusta migratoria* (Linneaus, 1758) and *Chortippus parallelus* (Zetterstedt, 1821) (respective accession numbers: X80245 and X95575), we designed primers on conserved regions over both species to amplify the COI gene in each of the five species under focus. Using primer pairs COI75F: 5′-GCATGAGCAGGAATAGTAGG-3′ and COI1524R: 5′-CTGAATATCTATGTTCTGCAGG-3′, we amplified sequences of the five species with different localities for *C. barbarus*, *C. wattenwylianus* and *C. italicus*. All sequences were submitted to Genbank (accession numbers GQ355945 to GQ355955 and GU326338).

These sequences were then aligned, and we determined a conserved region for the four *Calliptamus* species, but differentiated from the *Paracaloptenus* genus by some nucleotides in 3′. Moreover, for each species a specific primer region, containing at least two discriminating mutations, was screened in order to obtain species-specific amplification products of different lengths.

We designed a reverse primer (COI-1070-R) in the genus conserved region, as well as a forward species-specific primer for each species: CBA-260F for *C. barbarus*, CI-510F for *C. italicus*, CW-720F for *C. wattenwylianus* and CS-910F for *C. siciliae* (table 3).

For all individuals analysed, DNA extraction was performed as explained above: PCR amplifications were run in a 25 µl total volume using a Qiagen core kit (QIAGEN, Courtaboeuf, France), with 2.5–5.0 ng DNA. The PCR buffer contained 0.1 mM of each dNTPs, 1.5 mM $MgCl_2$, 1 U of Taq DNA polymerase and 0.4 µM of reverse common primer and 0.4 µM of each specific F-primer, except for CB, for which 0.8 µM was necessary. PCR reactions were performed on T-personal thermocycler (Biometra) using the following parameters: denaturation at 94°C for 5 min, followed by 35 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 52°C and elongation for 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. PCR products were then electrophoresed in 2% agarose gel for 40 min at 100 volts.

Results

Null amplification checked on two different samples of *P. bolivari* (Uvarov, 1942), and sequence identity confirmed that our method was specific to the *Calliptamus* genus.

Four different PCR products were obtained (see fig. 2), in which *C. barbarus* showed a PCR product of 610 pb, *C. italicus* of 560 pb, *C. wattenwylianus* of 350 pb and *C. siciliae* of 160 pb.

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Table 2. Tested samples and their origins.

The number of samples successfully amplified per number of tested samples is shown in the third column; the presence of brackets indicates a contradiction between morphological determination and the molecular diagnostic. All the samples used for tests were males, except where F is specified alongside the species name.

Species	Locality	Nb	Longitude	Latitude
CB	Corsica	3/3	15°22′27.29″ E	41°59′39.32″ N
	Var	3/3	06°47′46.15″ E	43°33′16.71″ N
	Bouche du Rhône	3/3	05°23′39.40″ E	43°39′37.84″ N
	Hérault (Aumelas)	3/3	03°37′32.89″ E	43°34′18.54″ N
	Hérault (Hortus)	3/3	03°49′00.69″ E	43°51′18.25″ N
	Hérault (Larzac)	2/3	03°23′25.30″ E	43°51′51.92″ N
	Tarn	2/2	01°59′46.75″ E	43°24′31.95″ N
	Gers	3/3	00°44′50.54″ E	43°45′19.35″ N
	Aude	3/3	02°51′02.80″ E	43°00′53.62″ N
	Ariège	10/12+3F/3F	00°51′03.83″ E	43°06′17.47″ N
	Pyrénées-Orientales	3/3	02°24′42.32″ E	42°35′07.84″ N
	Spain, Ballobar	3/3	00°09′46.15″ W	41°36′49.07″ N
	Italy	2/3	16°23′13.70″ W	41°57′58.90″ N
	Morocco	1+(6)/7	06°21′03.99″ E	32°06′35.69″ N
CI	Var	3/3	06°43′29.01″ E	43°34′29.89″ N
	Bouche du Rhône	3/3	05°35′01.54″ E	43°39′13.96″ N
	Hérault (Aumelas)	3/3	03°37′32.89″ E	43°34′18.54″ N
	Hérault (Hortus)	3/3	03°49′00.69″ E	43°51′18.25″ N
	Tarn	3/3	02°04′04.89″ E	43°27′59.21″ N
	Gers	3/3	00°10′38.86″ E	43°44′30.53″ N
	Aude	2/2	02°49′05.40″ E	42°59′58.01″ N
	Pyrénées-Orientales	3/3	02°24′42.33″ E	42°35′07.84″ N
	Deux-Sèvres	3F/3F	00°20′50.49″ W	46°06′56.94″ N
	Spain, Gerri de la Sal	1/1	01°03′57.45″ E	42°19′30.65″N
CW	Var	3/3+3F/3F	06°48′44.10″ E	43°30′47.15″ N
	Bouche du Rhône	3/3	05°01′36.28″ E	43°42′35.92″ N
	Hérault (Aumelas)	3/3	03°37′32.89″ E	43°34′18.54″ N
	Hérault (Hortus)	3/3	03°49′00.69″ E	43°51′18.25″ N
	Pyrénées-Orientales	2/2	03°06′24.61″E	42°30′34.23″ N
	Spain, Monegrillo	3/3	00°33′14.36″ W	41°33′27.90″ N
	Spain, Caspe	3/3	00°11′10.54″ W	41°15′04.27″ N
	Morocco	3/3	06°21′34.83″ W	32°6′34.75″ N
	Algeria	3/3	00°56′21.00″ N	36°26′24.00″ W
CS	Alpes-Maritimes	10/10	05°55′19.56″E	44°19′07.57″N

Species names are provided in the first column: CW, *C. wattenwylianus*; CB, *C. barbarus*; CI, *C. italicus*; and CS, *C. siciliae*. The second column indicates the sample's origin; French localities are indicated by department name and border samples (in bold) by country, sometime with other different localities such as Spain.

Table 3. Primer pairs developed for the molecular diagnostic.

Species	Primers	Sequence	Length (pb)
Common	COI-1070-R	5′-GATAATACATAATGGAAGTGTGC-3′	–
C. barbarus	CB-260F	5′-CTCCTCCTAATATCTTCCATAGTAG-3′	810
C. italicus	CI-510F	5′-CTACTACTTTTATCTTTACCAGTTT-3′	560
C. wattenwylianus	CW-720F	5′-CGAATCATTTGGAACCTTAGGA-3′	350
C. siciliae	CS-910F	5′-CGCTTTATGGAACAAAATTC-3′	160

From left to right: species names, primers names, primers' and length of the PCR products (in pairs of bases).

For 123 individuals tested, molecular diagnostics confirmed morphological determination for 113 samples, irrespective of their geographical origins. Four PCR samples failed to amplify, and six molecular diagnostics disagreed with the respective morphological identification. These discrepancies concerned *C. barbarus* specimens from Morocco, which were identified as *C. wattenwylianus* using our method. Additional verification of morphological traits revealed that these individuals did not display the typical morphological criteria proposed by Chopard (1943) and that it was difficult to distinguish between *C. barbarus* and *C. wattenwylianus*. To be certain of their species membership, we sequenced two individuals coming from Morocco, one identified as *C. barbarus* with morphological and molecular diagnostic and one morphologically undetermined sample revealed as *C. wattenwylianus* by the molecular diagnostic. Estimation of p-distance (p-distance = 0.39) between both sequences, using Mega v.4 (Tamura *et al.*, 2007) was enough

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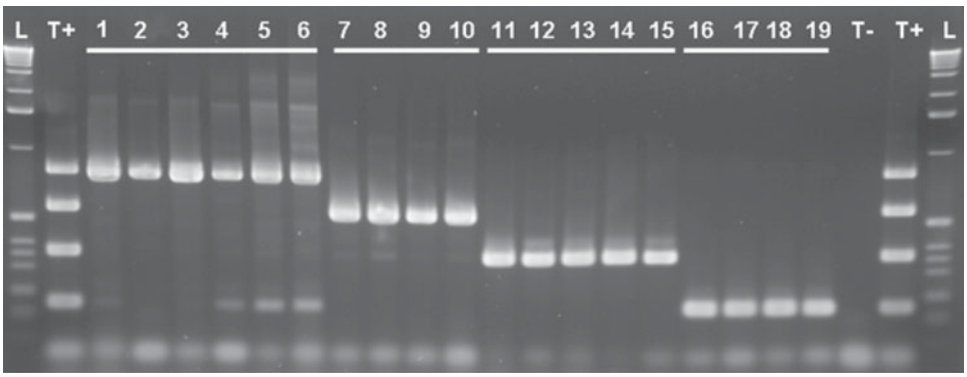

Fig. 2. Results of molecular diagnostics for the four *Calliptamus* species of southern France.

L on each side is Ladder (1 kb) Invitrogen. T+, DNA positive control; we gathered the four PCR Product types (160/350/560/810 pb) from our assays. 1–6: *C. barbarus* from Spain (1), Pyrénées-Orientales (2), Hérault (Aumelas) (3), Var (4), Corsica (5), Italy (6); 7–10: *C. italicus*, from Spain (7), Pyrénées-Orientales (8), Hérault (Aumelas) (9), Var (10); 11–15: *C. wattenwylianus* from Spain (11), Pyrénées-Orientales (12), Hérault (Aumelas) (13), Var (14), Morocco (15); 16–19: *C. siciliae* from Alpes-Maritimes; T−, negative control of PCR with only H_2O. *CB* gave a band at 810 pb, *CI* at 560 pb, *CW* at 350 pb and *CS* at 160 pb.

to confirm their membership to two different species. Moreover, the second sequence was closed to other *C. wattenwylianus*' sequences (p-distance < 0.01) confirming their membership to the *C. wattenwylianus* species.

All PCRs led to highly specific amplification products, although *C. barbarus* from the Var, Corsica, Algeria and Italy had a very slight secondary band present, which did not interfere with their identification.

All females were successfully amplified, and identification was the same using both morphological and molecular methods.

Discussion

Species identification of insects is not always straightforward. In particular, the absence of suitable morphological keys for immature stages often requires specialist knowledge, and it can be time-consuming or even impossible.

Consequently, molecular diagnostic tools are increasingly being developed for insects (Saccaggi *et al*., 2008; Ståhls *et al*., 2009) and other arthropods (Hinomoto *et al*., 2004; Hosseini *et al*., 2007). Not only do these enable differentiation between different species irrespective of the sex or larvae instars, but they also provide essential security for any additional investigation into population dynamics, development time in the wild and the possible coexistence of different species in the same habitat. Moreover, molecular identification methods may be helpful for the study of outbreak dynamics in the case of insect pests such as *C. italicus* or *C. wattenwylianus*.

Our molecular diagnostic is especially straightforward and fast (five hours), since it uses a simple multiplex PCR assay which does not involve an endonuclease restriction step.

The four failed amplifications were most probably explained by a poor DNA quality due to a problem of sample storage. Moreover, amplification of 13 samples over 15 of *C. barbarus* from Ariège ruled out the possibility of a primer mismatch, which strengthens the hypothesis of poor DNA quality. Even if slight non-specific amplification products were observed for particular samples of *C. barbarus*, the competition between the different primers during the multiplexed PCR always favoured the most specific amplification.

Although we cannot be certain that the specificity of our primers makes it possible to avoid the amplification of DNA mitochondrial copies (numt and possibly heteroplasmy), the following points explain why the potential presence of numt (or other mitochondrial copies) would probably not seem to affect the reliability of our method. The high number of different haplotypes obtained in the case of *C. italicus* in Song *et al*. (2008) shows that several different copies of COI can be found in the nuclear genome of this species. Nevertheless, the homology between all the haplotype sequences in Song *et al*. (2008) (EU589059, EU589086–EU589094) is very high (only a few points of difference). It follows that the presence of numt or mitochondrial copies would not lead to a wrong diagnostic in this case. Moreover, the absence of amplification failure during our tests shows that the primers' target regions were well conserved across samples. Even if some mitochondria copies were amplified, their lengths are probably very close. Moreover, all sequences obtained from *C. italicus* were close (p-distance = 0.004) to the COI' sequence from the whole mitochondrial genome (EU938373) given by Fenn *et al*. (2007). This high similarity allowed confidence about the origin of our sequences. All the more so, regarding p-distances values (> 0.03) between all pair species, it showed and strengthened their usefulness. This does not affect the ability to discriminate species since this is based on the length of the amplified DNA fragments.

Indeed, the 123 samples tested in this study, the length of the four amplification fragments was highly repeatable, and all the samples from the same species showed the same band length on agarose gel. It, therefore, appears that the potential presence of numt or mitochondrial copies is not a hindrance to applying the multiplex PCR method.

Although no test was performed on other *Calliptamus* species observed elsewhere, as for example *C. ictericus* or *C. tenuicercis* from North Africa (Chopard, 1943), the tests conducted on foreign samples seemed to match with morphological characteristics supplied in the different determination keys developed for each of these countries.

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The relevance of this method is further confirmed by the difficulty of distinguishing morphologically certain individuals of *C. wattenwylianus* and *C. barbarus* from North Africa.

However, while FCA discrimination power based on morphological characteristics (fig. 1) suggests that *C. siciliae* is more closely related to *C. wattenwylianus*, a slight non-specific amplification of *C. barbarus* with *C. siciliae* primers and sequence similarity indicated that *C. siciliae* could be closer to *C. barbarus* from some localities like Corsica. Species genetic proximity was confirmed by the estimation of p-distance while all species were significantly distinguished (p-distance > 0.03). According to the sequences presented here, analyses revealed a weaker p-distance (0.03) between *C. barbarus* and *C. siciliae* than between all other pair species ranging from 0.034 to 0.076. These conflicting observations derived from morphology (see FCA analysis) and DNA sequences highlight the need to study the molecular phylogeny of the *Calliptamus* genus in more detail.

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References

Behura, S.K. (2006) Molecular marker systems in insects: current trends and future avenues. *Molecular Ecology* **15**, 3087–3113.

Bensasson, D., Zhang, D.X. & Hewitt, G.M. (2000) Frequent assimilation of mitochondrial DNA by grasshopper nuclear genomes. *Molecular Biology and Evolution* **17**, 406–415.

Blaxter, M.L. (2004) The promise of a DNA taxonomy. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* **359**, 669–679.

Chopard, L. (1943) *Orthoptéroïdes de l'Afrique du Nord*. 450 pp. Paris, France, Larose.

Chopard, L. (1951) *Faune de France n°56: Orthoptéroïdes*. 359 pp. Paris, France, Paul Lechevalier.

Contreras-Diaz, H.G., Lopez, H., Oromi, P. & Juan, C. (2006) Microsatellite loci development in endangered pamphagid grasshoppers endemic to the Canary Islands (Orthoptera). *Conservation Genetics* **7**, 767–771.

COPR (1982) *The Locust and Grasshopper Agricultural Manual*. 690 pp. London, UK, Centre for Overseas Pest Research.

Doyle, J.J. & Doyle, J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin* **19**, 11–15.

Fenn, J.D., Cameron, S.L. & Whiting, M.F. (2007) The complete mitochondrial genome sequence of the Mormon cricket (*Anabrus simplex*: Tettigoniidae: Orthoptera) and an analysis of control region variability. *Insect Molecular Biology* **16**, 239–252.

Fontana, P., Buzzeti, F.M., Cogo, A. & Odé, B. (2002) *Guida al Riconoscimento e allo Studio di Cavallette, Grilli, Mantidi e Insetti Affini del Veneto*. 592 pp. Vicence, Italia, Museo Naturalistico Archeologico.

Harz, K. (1975) *Die Orthopteren Europas. The Orthoptera of Europe*. 939 pp. Den Haag, The Netherlands, Dr W. Junk NV.

Hinomoto, N., Muraji, M., Noda, T., Shimizu, T. & Kawasaki, K. (2004) Identification of five *Orius* species in Japan by multiplex polymerase chain reaction. *Biological Control* **31**, 276–279.

Hosseini, R., Keller, M.A., Schmidt, O. & Framenau, V.W. (2007) Molecular identification of wolf spiders (Araneae: Lycosidae) by multiplex polymerase chain reaction. *Biological Control* **40**, 128–135.

Jago, N.D. (1963) A revision of the genus *Calliptamus* Serville (Orthoptera: Acrididae). *Bulletin of the British Museum, Natural history (Entomology)* **13**, 290–350.

Nolan, D.V., Carpenter, S., Barber, J., Mellor, P.S., Dallas, J.F., Mordue, A.J. & Piertney, S.B. (2007) Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. *Veterinary Microbiology* **124**, 82–94.

Olmo Vidal, J.M. (2006) *Atlas dels Ortòpters de Catalunya i llibre vermell*. 428 pp. Barcelona, Spain, Generalitat de Catalunya.

Phuc, H.K., Ball, A.J., Son, L., Hanh, N.V., Tu, N.D., Lien, N.G., Verardi, A. & Townson, H. (2003) Multiplex PCR assay for malaria vector *Anopheles minimus* and four related species in the Myzomyia Series from Southeast Asia. *Medical and Veterinary Entomology* **17**, 423–428.

Saccaggi, D.L., Kruger, K. & Pietersen, G. (2008) A multiplex PCR assay for the simultaneous identification of three mealybug species (Hemiptera: Pseudococcidae). *Bulletin of Entomological Research* **98**, 27–33.

Song, H., Buhay, J.E., Whiting, M.F. & Crandall, K.A. (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 13486–13491.

Ståhls, G., Vujic, A., Pérez-Bañon, C., Radenkovic, S., Rojo, S. & Petanidous, T. (2009) COI barcodes for Identification of *Merodon* hoverflies (Diptera, Syrphidae) of Lesvos Island, Greece. *Molecular Ecology Resources* **9**, 1431–1438.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599.