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Genetic diversity, population structure and taxonomy of *Calopteryx splendens* (Odonata: Calopterygidae): An AFLP analysis

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Abstract. *Calopteryx splendens* is a widely distributed palaearctic damselfly with a remarkably uniform morphology. Variation in the size and shape of the pigmented spot on the wing is the main diagnostic character used to discriminate subspecies across its huge geographic range. Here, AFLP analysis was used to assess the genetic structure and diversity of nine populations representing 3 putative subspecies and evaluate the pigment spot as a taxonomic marker. Genetic diversity was high, with the number of polymorphic loci per population ranging from 141 to 280 out of a total of 333 variable sites (42.3–84.1%) and Nei's gene diversity from 0.160 to 0.283 (overall 0.299). Overall population genetic differentiation (F_{ST} = 0.2766) suggests limited gene flow and adaptation to local environments. Restricted gene flow and genetic differentiation among populations are supported by significant F_{ST} estimates. High levels of gene flow (N_m >1) were only recorded among three Asian populations (Russia – Kazakhstan – Turkey). The patterns of genotypic diversity suggest that a given wing spot size and shape may arise from the hybridization of a limited number, possibly not more than four, ancestral gene pools in different ways and at different times. Clearly, the sample analyzed was not sufficient to capture all of the complex history of *C. splendens*, but sufficient to indicate the taxa *ancilla*, *waterstoni*, and *orientalis* possibly represent three of the four ancestral gene pools, and originated in western Asia. The origin of the fourth, *xanthostoma*, is the western Mediterranean.

INTRODUCTION

Calopteryx (Odonata: Calopterygidae) is a genus of large and colourful damselflies with bright metallic bodies. Its members are restricted to running water and migrate over short distances, i.e. they are, on average, poor dispersers (Dumont, 1975; Stettmer, 1996). The wings of males of *Calopteryx splendens* Harris have iridescent blue veins, usually partly covered by a spot that is a secondary sexual character, which plays an important role in the courtship display (Askew, 2004). Males court females by flashing their spotted wings during their courtship flight (Askew, 2004; Corbet, 2004).

Studies on a *Calopteryx* of the *splendens*-group (*C. xanthostoma*) have shown that the extent of wing pigmentation is heritable but also correlates to some degree with the fat reserves and number of parasites hosted (Siva-Jothy, 2000; Cordoba-Aguilar et al., 2002; Koskimaki et al., 2004). Hopeman & Abramson (2005) found no correlation between degree of wing pigmentation and male mating or territorial success in *C. maculata*, but most results suggest that wing pigmentation is a signal of male quality (Grether, 1996; Tynkkynen et al., 2004; Hopeman & Abramson, 2005). Waage (1975) and Grether (1996) found that visual discrimination based on wing pigmentation is a major component of behaviour-based reproductive isolation between species.

In Europe, there are three well-defined, reproductively (almost) fully isolated species (*C. virgo*, *C. splendens*, and *C. haemorrhoidalis*) that can be identified by wing shape and colour, and colour of the ventrum of the male terminalia. No structural differences in the sexual organs exist. Of these, *C. splendens* is taxonomically the most complex. It also occupies the widest range, extending from North Africa to Europe and West-Central Asia as far north as 60°N, bounded in the East by the south end of Lake Baikal and the extreme south-west of Mongolia and of Xinjiang. More than a dozen "subspecies" and subspecific ranges have been catalogued in the last hundred and fifty years (Dumont, 1972). None of these can be separated structurally; they are identifiable only by the extent of the wing spot in males and by geographic range (Dumont et al., 1987, 1992, 1997, 2005), but whether the wing spot alone is sufficient for the delimitation of subspecies is uncertain. Studies on ethological and especially genetic differences for characterizing such infra-specific categories are necessary. Yet, to our knowledge, only two formal population genetic studies of *C. splendens* have to date been done, one in Sweden and one in France (Svensson et al., 2004; Chaput-Bardy et al., 2008) and both at a geographic scale too local to be of taxonomic significance.

Wing spots in males vary between absence (e.g. in ssp. *waterstoni* and *hyalina*) and almost complete coverage of the wings (only the wing base hyaline, e.g. in ssp.

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ancilla), with sometimes the tip of the wing slightly hyaline and females either with (androchrome) or without (gynochrome) a wing spot. Two further forms are *xanthostoma* (wing spot from near nodus to tip of wing), found only in the West Mediterranean although similar phenotypes occur in the Balkans and Western Asia, and *orientalis* (wing spot commencing halfway between nodus and wing tip and extending to wing tip). The form *ancilla*, also known under a variety of younger names (*intermedia*, *balcanica*, *caprai*, *faivrei*), is the most widely distributed phenotype. It occurs in the south of France, Italy, most of the Balkans south of Croatia, Poland, the Baltic States, extends into Russia and much of the Ob catchment, including the Irtysh subbasin, and is present in Anatolia, much of Iran, Afghanistan and parts of Uzbekistan. In contrast, *orientalis* is limited to the southern fringe of the Caspian Sea, and the related *syriaca* lives only in some Levantine valleys like those of the Jordan and Litani Rivers. Pure *waterstoni* is only found in a restricted coastal fringe along the south-eastern Black Sea. Forms with a reduced wing spot are named *taurica*, *tchaldyrica*, *mingrelica*, and *cartvelica*. They grade into *C. splendens splendens*, where the wing spot is centrally situated and there is a broad hyaline wing tip.

All adjacent wing-spot types hybridize and are easily identified where clines in wing spot extent occur. Hybrid populations can be spotted in the field if they are intermediate between the parental taxa, but experimental hybridization has shown that this is not always the case (Lindeboom, 1998): some hybrids look like one of the parent subspecies, and thus, molecular discrimination is necessary as wing spot analysis captures only part of the underlying genetic variation.

The goals of the present study were to: (1) characterize the genetic diversity, structure and population differentiation of *Calopteryx splendens* across a broad geographic area of Eurasia; (2) assess the geographic distribution of genetic diversity within and between *C. splendens* populations; (3) compare the prevalence of intraspecific gene flow with morphological differentiation.

To do this, specimens from nine populations across Eurasia were genotyped using the amplified fragment length polymorphism (AFLP) technique, developed by Vos et al. (1995). This technique continues to be widely used in analyses of genetic variation below species level, particularly in investigations of population structure and differentiation (Mueller & Wolfenbarger, 1999; Bensch & Åkesson, 2005; Lowe et al., 2003).

MATERIALS AND METHODS

Animal material

This study was performed on *Calopteryx splendens* (Har.) s.l. from Eurasia. Specimens were collected by the authors and their colleagues. Table 1 summarizes the characteristics of the populations sampled, including their geographical location and coordinates (Fig. 1). Each specimen was preserved initially in 70% ethyl alcohol, cleaned and then preserved separately in fresh 70% ethyl alcohol after examining them in the laboratory. In total, 114 male specimens from nine populations were used in the analysis.

Genomic DNA extraction

DNA was extracted from thoracic muscle using the protocol of the Puregene DNA Isolation Kit type D-5000A (Gentra Systems, Inc., BIOzym, Landgraaf, The Netherlands).

AFLP procedure

Five hundred nanograms of genomic DNA per individual were digested simultaneously with 0.5 µl (5 unit/µl) of *Eco*RI (Invitrogen, Carlsbad, CA, USA) and *Mse*I (New England Biolabs, Ipswich, MA, USA) at 37°C for 1 h. Following digestion, *Eco*RI and *Mse*I adapters were ligated to restricted fragments at 37°C for 3 h. A 1: 4 dilution of the restricted and adapter-ligated DNA was used as a template in the pre-amplification reactions. Pre-amplification products were generated using an *Eco*RI-primer with one selective nucleotide (*Eco*RI + T) and *Mse*I-primer with another selective nucleotide (*Mse*I + C).

Pre-amplification was performed by a thermocycler (Techne-Progene, Rockville, MD, USA) using pre-heating, 94°C for 2 min and then 20 cycles: 94°C for 30 s, 56°C for 30 s, 72°C for 60 s and finally post-heating, 72°C for 10 min. After estimating the concentration of the PCR product using 1% agarose gel electrophoresis, a second round of selective amplification was performed. At this stage, a 1 : 10 or 1 : 5 diluted pre-amplified DNA (related to the PCR product concentration at pre-amplification) was amplified using two fluorescently labelled *Eco*RI primers carrying three selective nucleotides (FAM-*Eco*RI primer + TAG, NED-*Eco*RI primer + TGA) in combination with *Mse*I primers including three selective nucleotides (*Mse*I primer + CAC, *Mse*I primer + CCG). Our choice of pre-selective and selective primer pairs was based on the study of Svensson et al. (2004) and results of a pilot study using seven different primer combinations.

TABLE 1. Number of specimens, where and by whom they were collected.

Population (Country)	No.	Locality	Collector
Azerbaijan	13	Kura valley, Agsu, 8.ix.2004, (40:34 N, 48:23 E)	H.J. Dumont
France	12	Canal au bord du Rhone, Gard, 12.vii.2004	H.J. Dumont & M. Papazian
Finland	13	Espoo, S Finland, 25.vi.2004 (60:12 N, 24:39 E)	M. Hamalainen
Iran	12	Cheshmeh Belgheis, Dehdasht, 14.viii.2003 (30:43 N, 50:31 E)	S. Sadeghi & A. Johargholizadeh
Kazakhstan	8	Irtysh River, China Border, 11.vii.2004,	H.J. Dumont & A. Haritonov
Russia	17	South Ural, 18.vii.2004 (52:41 N, 58:54 E)	H.J. Dumont & Haritonov
Slovenia	19	Rakov Skocjan, near Rakek, 2005 (45:47 N, 14:17 E)	H.J. Dumont & A. Brancelj
Spain	3	Ponte Caldelas, Vendugo River, 13.vii.1999 (42:23 N, 8:29W)	A. Cordero
Turkey	17	Hakkari, W Yuksekova, 24.vi.1998 (37:34 N, 43:45 E)	H.J. Dumont

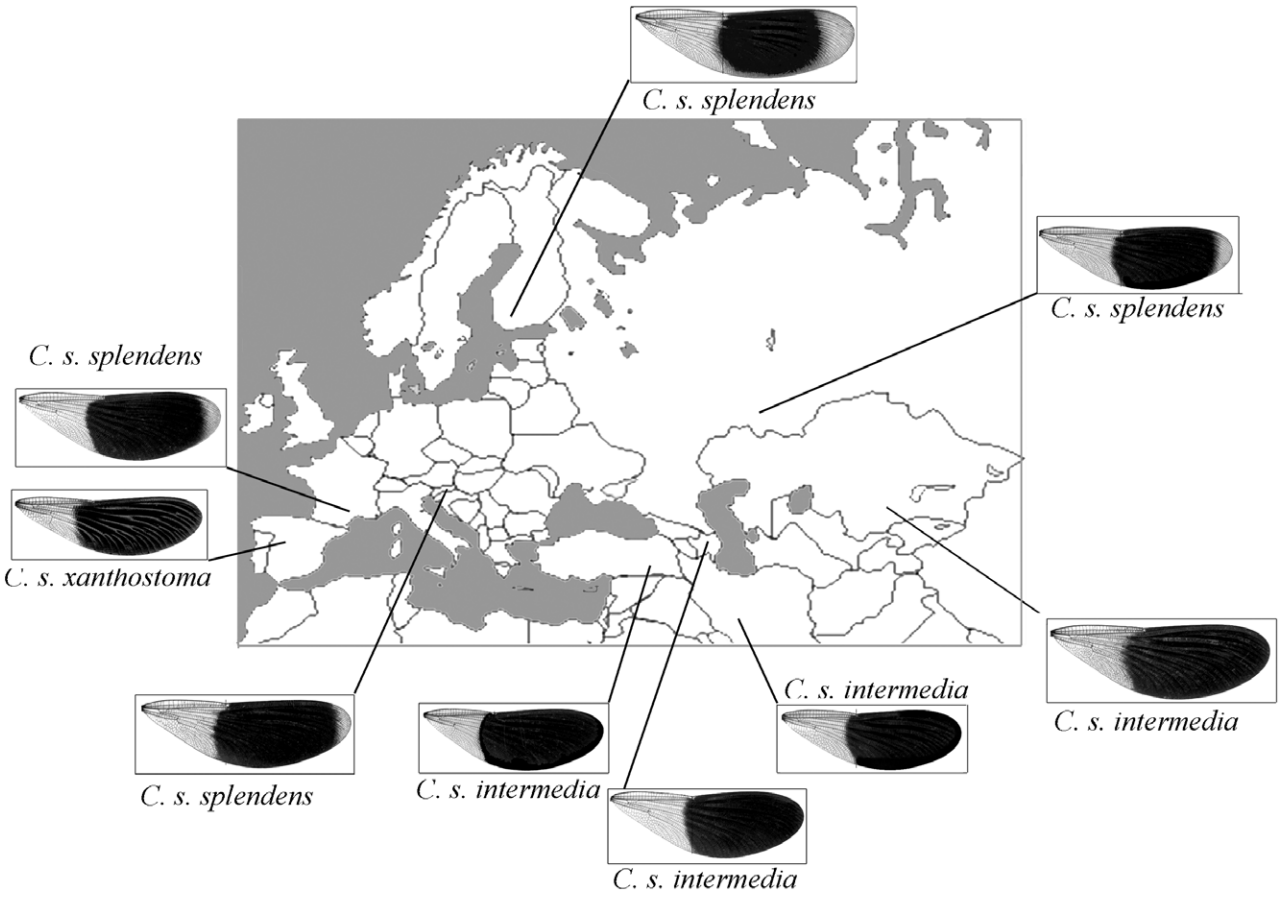

Fig. 1. The localities of the populations studied and photographs of the general form of the left fore wing of an individual from each the populations.

Capillary electrophoresis

Following amplification, samples were loaded onto the 16-capillary system of the Applied Biosystems 3130 *xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), in a 10 µl volume containing 0.5 µl of the selective amplification product mixed with 0.8 µl of the gene scan 600 LIZ size standard (Applied Biosystems P/N 4366589) and 8.7 µl of Hi-Di formamide (Applied Biosystems P/N 4311320). A 50 cm capillary array (Applied Biosystem P/N 4315930) and 3130 pop-7 polymer (Applied Biosystem P/N 4363785) were used. In total, the four primer pairs generated 333 polymorphic loci (AFLP markers), which were used in the analysis.

Data analysis

The AFLP markers were scored as present/absent in each individual by the GeneMapper software of the 3130 Genetic Analyzer. The resulting binary data matrix was analyzed using the NTSYS-pc software package (Version 2.11; Rohlf, 2004) to determine similarities among pairs of individuals. Genetic similarities based on Jaccard's coefficient (Jaccard, 1908) were calculated using the SIMQUAL module of NTSYS-pc. From this similarity matrix an Unweighted Pair Group Method (UPGMA; Sokal & Michener, 1958) dendrogram was generated using NTSYS-pc. Principal co-ordinate analysis (PCoA) was performed to represent inter-individual and inter-group relationships using the DCENTER and EIGEN modules of NTSYS-pc. A three dimensional plot was produced to illustrate the genetic similarities between individuals.

Genetic diversity and population genetic structure were evaluated using the program AFLP-SURV (Version 1.0; Vekemans, 2002), which computes the genetic distance between populations based on two approaches: that of Lynch & Milligan (1994) or Nei's gene diversity, and that of Clark & Lanigan (1993) for RAPD loci, extended for AFLP by Innan et al. (1999). Allelic frequencies were computed from the observed frequencies of fragments using the Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999) for diploid species, assuming Hardy-Weinberg genotypic proportions. Nei's (1973) gene diversity (also known as expected heterozygosity), global and pairwise genetic differentiation (F_{ST}) values were computed. Significance of the genetic differentiation between groups was tested by comparison of the observed F_{ST} with a distribution of values assuming no genetic structure, obtained by means of 1000 random permutations of individuals among populations.

Analysis of molecular variance (AMOVA; Excoffier et al., 2005) in ARLEQUIN (Version 3.1, 2005; Schneider et al., 1996) was performed to test the distribution of genetic variability among and within populations. This approach involves a hierarchical partitioning of the observed genetic variation, evaluated at three levels: differences between groups, differences among populations within groups and differences within populations (genetic variation attributable to individuals). A non-parametric procedure with 1023 permutations was used to test the significance of variance components associated with the different levels of genetic structure. The average level of gene flow (N_m) among populations was calculated based on the F_{ST} value (Slatkin, 1989, 1991) according to the equation

$$N_m = \frac{1}{4}\left(\frac{1}{F_{ST}} - 1\right)$$

TABLE 2. Number of polymorphic markers, polymorphism rate (%), and gene diversity within the 9 populations of *C. splendens* analyzed, based on 333 AFLP markers.

Population	Polymorphic loci	% polymorphic loci	Nei's Gene diversity	SE (Standard Error)
1 – Azerbaijan	280	84.1	0.283	0.0096
2 – France	278	83.5	0.267	0.0090
3 – Finland	141	42.3	0.172	0.0098
4 – Iran	178	53.5	0.208	0.0108
5 – Kazakhstan	178	53.5	0.161	0.0096
6 – Russia	198	59.5	0.217	0.0103
7 – Slovenia	147	44.1	0.160	0.0102
8 – Spain	199	59.8	0.235	0.0099
9 – Turkey	225	67.6	0.246	0.0100

using ARLEQUIN, where N_m is the absolute number of migrants exchanged between two populations.

Structure

To infer genetic structure within the range of population samples and define genetic relationships between populations with many morphological similarities, genetically homogenous populations ("gene pools") were identified by an ad hoc designed clustering approach implemented in the software STRUCTURE (Version 2.2, 2007, http://pritch.bsd.uchicago.edu/structure.html). The program uses a Markov Chain Monte Carlo (MCMC) algorithm to cluster individuals into gene pools on the basis of multilocus genotype data (Pritchard et al., 2000; Falush et al., 2003); it has also been applied to problems such as identifying cryptic population structure (Pritchard et al., 1999), detecting migrants or admixed individuals and inferring historical population admixture (e.g. Rosenberg et al., 2002; Falush et al., 2003, 2007; Albert et al., 2006). STRUCTURE assumes that all the genetic material of the individuals sampled comes from one or more of *K* unobserved populations or gene pools (Falush et al., 2007). The software places specimens in *K* clusters that have distinct marker frequencies, where *K* is chosen in advance and can be varied across different runs. This method, therefore, attempts to assign individuals to gene pools on the basis of their genotypes, while simultaneously estimating population allele frequencies.

On the presence/absence matrix an admixture ancestry model was used and allele frequencies were correlated with a burn-in period of 100,000 generations and MCMC simulations of 100,000 iterations. Then "STRUCTURE" was run ten times for each *K* value, where *K* is the potential number of gene pools in the overall sample of individuals. The most likely number of gene pools was determined by Δ (*K*) as described in Evanno et al. (2005). Then individuals of the nine populations were assigned probabilistically to the inferred gene pools. The outputs of STRUCTURE (individuals and population Q matrices) were displayed using Distruct software, Version 1.1, 2007 (Rosenberg, 2004).

Mantel test

The Mantel test (Mantel, 1967), a permutation test for estimating the correlation between two distances or similarity matrices (see Mantel, 1967; Sokal & Rohlf, 1995; Sokal, & Wartenberg, 1983), was used to evaluate the relationship between geographic distance and genetic differentiation of population pairs. The shortest geographic distances between populations were calculated using an online distance calculator (http://www.infoplease.com/atlas/calculate-distance.html). Geographic distances were then compared with F_{ST} values between populations using the MXCOMP module of NTSYS-pc (Rohlf, 2005).

RESULTS

AFLP marker profile

A total of 333 AFLP markers ranging in size from 50 to 400 bp were generated using four primer combinations. The presence/absence of these markers was assessed across the entire collection (114 demoiselles, 9 localities). The number of scored individuals, number and percentage of polymorphic loci and their genetic diversity, calculated using AFLPsurv, are summarized in Table 2.

The Azeiri population with 280 variable loci (84.1%) and Finnish population with 141 variable loci (42.3%) were the most and least variable populations, respectively. Nei's gene diversity (or expected heterozygosity within the population) ranged between 0.160 (Slovenia) and 0.283 (Azerbaijan).

Principal co-ordinate analysis

PCoA partitioned the genetic variance among the principal co-ordinates of the complete data set. The first three principal co-ordinates (Fig. 2) explain 23.4%, 9%, and 7.6% of the total variance (40.02% cumulative).

The plot shows three distinct clusters. The first two co-ordinates separate two groups at the two ends of the field and one mixed group in between. The group largely includes individuals from the Azeiri and French populations, which are separated by the third co-ordinate. On the right hand side a group consisting of Finnish and Slovenian individuals is formed. Turkish, Russian, Spanish, and Iranian populations form the middle cluster. The third axis separates the main populations within these groups. There is limited mixture of Turkish and Azeiri populations, and Iranian and Slovenian individuals. Unexpectedly, the Spanish individuals are not well separated in this plot, possibly due to the small sample size (N = 3).

Structure

The model-based clustering method of Pritchard et al. (2000) using an admixture model with correlated allele frequencies was applied to the data set without using prior information on the number of populations. This STRUCTURE analysis identified 7 distinct clusters (Fig. 3).

The most obvious pattern is the close genetic relationship of the Kazakhstan and Russian samples on the one hand, and the Finnish and Slovenian populations on the

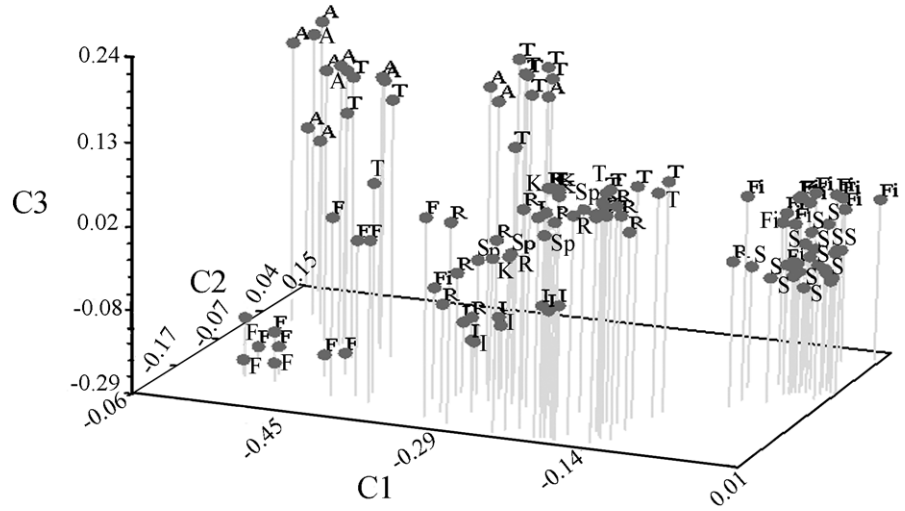

Fig. 2. Principal co-ordinate plot based on genetic similarity between 114 individuals from nine populations (1–9), A – Azerbaijan, F – France, Fi – Finland, I – Iran, K – Kazakhstan, R – Russia, S – Slovenia, Sp – Spain, T – Turkey.

other hand, although both contained traces of the other's gene pools. Azerbaijan, France, and Spain showed independent gene pools with some admixture from the Russia-Kazakhstan gene pool and a very slight admixture from other gene pools. The Iranian population too had a largely independent gene pool, including some admixture from Finland-Slovenia, and the Spanish population had an independent gene pool with traces of the Russian-Kazakh gene pool. The Turkish population contained elements from the Azerbaijani and Russian-Kazakhstani gene pools. The 7th gene pool was present as a trace in some populations. Azerbaijan and France were close to each other in the cluster analysis (see below) but in the K = 7 analysis, they had separate gene pools.

The assessment of the distribution of genetic variability among and within the 9 geographically distant populations, estimated by the Lynch and Milligan method using AFLP-SURV 1.0., gave a total gene diversity (H_t) of 0.2993, with the mean gene diversity within (H_w, analogous to Nei's H_s) populations 0.2165 (SE 0.015) and among ($_{Hb,}$ analogous to Nei's D_{st}) populations 0.0827 (SE 0.009). Wright's fixation index (F_{ST}) (Wright, 1951) was 0.2766 (SE 0.091). The observed value of F_{ST} was significantly genetically differentiated from random assemblages of individuals ($P < 0.0001$). Pairwise F_{ST} values were calculated between all populations, and a UPGMA tree was computed from the matrix of distances (Fig. 3). The collections from Russia and Kazakhstan were much more similar to each other than to those from Slovenia and France.

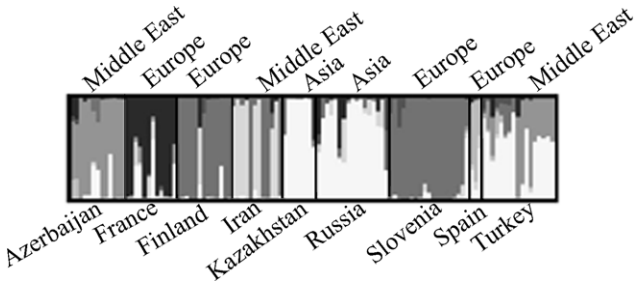

Fig. 3. Estimated population structure for all individuals. Each individual is represented by a vertical line of a different shade of grey, partitioned into K segments that represent the individual's estimated membership of K = 7 gene pools.

Cluster analysis within and among populations

To test the findings from the F_{ST} statistic an Analysis of Molecular Variance (AMOVA) was carried out using Arlequin version 2.001 (Schneider et al., 2000). On the basis of the haplotype scores, the genetic differentiation (φ_{st}) between all pairs of taxa was estimated using a phenetic method (Table 5, model 1). About 60.8% of total genetic variability among samples could be attributed to genetic differentiation within the nine populations, the remaining 39.2% was due to genetic differences between populations (φ_{st}= 39.2%). Pairwise comparisons of φ_{st} values (Table 3) among populations confirmed the presence of significant genetic differentiation between all the populations analyzed.

The Mantel test revealed no significant correlation ($P > 0.05$) between genetic differentiation (φ_{st}) and geographical distance matrices based on 100,000 random permutations. The lack of correlation between the geographic distance between populations and genetic (dis)similarity implies that factors other than isolation-by-distance are involved in the genetic isolation of the populations.

In the UPGMA dendrogram based on AMOVA-derived pairwise φ_{st} distances (Fig. 4), four clusters can be identified. Group I combines the populations from Azerbaijan and France, although at relatively large distances. Group II includes four Asian populations: Iran, Kazakhstan, Russia and Turkey. The Spanish population appears in a separate branch as group (III), while group IV clusters the Finnish and Slovenian populations. In the UPGMA dendrogram, the group IV and the Spanish population are most divergent from the other populations. The Russian and Kazakh populations are the most genetically similar, as expected from their geographical relationship. This

TABLE 3. Pairwise geographic distance (above the diagonal) and genetic differentiations (φ_{st} values) (below the diagonal) from the AMOVA. All φ_{st} values are significant ($P < 0.01$) after 1000 permutation; maximum and minimum values are in bold type.

Populations	Azerbaijan	Iran	Turkey	Kazakhstan	Russia	Spain	Slovenia	Finland	France
Azerbaijan	–	1138	**487**	2949	1864	4675	2809	2729	3591
Iran	0.42890	–	1022	3365	2868	5434	3629	3846	4353
Turkey	0.26128	0.29116	–	3424	2347	4477	2639	2875	3390
Kazakhstan	0.48886	0.40605	0.22097	–	1910	6870	5153	3980	5108
Russia	0.42523	0.26089	0.18491	0.07675	–	4967	3309	2127	4048
Spain	0.42729	0.40532	0.32252	0.54739	0.37926	–	1668	2994	1088
Slovenia	0.57441	0.28285	0.38606	0.47088	0.36520	0.53782	–	1742	796
Finland	0.53434	0.38174	0.36787	0.49105	0.38853	0.55128	0.23466	–	2263
France	0.29502	0.36255	0.33475	0.44985	0.35846	0.39850	**0.58354**	0.56575	–

grouping corresponds to the UPGMA dendrogram of all samples based on Jaccard's similarity coefficient, showing the relationships between all 114 individuals and the PCoA-plot (Fig. 2).

A dendrogram based on Nei's genetic distance between populations (results not shown) reveals a similar clustering, except for an exchange of position of groups I and IV. The Spanish population of *C. s. xanthostoma* (Dumont, 1972), often considered a valid species, is a distinct cluster. Surprisingly, the French and Azeiri populations (group I) show a close relationship to each other and the Finnish and Slovenian populations are the most divergent group of all.

An estimation of gene flow between populations (N_m) (Table 4) was obtained from the AMOVA-derived pairwise φst distances. Populations with $N_m > 1$ are considered to be genetically homogeneous in the absence of counteracting forces. These results reveal the presence of a high gene exchange rate ($N_m = 3.0073$) between Russian and Kazakh populations. The only other exchange with a value above one is between the Turkish and Russian populations ($N_m = 1.1021$). The lowest gene flow ($N_m = 0.1784$) is between the Slovenian and French populations.

Together, these results establish a lack of differentiation between Russian and Kazakh populations, and their close relationship with Turkish populations. On the other hand, the Slovenian and the Finnish populations are closely related, but well differentiated from French and Spanish populations.

To further explore the data, a three-level hierarchical analysis in which variation was partitioned at different levels (between groups, between populations within groups, and within populations) was applied. First, we placed all populations into a number of groups on the basis of their wing spot phenotype (model 2) or their geographical origin (model 3). Grouping individuals by geographic locality can be problematic because boundaries may be arbitrary and population allele frequencies may be biased by migration and inter-population hybridization (Allendorf & Luikart, 2007). When grouping the populations on the basis of the general appearance of their wing spots a statistically non-significant differentiation (1.13%) between groups was found (Table 5, model 2). This indicates that among the populations studied no genetically defined taxa based on wing spot variation could be identified.

In contrast, grouping on the basis of geographical differentiation revealed a striking difference between groups and between populations within groups. Although no prominent differences were present between the European and the Asian groups (Group 1: Asia; Group 2: Europe; model 3, Table 5, model 3), it seems that a deep genetic differentiation (16.95%) is present between populations from Asia, Western Europe (France and Spain), and Central Europe (Finland and Slovenia) (Table 5, model 4). The fifth model was based on the grouping seen in the PCoA plots and UPGMA-trees (Figs 2, 4). AMOVA showed that this population grouping correlates with the maximum amount of differentiation between groups (23.26).

TABLE 4. Matrix of estimated gene flow (N_m); maximum and minimum values are in bold type.

Populations	Azerbaijan	France	Finland	Iran	Kazakhstan	Russia	Slovenia	Spain	Turkey
Azerbaijan	–								
France	0.5947	–							
Finland	0.2179	0.1919	–						
Iran	0.3329	0.4396	0.4049	–					
Kazakhstan	0.2614	0.3058	0.2591	0.3657	–				
Russia	0.3379	0.4475	0.3935	0.7083	**3.0073**	–			
Slovenia	0.1853	**0.1784**	0.8154	0.6339	0.2809	0.4346	–		
Spain	0.3351	0.3774	0.2035	0.3668	0.2067	0.4092	0.2149	–	
Turkey	0.7069	0.4969	0.4296	0.6087	0.8814	1.1021	0.3976	0.5252	–

TABLE 5. Results of analysis of molecular variance (AMOVA) based on haplotype scores of 333 AFLP loci among 9 populations of *C. splendens*.

	AMOVA model analyzed				
	Model 1	Model 2	Model 3	Model 4	Model 5
	G: Az, Ir, Tu, Ru, Ka, Fr, Sp, Sl, Fi	G1: Az, Ir, Tu, Sp G2: Sl, Fi, Fr, Ru, Ka	G1: Az, Ir, Tu, Ru, Ka G2: Sp, Fr, Fi, Sl	G1: Az, Tu, Ir, Ru, Ka G2: Fi, Sl G3: Fr, Sp	G1: Tu, Ir, Ru, Ka G2: Az, Fr G3: Fi, Sl G4: Sp
	AMOVA results as % genetic variance				
Between groups	—	1.13	0.7	16.95**	23.26**
Between populations wthin groups	39.20**	38.38**	38.69**	25.97**	19.60**
Within populations	60.80**	60.49**	60.61**	57.08**	57.14**

** $P < 0.01$

DISCUSSION

In recent years, molecular genetic studies have lead to novel insights into the spatial genetic structure of aquatic insects (Kelly et al., 2002; Hughes et al., 2003; Wilcock et al., 2003).

In many cases, molecular results have supported classical morphological work, but there are cases where unexpected novelty emerges (Parkes et al., 2009).

Parkes et al. (2009) resolved the longstanding debate about the sub-specific level of *Sympetrum striolatum* and *S. nigrescens* using AFLP. They found no evidence to support a specific difference between them, but show that there is a clear pattern of isolation and population groupings caused by a salt water barrier to gene flow.

Here, we explore the genetic structure of nine geographically separated populations of *C. splendens*, a complex damselfly that has been the subject of much debate in past decades. We investigated whether wing spot differences (size and shape) between these populations (or subspecies), correspond to genetic differentiation, suspecting that traditional wing spot taxonomy might simplify reality too much, given that almost any wing spot can result from the hybridization of several possible parental forms.

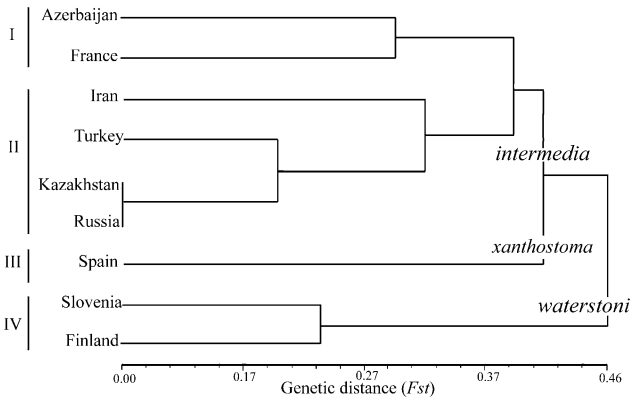

Fig. 4. UPGMA dendrogram based on the AMOVA-derived F_{ST} distance between populations of nine *C. splendens* populations based on AFLP data.

Populations within species can currently be identified using multilocus allele frequencies and statistical approaches for clustering individuals or populations (Allendorf & Luikart, 2007). For assessing the genetic relationships among our *C. splendens*, AFLP was used because this method allows the analysis of multiple loci in genomes with an unknown sequence composition.

Pairwise population comparisons of Nei's genetic distance and F_{ST} are interpreted as standardized inter-population distances between populations. Our AMOVA result for the fifth model ($\varphi_{st} = 0.4286$) explained 57% of the variation within populations and 20% of the variation among populations within groups, possibly due to little gene flow between collection sites or insufficient time for populations to reach equilibrium. Although F_{ST} is influenced by the number of populations analyzed and by the way in which individual populations are defined and sampled (Jorde, 1980; Urbanek et al., 1996) we identified striking differences between our samples.

The genetic differentiation of our samples is larger than that found by Svensson et al., (2004) in Sweden (overall $F_{ST} = 0.054$). They report much more genetic variation within populations (94.57%), which is explained by the geographical nearness of their populations. It is also larger than that reported by Chaput-Bardy et al. (2008) for populations about 500 km apart, whereas our study encompassed the entire width of the geographic range of the species. Compared with other groups of insects, our results are similar to φ_{st} values for winter pine moth in the Mediterranean region (Salvato et al., 2002), which ranges from 0.243 to 0.480, but higher than in tephritid flies (*Urophora cardui*) (0.096 to 0.161) (Eber & Brandl, 1997) and caddisflies (*Plectrocnemia conspersa*) in Europe (0.1260) (Wilcock et al., 2003).

Based on Wright's formula ($F_{ST} = 1 / (1 + 4 N_m)$, a combination of parameters reveals the relative strength of gene flow and genetic drift. Genetic drift generates substantial local differentiation if $N_m < 1$ but not if $N_m > 1$ (Slatkin, 1987). Our analysis found little gene flow between most populations except those of the Asian group. A better understanding of the relatedness between Russian and Kazakh populations and between Turkish

and Iranian populations, and the climatic history of Central Asia, may provide relevant clues.

Currently this area is largely arid or semi-arid, with the Caspian Lake level at –25 to –28 m, but a more humid climate in the past may have prompted genetic mixing. In fact, the humidity of the Pontocaspian area has continuously fluctuated over most of the Pleistocene, with the Caspian, Black, and Aral seas repeatedly united into a single lake with surface level at c. +45 m. One such episode occurred shortly before the last glaciation (40–70 kyr ago) (Kosarev & Yablonskaya, 1994). At that time, all major regional rivers, starting with the Danube in the west, the Volga, Ural, and the Amu and Syr Darya in the east, emptied in a single giant Ponto-caspian water body, and dispersal of aquatic insects across this enormous basin was facilitated. Contacts with the Baltic in Eastern Europe and the Ob and Irtysh rivers in Western Siberia, via the upper reaches of Uralian rivers, must have been relatively easy. They are still reflected in reduced genetic differentiation between the regional *Calopteryx* subspp., which is consistent with the definition of *ancilla-intermedia*, even if in the Turkish and, especially Iranian populations we suspect a presence of the *orientalis* genome. The Finnish and Slovenian populations could be part of another wave of dispersal that used the Danube or Dniepr as a pathway, as their genome is enriched with alleles that could be derived from *waterstoni*.

The *C. splendens* in France is genetically distinct from Spanish *C. xanthostoma*, although *xanthostoma* also occurs in the south of France. Such a differentiation is consistent with previous morphological and phylogenetic studies by Dumont (1972), Weekers et al. (2001) and Dumont et al. (2005a), who conclude that the west Mediterranean refugium allowed *xanthostoma* to survive the glaciations. Hybridization between the taxa *splendens* and *xantostoma* is reported: Papazian (1995) claims there is little contact between them in the south of France, but Pavesi (pers. com.), conversely, finds that in Northern Italy, *xanthostoma* is being introgressed by *splendens* at a rate measurable in terms of a human lifetime.

Curiously, there is a remote relationship between the "*ancilla*" cluster of populations and *C. xanthostoma,* which is closer than that with the "*waterstoni*" cluster. This might revive an old hypothesis by Bodenheimer (1935) that a corridor suitable for *Calopteryx* dispersal once existed across a more humid North Africa. To test this idea, an analysis of "*intermedia*" populations from Northern Syria-Hatay and Northern Iraq, as well as from the Jordan-Litani valleys (*Calopterxyx syriaca)* (Dumont, 1975a) will be required.

The relationship between the French and Azeiri (Kura) populations, with regard to their geographical position, is puzzling. Both populations are situated near the foot of mountain chains (the Pyrenees and Caucasus). Such mountain chains no doubt impede the rapid spread of the first invaders during inglacials (Hewitt, 2001). Afterwards, in contrast, they may have functioned as microrefugia that conserved some old genomes, perhaps dating back to the early Würm III deglaciation. These are currently, but slowly, invaded by "new" genes. Indeed, our results illustrate that while some clear groupings can be distinguished within the nine populations studied, virtually all populations have some gene-flow with other groups. This suggests a complex history of expansion and contraction of populations, with wave upon wave rolling over previously inhospitable territory. *Calopteryx* is considered a "slow" disperser, with many individuals spending their adult life within a few hundred meters of their place of emergence. Yet, a few individuals may travel several kilometers and cross the divide between adjacent streams (Stettmer, 1996; Ward & Mill, 2005). In around one millennium, consecutive generations of such individuals may carry their particular genome across the entire range of the species. The low sea levels around 18,000 BP and in the few millenia thereafter, making it much easier to cross from Anatolia to Greece, into Italy, and finally to southern France, make this a plausible scenario.

With this in mind, it becomes understandable why Russian, Kazakh, and the Turkish populations show high gene flow and low genetic differentiation while their wing spots differ. Conversely, French and Slovenian populations have a high genetic differentiation but a similar wing spot. These observations imply that genetically defined groups of *C. splendens* may, but do not necessarily, correspond to traditionally recognized taxa based on wing spot characteristics. Local adaptation (Guppy, 1986; Brakefield & Reitsma, 1991), character displacement due to coexistence with related species (Waage, 1975, 1979; Tynkkyen et al., 2004), parasites hosted (Siva-Joty, 2000; Koskimaki, 2004) and environmental factors (Kolyer, 1966, Torres et al., 1992; Hooper et al., 1999) may affect size and density of wing pigmentation. Therefore, wing spot similarity cannot capture the full genetic grouping of populations. Finally, nothing has been said so far about *C. splendens splendens*, the type subspecies, found in Western Europe and the British Isles. From the present analysis it follows, however, that no single genome adequately represents this taxon. It probably is a complex hybrid, containing elements of the gene pools of *waterstoni* and *ancilla*.

General conclusion

Morphological differentiation of *C. splendens* is restricted to wing spot features. This character has been used extensively by morpho-taxonomists, but is not sufficiently powerful for subspecific delineation; in contrast, the genetic structure of populations revealed in this study, although not covering the full genetic variation now suspected to be present, show that genetic differences between and within populations are a more informative criterion in subspecific or specific classification.

Based on the results of this study it is suggested there are four atavistic (probably pre-Pleistocene) gene pools (more may exist but currently there is no evidence for this): *waterstoni*, *ancilla* (= *intermedia*), *orientalis*, and *xanthostoma*. Individuals of all four gene pools may hybridize when they meet, as they expand their range along rivers as climate allows. From late-Pleistocene they

first dispersed from refugia in western Asia (*ancilla*, *orientalis*, and *waterstoni*) or North Africa-Iberia (*xanthostoma*), partly mixed in the Ponto-Caspian basin and then invaded Europe and Siberia, in consecutive waves. Contact between the populations that resulted from these invasions resulted in a variety of phenotypes that can only partly be characterized by size and shape of the wing spot in males, but their genetic basis is revealed by AFLP analysis.

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