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ORIGINAL ARTICLE

Molecular taxonomy of the *Sympetrum vulgatum* (Odonata: Libellulidae) complex in the West Palaearctic

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Abstract. The *Sympetrum vulgatum* (Linnaeus, 1758) complex is composed of the subspecies *S. vulgatum vulgatum*, *S. vulgatum decoloratum* (Selys, 1884) and *S. vulgatum ibericum* Ocharan, 1985 in the West Palaearctic. These taxa have parapatric distributions and noticeable morphological differences in colour and body size, and their taxonomic status is debated. Here we revise the systematics of this group using molecular taxonomy, including molecular analyses of mitochondrial (*cytochrome c oxidase subunit I*, *COI*) and nuclear (*internal transcribed spacer, ITS1*) DNA taking into account known morphological differences. Each subspecies has a unique and differentiated *COI* haplotype, although divergences among them are low (0.4% maximum uncorrected p-distance). The subspecies are not differentiated by the nuclear marker *ITS1*. The genetic results for these taxa contrast with the deep divergence of the sister species *S. striolatum* (Charpentier, 1840). Given current evidence, we propose to maintain the subspecific status of the *S. vulgatum* complex and hypothesize their biogeographical history. It is likely that the three subspecies became isolated during one of the latest glacial periods, each in a different refugium: *S. vulgatum ibericum* possibly occupied the Iberian Peninsula, *S. vulgatum vulgatum* the Balkan Peninsula or territories further east and *S. vulgatum decoloratum* Anatolia.

INTRODUCTION

Until the introduction of molecular systematics, the genus *Sympetrum* Newman, 1833 (Anisoptera: Libellulidae) included over 60 species divided into subgroups according to morphological criteria, especially the secondary genitalia of the male and the female vulvar scale. These groups are now considered artificial due to a lack of synapomorphies and the long-standing debate over which taxa should be included in the genus. Pilgrim & von Dohlen (2012), combining molecular and morphological methods, provided evidence for the monophyly of the genus in spite of the existence of some dubious taxa and proposed the use of *Sympetrum* sensu lato and sensu stricto. They divided the genus into six species-groups, but pointed out that the relationships between them were not satisfactorily settled. They conclude that the genus arose about 50 mya and that both dispersal and vicariance might have played an important role in its biogeographical history. Their preliminary estimates provide a divergence time for the species-groups of approximately 32–38 myr, possibly influenced by climate cooling and drying in the late Eocene and early Oligocene, leading to the fragmentation of populations. One of these groups is the *vulgatum* group, which has a Holarctic distribution and is formed by *S. vulgatum* (Linnaeus, 1758), *S. meridionale* (Selys, 1841), *S. sanguineum* (Müller, 1764), *S. striolatum* (Charpentier, 1840), *S. signiferum* Cannings & Garrison, 1991 and *S. vicinum* (Hagen, 1861). The *vulgatum* group started diverging approximately 32 mya, whereas the species *S. vulgatum* differentiated about 14 mya in the Miocene (Pilgrim & von Dohlen, 2012).

In addition, there are several species of *Sympetrum* that only differ in colouration or body size, which is a reason for doubting their validity. Molecular studies (Sawabe et al., 2004; Parkes et al., 2009) and others combining molecular and morphological analyses (Pilgrim & von Dohlen, 2007) conclude that in most cases there is no reason to maintain the specific status of some of them. However, there are still some cases that need clarification (Boudot & Kalkman, 2015), including the status of the *S. vulgatum* subspecific complex.

Sympetrum vulgatum has a wide distribution in Eurasia, from Western Europe to the oriental part of Russia. It sometimes reaches as far as Britain in the west and Japan in the east (see distribution map in Boudot & Kalkman, 2015). Most of this huge distribution area is occupied by the nominotypical subspecies, but in the south it is replaced

Fig. 1. Males (above) and females (below) of *Sympetrum vulgatum vulgatum* (left), *S. vulgatum ibericum* (middle) and *S. vulgatum decoloratum* (right).

by paler forms (Dumont, 1977; Ocharan, 1985). Thus, on the Iberian Peninsula and a minute part of southern France *S. vulgatum ibericum* Ocharan, 1985, and from Turkey to central Asia *S. vulgatum decoloratum* (Selys, 1884) occur (Boudot & Kalkman, 2015). In addition, there are two subspecies in East Asia: *S. vulgatum fuscopterum* (Belyshev, 1971) and *S. vulgatum imitans* (Selys, 1886).

Sympetrum vulgatum ibericum is restricted to thirty UTM 10 × 10 squares on the northern Iberian Peninsula, specifically in the eastern part of the Iberian System mountain range, the Pyrenees and Pre-Pyrenees and the northern sub-plateau (see distribution map in Díaz Martínez & Evangelio Pinach, 2015). Several formerly published records originated from confusion with *Sympetrum fonscolombii* and *S. sinaiticum* (Díaz Martínez & Evangelio Pinach, 2015). It is also present in one square in Andorra (Grand, 2004) and in three in the Pyrénées-Orientales department in France (Grand et al., 2007). Its distribution does not seem to overlap that of *S. vulgatum vulgatum*. Its habitat preferences are sunny stagnant waters such as marshlands, mountain lakes and gravel pits. It is believed to be significantly threatened (Boudot & Kalkman, 2015).

The distribution areas of *S. vulgatum decoloratum* and the nominotypical subspecies seem rather intricate and difficult to delineate. They possibly overlap in Georgia and Central Asia and there are individuals showing intermediate colouration in Kyrgyzstan (Schröter, 2010). In these geographical areas it occurs in a variety of shallow water bodies with clayey bottoms and abundant vegetation like floodplains and the littoral zones of water reservoirs (Schröter, 2010) and in Turkey in headwater regions above the treeline (Ikemeyer & Schneider, 2014).

Several authors have highlighted differences in colouration and size of these subspecies (Ocharan, 1985; Dijkstra & Lewington, 2006; Grand et al., 2007), which indicate the need to clarify the taxonomic status of both taxa by means of molecular analyses (Grand et al., 2007; Dijkstra & Kalkman, 2012; Boudot & Kalkman, 2015). These differences are summarized in Table 1 and illustrated in Fig. 1.

The aim of this study is to clarify the phylogenetic relationships between the European and Near East subspecies: *S. v. vulgatum*, *S. v. ibericum* and *S. v. decoloratum*, based on mitochondrial and nuclear DNA markers in order to shed light on their biogeographical history and if needed to revise their taxonomic rank.

MATERIAL AND METHODS

Specimens

Nuclear (*internal transcribed spacer*, *ITS1*) and/or mitochondrial (*cytochrome c oxidase subunit I*, *COI*) DNA fragments from a total of 30 individuals of *S. vulgatum* were sequenced for this study (Table 2). All specimens were preserved in 95% ethanol after collection, except those of *S. v. decoloratum*, which were dried in acetone, and stored at –20°C. These samples are deposited in the DNA and Tissues Collection of the Institute of Evolutionary Biology (IBE), Barcelona, Spain. Three *COI* and three

Table 1. Main differences in size and colouration of *Sympetrum vulgatum vulgatum*, *S. v. decoloratum* and *S. v. ibericum*.

Subspecies	Total length (mm)	Colour			
		General	Legs	Black thoracic lateral sutures	Black at base of frons
S. v. vulgatum	35–40	Darker	Black with yellow longitudinal markings	Marked with thick line	Extends along anterior margin of eyes
S. v. decoloratum	34–38	Much paler	Pale brownish	Marked with very narrow line	Barely visible
S. v. ibericum	30–36	Paler	Pale brownish with darker markings on inner sides	Marked with narrow line	Does not extend along margin of eyes

Table 2. Samples used in this study with the specimen codes, original localities and GenBank accession numbers.

Sample ID	Taxon	Locality	*COI*	*ITS1*
RVcoll16A404	*Sympetrum vulgatum vulgatum*	St.-Martin-de-Belleville, France	LT634113	LT634127
RVcoll16A405	*Sympetrum vulgatum vulgatum*	St.-Martin-de-Belleville, France	LT634114	LT634128
RVcoll16A406	*Sympetrum vulgatum vulgatum*	Montgellafrey, France	–	LT634129
RVcoll16A407	*Sympetrum vulgatum vulgatum*	Espoo, Finland	–	LT634130
RVcoll16A408	*Sympetrum vulgatum vulgatum*	Espoo, Finland	–	LT634131
RVcoll16A409	*Sympetrum vulgatum vulgatum*	Espoo, Finland	LT634115	LT634132
RVcoll16A410	*Sympetrum vulgatum vulgatum*	Espoo, Finland	–	LT634133
RVcoll16A411	*Sympetrum vulgatum vulgatum*	Espoo, Finland	–	LT634134
RVcoll16A412	*Sympetrum vulgatum vulgatum*	Espoo, Finland	LT634116	–
RVcoll16M104	*Sympetrum vulgatum vulgatum*	Ségur-les-Villas, France	LT898338	LT898344
RVcoll16M107	*Sympetrum vulgatum vulgatum*	Ségur-les-Villas, France	LT898339	LT898341
RVcoll16A413	*Sympetrum vulgatum ibericum*	Puebla de Lillo, Spain	–	LT634135
RVcoll16A414	*Sympetrum vulgatum ibericum*	Puebla de Lillo, Spain	LT634117	LT634136
RVcoll16A415	*Sympetrum vulgatum ibericum*	Puebla de Lillo, Spain	–	LT634137
RVcoll16A416	*Sympetrum vulgatum ibericum*	Puebla de Lillo, Spain	LT634118	LT634138
RVcoll16A417	*Sympetrum vulgatum ibericum*	Puebla de Lillo, Spain	LT634119	LT634139
RVcoll16A418	*Sympetrum vulgatum ibericum*	Puebla de Lillo, Spain	LT634120	LT634140
RVcoll16A419	*Sympetrum vulgatum ibericum*	Das, Spain	LT634121	LT634141
RVcoll16A420	*Sympetrum vulgatum ibericum*	Das, Spain	–	LT634142
RVcoll16A421	*Sympetrum vulgatum ibericum*	Das, Spain	LT634122	LT634143
RVcoll16A422	*Sympetrum vulgatum ibericum*	Das, Spain	LT634123	LT634144
RVcoll16A423	*Sympetrum vulgatum ibericum*	Das, Spain	LT634124	LT634145
RVcoll16M100	*Sympetrum vulgatum ibericum*	Puyvalador, France	LT898333	LT898348
RVcoll16M101	*Sympetrum vulgatum ibericum*	Puyvalador, France	LT898336	LT898347
RVcoll16M102	*Sympetrum vulgatum ibericum*	Puyvalador, France	–	LT898346
RVcoll16M103	*Sympetrum vulgatum ibericum*	Puyvalador, France	–	LT898345
RVcoll16M109	*Sympetrum vulgatum ibericum*	Estaing, France	LT898334	LT898342
RVcoll16M110	*Sympetrum vulgatum ibericum*	Estaing, France	LT898337	LT898343
RVcoll16M111	*Sympetrum vulgatum ibericum*	Estaing, France	LT898335	LT898340
RVcoll16A424	*Sympetrum vulgatum decoloratum*	Aspindza, Georgia	LT634125	–
RVcoll16A425	*Sympetrum vulgatum decoloratum*	Aspindza, Georgia	LT634126	LT634146
ST20	*Sympetrum striolatum*	Unknown	EF636244	EF636365
ST21	*Sympetrum striolatum*	Unknown	EF636245	EF636366
RF1852	*Sympetrum eroticum*	Ishikawa, Nomi, Japan	AB709153	AB707259

ITS1 additional sequences, from two *S. striolatum*, the sister species according to Pilgrim & von Dohlen, 2012, and one individual of *S. eroticum* were retrieved from GenBank.

COI and *ITS1* sequencing

DNA extraction was done following the protocol described in Vodă et al. (2015).

LCO1490 and Nancy primers (GGTCAACAAATCATAAAGATATTGG and CCCGGTAAAATTAAAATATAAACTTC, respectively) were employed for the *COI* amplification, and 21 sequences of 676 bp were obtained. Conditions were: first denatured at 92°C for 60 s, then 92°C for 15 s, 48°C for 45 s and 62°C for 150 s in 5 cycles and another 30 cycles in which the annealing temperature was 52°C with the final extension step at 62°C for 7 min.

ITS1 was amplified with 18S-forward and 5.8S-reverse primers (GATTACGTCCCTGCCCTTTG and CGATGATCAAGTGTCCTGCA, respectively) under the following conditions: first denatured at 94°C for 150 s, then 94°C for 30 s, 46°C for 60 s and 72°C for 60 s in 5 cycles and another 30 cycles in which the annealing temperature was 52°C with the final extension step at 72°C for 10 min. As a result, 29 sequences were obtained and the longest fragments obtained were 481 bp.

PCR products were purified and Sanger sequenced by Macrogen Inc. Europe (Amsterdam, the Netherlands). All sequences have been deposited in GenBank (accession nos in Table 2).

Phylogenetic reconstruction

DNA sequences were aligned with MAFFT v7.304 (Katoh & Standley, 2013). The best fitting nucleotide substitution models according to jModelTest v2.1.7 (Darriba et al., 2012) were HKY+I for *COI* and TPM2 for *ITS1*, both under BIC. We performed a Maximum Likelihood phylogenetic inference using raxmlGUI v1.3 (Silvestro & Michalak, 2012) selecting 100 runs and 1000 replicates. A Bayesian inference phylogeny was also obtained using Mr. Bayes v3.2.6 (Ronquist et al., 2012) with 1,000,000 generations and sampling every 1000 generations.

We constructed a *COI* haplotype network using the TCS Network method in PopART v1.7 (Clement et al., 2002). Genetic distances between subspecies were calculated for *COI* using MEGA v7.0.14 (Kumar et al., 2016) with the bootstrap method to estimate variance and using uncorrected p-distances (Collins et al., 2012; Srivathsan et al., 2012).

RESULTS AND DISCUSSION

The phylogenetic tree based on the mitochondrial *COI* marker (Fig. 2) recovered all *Sympetrum vulgatum* sequences in a highly supported clade. Within this clade, the subspecies *S. vulgatum ibericum* and *S. vulgatum decoloratum* were recovered as monophyletic (albeit with low support), and each taxon displayed a single haplotype, as also reflected in the *COI* haplotype network (Fig. 3). *Sympetrum vulgatum ibericum* specimens were differentiated from the rest by two diagnostic nucleotide changes, and *S. vulgatum decoloratum* displayed a single private mutation. Thus the maximum genetic distance was that of *Sympetrum vulgatum ibericum* with respect to *S. vulgatum decoloratum* (3 mutations, 0.4% uncorrected p-distance),

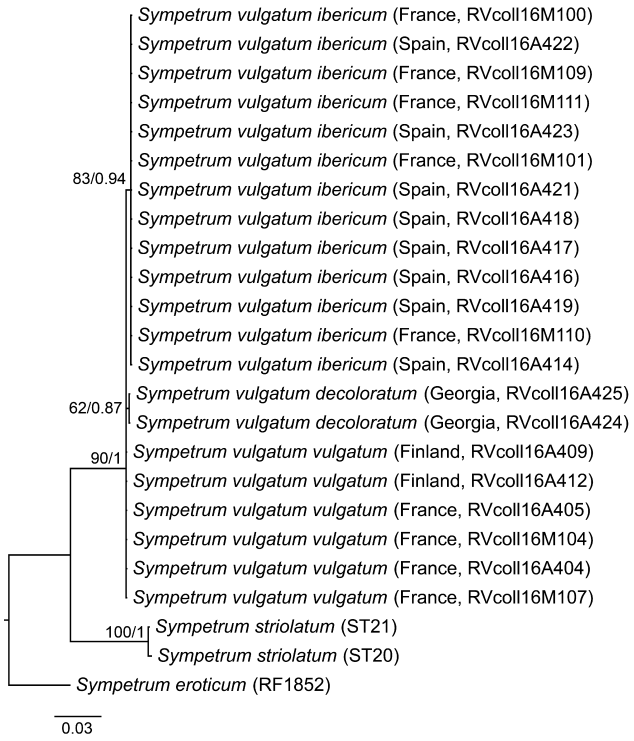

Fig. 2. *COI* gene tree obtained using Bayesian inference. Maximum likelihood bootstrap values and Bayesian posterior probabilities are indicated in this order for the key nodes. Scale units are presented in substitutions per site.

much lower than the typical divergences between generally accepted Odonata sister or closely related species (Rach et al., 2007; Damm et al., 2010; Froufe et al., 2013; Ferreira et al., 2016) and in concordance with documented intraspecific variation (Rach et al., 2007; Damm et al., 2010). In fact, genetic distances among the three taxa studied were minimal compared to that of the sister species *S. striolatum*, for which the minimum interspecific distance was 10.6%.

In the phylogeny based on *ITS1* sequences (Fig. 4) *S. vulgatum* formed a highly supported clade, but monophyly was not retrieved for any subspecies, indicating a close evolutionary relationship among all the individuals of this species. In fact, there were only two sequences with one variant each that did not correlate with the subspecies. *Sympetrum striolatum* sequences were once again highly divergent (6.3%) from those of *S. vulgatum*.

There is a remarkable divergence of more than 10% in *COI* of *S. vulgatum* and *S. striolatum*. Based on the data available *S. vulgatum ibericum* and *S. vulgatum decoloratum* cannot be treated as different species and it is proposed that the current subspecies status should be maintained. There are morphological differences and they could indeed be compatible with both species and subspecies status, but the low genetic differentiation detected, especially when compared to that of the sister species, suggests a very recent origin for these populations. It is suggested that light-

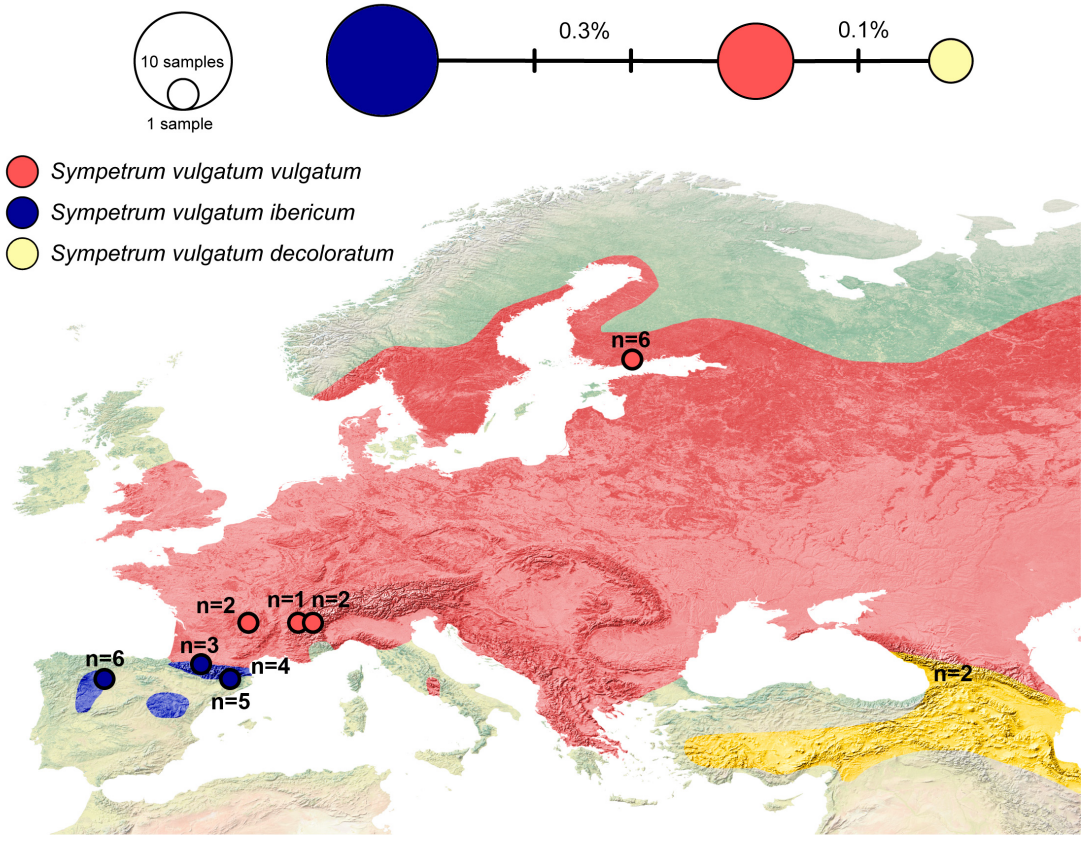

Fig. 3. Haplotype network based on *COI* with the minimum uncorrected p-distances between the subspecies indicated. Every mutation is marked with a bar and the circle size is proportional to the number of samples represented. Sample locations are marked with dots coloured to correspond with the haplotype. The number of samples used for *ITS1* and *COI* is indicated if there is more than one per locality. The map also shows the approximate distribution of the three subspecies: *S. v. ibericum* in blue, *S. v. vulgatum* in red and *S. v. decoloratum* in yellow, based on Boudot & Kalkman (2015) and Díaz Martínez & Evangelio Pinach (2015).

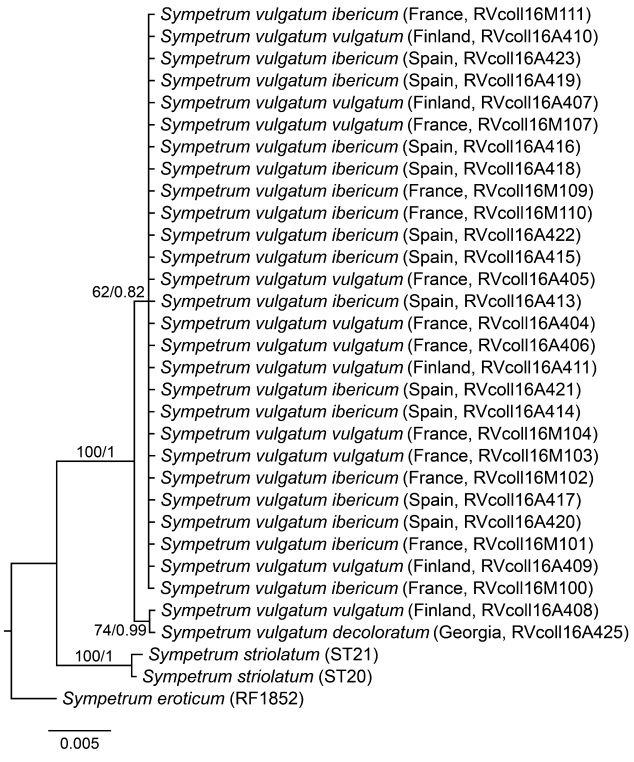

Fig. 4. *ITS1* gene tree obtained using Bayesian inference. Maximum likelihood bootstrap values and Bayesian posterior probabilities are indicated in this order. Scale units are presented in substitutions per site.

coloured insects may be favoured in warmer climates for thermoregulatory reasons (Zeuss et al., 2014; Pinkert et al., 2016) and this might be one plausible explanation for the pale colour of the meridional *S. vulgatum* subspecies. The results presented indicate that it is likely these populations diverged in the Upper Pleistocene. We hypothesize that they became isolated during one of the latest glacial periods in different refugia, *S. vulgatum ibericum* on the Iberian Peninsula, *S. vulgatum vulgatum* on the Balkan Peninsula or further east, and *S. vulgatum decoloratum* possibly in Anatolia. In the current interglacial, their ranges expanded to the present-day generally parapatric distributions. It is possible that the limited extent of secondary sympatry that is now observed is a result of a certain degree of hybrid depression in crosses between subspecies or to some degree of ecological specialization, but assessing this would require additional studies at the contact zones.

Overall, we found a low genetic differentiation among *S. vulgatum* putative subspecies in the mitochondrial marker *COI* and no divergence in the nuclear *ITS1*, with values below the estimated thresholds for reproductive isolation in Odonata (Sánchez-Guillén et al., 2014). These results sharply contrast with those obtained at the interspecific level, as there is a remarkable divergence of more than 10% in *COI* in the sister species *S. vulgatum* and *S. striolatum*. Based on the data available *S. vulgatum ibericum* and *S. vulgatum decoloratum* cannot be treated as different species and we propose that the current subspecies status should be maintained. It is important to obtain new evidence that sheds light on debated taxonomic questions in order to understand the evolutionary history of taxa, as well as to properly prioritize conservation efforts. In this regard, obtaining and combining ecological, morphological and molecular data is key to eventually reaching a consensus and stability in the systematics of Palaearctic Odonata.

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