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Coccinella septempunctata (Coleoptera, Coccinellidae): a species complex?

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Coccinella septempunctata L., the seven spot ladybird is widely distributed across the Palearctic region. Based on a few morphological characteristics and geographical origin, some populations are recognized as distinct species, e.g., the North African *Coccinella algerica* Kovář and Japanese *Coccinella brucki* Mulsant later considered to be a subspecies – *C. septempunctata brucki*. The objective of this study is to discuss the taxonomic status of the seven spot ladybird, in particular whether it is a complex of species or the same species throughout the Palearctic region. The relationship between populations was clarified by using a combination of molecular and limited morphometric data and assessing potential reproductive barriers by means of cross breeding. Although there is considerable variation in the size of the spots on the elytra, the results of this study confirm that all studied populations belong to the same species.

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Introduction

Species delimitation ('the process of identifying and delineating distinct organismal entities in nature', Roe & Sperling 2007) is essential for assessing biodiversity, conservation or biological control projects and generally understanding the natural world (Wheeler *et al.* 2004). The current taxonomy, especially for invertebrate groups, is mainly based on morphological criteria. However, phenotypic differentiation alone may fail to correctly delimit species and reconstruct phylogeny. First, it requires a high level of expertise and frequently results in false identifications (Hebert *et al.* 2003); in addition, the criteria used are often adult characteristics and unsuitable for the identification of immature stages. Second, as phenotypes are subject to selection, convergent evolution of morphological characteristics (homoplasy) is common. Finally, it does not take into account cryptic species, which are widely distributed in different taxonomic groups (Jarman & Elliott 2000). Although, the concept of DNA taxonomy has been hotly debated (Avise & Wollenberg 1997; Will & Rubinoff 2004; Hey 2006), it is undeniable that molecular data from nuclear and mitochondrial DNA can provide an extensive source of information (Vogler & Monaghan 2006; Hou *et al.* 2007). Today, Integrative Taxonomy, which uses a wide range of information, from morphology or behaviour to DNA sequences, is considered a more rigorous means of species delimitation (Roe & Sperling 2007).

Species delimitation in a widely distributed species complex, which experiences a range of environments and exhibits environmentally related phenotype differences, is particularly difficult. The phenotypic plasticity and population differentiation may be environmentally induced or inherited due to geographical isolation and resultant low or inexistent gene flow between populations. Both these evolutionary forces, genetic drift and natural selection,

may contribute to the often-rapid divergence between populations creating a reproductive isolation (Friesen *et al.* 2007) and, due to the development of pre and/or postzygotic barriers (Wu & Ting 2004), eventually lead to speciation. In such species it is often difficult to assess the status of the different populations.

The Coccinellidae (Coleoptera), commonly called ladybirds or ladybugs, are a species rich family (6000 species, Vandenberg 2002), distributed worldwide. Although many species have specific colour patterns on their elytra and pronotum, variability within species is frequent (Honěk 1996). This variability can be a characteristic of a species throughout its distribution or correlate with geographical location or seasonal variation (Majerus 1994). In many species, the variation is not gradual but discontinuous (Dobzhansky 1933; Honěk 1996). Identification of the ladybirds based on morphological characters, particularly colour patterns, often lead to errors: for example, the highly polymorphic *Harmonia axyridis* (Pallas) was previously split into 15 different species belonging to 5 genera (Lewis 1873; Iablokoff-Khnzorian 1982). Nowadays, the classification of this highly diverse family is based not only on external characters but also on the structure of genitalia of both sexes (e.g., Iablokoff-Khnzorian 1982; Ślipiński 2007). Nevertheless, misidentifications still occur (e.g., Hoelmer & Pickett 2003; Wanntorp 2004). Moreover, the status of some species of ladybirds remains controversial, as is the case within the genus *Adalia* (L.) (Palenko *et al.* 2004) or *Coccinella* L. (Iablokoff-Khnzorian 1982). A classification based only on morphological criteria may not reflect true relationships.

Coccinella septempunctata L., the seven spot ladybird, is one of the best known insects in the world. It is widely distributed across the Paleartic region, from the Iberian Peninsula in the West to Japan in the East, to the Sahara in the South and the tundra in the North. This species, however, is apparently absent in the area between Lake Baïkal and the Okhostsk region, which means that the Kamchatka, Sakhaline, Japan and South China populations are isolated from those in the West (Iablokoff-Khnzorian 1982). Moreover, this species was introduced into USA and Canada from 1957 to 1986 (Omkar & Pervez 2002) and became widespread and dominant throughout North America (Schaefer *et al.* 1987; Evans 2000). Such a large and discontinuous distribution raises the possibility of the different selective pressures operating in the various geographical locations together with a limited gene flow between populations, leading to phenotypic differentiation. In addition, some isolated populations have long been known to have distinct phenotypic characteristics, namely in the pattern of spots on the elytra (e.g., Dobzhansky & Sivertzev-Dobzhansky 1927; Rao 1962). Based on a few morphological characteristics and geographical origin, some populations have been recognized as distinct species as is the case for *Coccinella algerica* Kovář (Kovář 1977) in North Africa and *Coccinella brucki* Mulsant (Mulsant 1866) in Japan, which was later considered a subspecies – *C. septempunctata brucki* (Dobzhansky & Sivertzev-Dobzhansky 1927; Korschefsky 1932).

The objective of this study is to discuss the taxonomic status of what hereafter will be referred to as the seven spot ladybird. Does it consist of a complex of species or is it the same species throughout the Paleartic region? In order to answer to this question, the morphological and genetic relationships between 11 populations covering most of its original geographic range were characterized. Particular attention was paid to the Japanese and the Algerian populations, traditionally considered as separate taxonomic groups. To clarify the relationships between populations, the variation in neutral genetic markers was compared with the morphometric pattern. For each individual, polymorphic bands in the highly informative Inter Simple Sequence Repeats (ISSR), and the dominant multilocus markers, which evolve rapidly and are widely used in phylogenetic relationships at intra- and inter-specific level, were identified (Joshi *et al.* 2000; Wolfe & Randle 2001; Datwyler & Wolfe 2004). The morphometric approach involved measuring the size of the spots on the elytra, a conspicuous character, which together with other less obvious characters, are used to differentiate Algerian and Japanese populations from other seven spot ladybirds (Kovář 1977; Iablokoff-Khnzorian 1982; Bensusan *et al.* 2006). In addition, the potential reproductive barriers between populations from both extremes of the geographic distribution, i.e. a French and a Japanese populations, was assessed by means of breeding tests, which are particularly important if the sibling species are not sympatric (Mayr 1970).

Materials and Methods

Biological material for the morphometric and molecular studies

Specimens were collected in the field from 12 populations of the seven spot ladybird scattered throughout its actual range and then preserved in 95% ethanol or frozen at −80 °C. The origin of the samples, together with the name of the collectors, is given in Table 1.

Morphometric study

The surface area of the elytral spots (*maculas*: *m. lateralis*, *m. discalis*, *m. scutellaris* and *m. marginalis*, Fig. 1) was measured relative to that of total area of the elytra for each individual. In total, 4–10 right hand elytra were analysed for each population (except for the Georgian individuals,

Table 1 Specimen geographical origin, code used in the present paper, number of collected individuals and collectors

Origin	Code	Individuals	Collectors
India (Lucknow city)	IL	15	Dr Omkar
India (Shimla)	IS	15	Dr D.C. Gautam
Iran	Ir	30	Dr S. Moharramipour Mrs Kianpour
Georgia (Doesi)	Ge	2	Dr S. Barjadze
Portugal (Lisbon)	P	19	Dr J.-C. Franco
France (Pyrénées)	FP	25	The authors
France (Toulouse)	FT	15	The authors
Belgium	B	9	Dr J.-F. Godeau
Greece (Athens)	G	15	The authors
Japan (Fuchu)	Jap	23	Dr M. Majerus
Algeria (Alger)	Alg	15	Dr L. Saharaoui
USA (Utah)	USA	15	Dr E. Evans

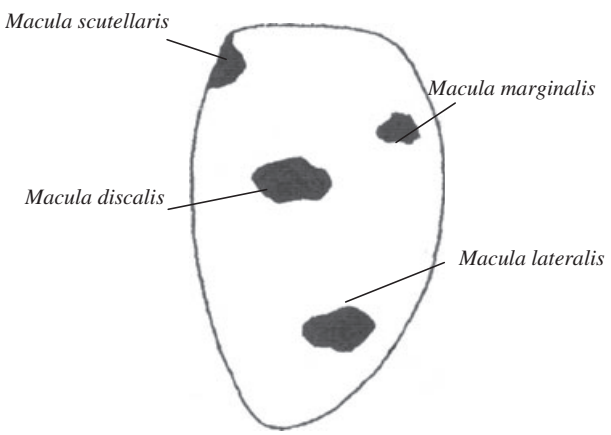

Fig. 1 General pattern of the right elytron of a seven spots ladybird, following Dobzhansky & Sivertzev-Dobzhansky (1927).

which were no longer available and were studied only from a molecular point of view).

As the surface of the elytra is convex, they were flattened prior to measurement. Each elytron was placed in a solution of 10% KOH for 30 min, which softens them. After this, two incisions were made in each elytron, which was then placed between two standard microscope slides. This dry mount was photographed alongside a slide micrometer (Graticule LTD, Tumbridge, Kent, UK, 100 × 0.05 = 5 mm), which was used to calibrate the photographs. An Olympus SF 10 camera (Rungis, France) coupled with a Nikon SE stereo microscope (Champigny-sur-Marne, France) was used. Photographs were analysed using the software ImageJ 1.38x (Maryland, USA), which determines the surface area of each spot and the total surface of the elytron. The relative surface area of each spot was then calculated. A principal component analysis (PCA) of the arcsine transformed data (Zar 1996) was performed using software R 2.4.0.

Molecular study

Experimental protocols. Total genomic DNA was extracted from whole beetles (minus elytra) using DNeasy Blood and tissue Kit from QIAGEN (Courtaboeuf, France) with PBS protocol according to the manufacturers instructions.

For each mitochondrial (CO sub-unit I, 12S, 16S) and nuclear RNA gene (18S and 28S) specific primers were used to amplify a region of approximately 710, 370, 360, 850 and 340 bp, respectively (Whiting *et al.* 1997; Kergoat *et al.* 2004; Orsini *et al.* 2007; Magro *et al.* 2010). Polymerase chain reactions, carried out in a T3 Thermocycler Biometra (Goettingen, Germany), were performed with 50 ng of DNA in a 25-μL volume containing a final concentration of 1× PCR buffer, 0.2 μM of each primer, 0.2 mM of each dNTPs, 1.5 mM of $MgCl_2$ and 1 U of Taq polymerase. PCR conditions involved initial denaturing of 5 min at 95 °C, followed by 35 cycles (Touch Down PCR 55–50° for the first 5 cycles for the 12, 18 and 28S genes and 45–40° for the 16S gene) of 1 min at 95 °C, 1 min at 50 °C (40 °C for the 16S), 1 min at 72 °C and 10 min extension at 72 °C. After electrophoresis on a 1% agarose gel, the PCR products were purified (QIAGEN PCR purification kit) before sequencing. The amplification of the cytochrome oxidase subunit I (COI), from universal primers (LCO 1490-HCO 2198: Folmer *et al.* 1994), involved initial denaturing at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 48 °C for 1 min, 72 °C for 1.5 min, a final extension at 72 °C for 10 min.

From an initial screening of 11 ISSR primers, six produced banding patterns (Table 2). The optimal temperature was determined for each primer independently, using an Eppendorf Mastercycler Gradient thermocycler (Le Pecq, France). The final reaction mixture (25 μL) contained 60 ng of DNA with the final concentration of 1× PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4–0.8 μM each primer and 2 U of Ampli Taq Gold (Applied Biosystem, Courtaboeuf, France). PCRs were performed, with the following conditions: initial denaturation of

Table 2 Oligonucleotide primers used for ISSR amplifications and number of polymorphic fragments in the total species

Primers	Sequence (5′ to 3′)	Amplified samples	Polymorphic bands
(CA+)	$(CA)_7RY$	60	41
(+CA)	$RY(CA)_7$	57	86
(ACA+)	$(ACA)_5BDB$	58	50
(+ACA)	$BDB(ACA)_5$	17	None
(GACA+)	$(GACA)_4WB$	12	None
(+GACA)	$WB(GACA)_4$	58	46

B = C, G or T; D = A, G, or T; S = C or G.

10 min at 94 °C followed by 40 rounds of three stages: 94 °C for 45 s, 50 or 48 °C according to the primers for 45 s, 72 °C for 2 min, then 72 °C for 10 min. The PCR products were visualized by electrophoresis on a 2% agarose gel (2/3 Nu Sieve, 1/3 standard agarose) in 0.5× buffer (Tris acetate EDTA).

Analyses of genetic diversity

COI and ribosomal genes. Sequences were first corrected and aligned using the software BioEdit 7 (Hall 1999). Genetic distances were estimated taking into account the rate of transversions and transitions and a heterogeneous rate of substitution between bases, using the Tamura & Nei (1993) test and MEGA 3.1 (Kumar *et al.* 2004). The aligned sequences for the phylogenetic reconstruction were analysed using maximum likelihood (ML) analyses and Bayesian inferences (BI).

The ML analyses were performed with PHYML 2.4.4 (Guindon & Gascuel 2003). Best-fit models of evolution for each dataset were determined using the Akaike information criterion, as implemented in MODELTEST version 3.7 (Posada & Crandall 1998): General Time Reversible with a proportion of invariants sites and distribution gamma (GTR + I + Γ). Bootstrap analysis (Felsenstein 1985) with 1000 replications was used to estimate the robustness of each node. Bayesian inference analyses were conducted using MRBAYES 3.1.2 software (Huelsenbeck & Ronquist 2001). Two independent runs using four Markov chains were conducted for 5 million generations with a sampling of trees and parameters every 100 generations (50 000 trees). For the two reconstruction methods, four species of the genus *Coccinella* were used as an out-group: *Coccinella quinquepunctata* L., *Coccinella undecimpunctata* L., *Coccinella magnifica* (Redtenbacher) and *Coccinella miranda* Wollaston.

Identical COI haplotypes were identified through the construction of a distance matrix. A minimum spanning network among haplotypes was constructed using the ARLEQUIN 3 software (Excoffier *et al.* 2005) to visualize the phylogenetic relationships among the different COI haplotypes and the number of mutational steps between the haplotypes.

Inter Simple Sequence Repeat (ISSR) patterns. Band scoring and sizing were carried out using BIO 1D + + V97.06 software and coded for the establishment of a binary matrix of presence (1)/absence (0). Only unambiguous bands that were present in at least two samples were used in the analysis of ISSR patterns. To assess the reproducibility of the ISSR assays, PCR reactions were repeated at least twice for a great number of samples.

Several similarity coefficients were estimated as the simple correlation coefficient [Simple Matching Coefficient (SMC)], the Jaccard's coefficient (J) and the Nei & Li's coefficient (NL) (Lamboy 1994) (Table 3). To construct the matrices of pairwise genetic distances, (1 – SMC), (1 – J) and (1 – NL) were used. The NL coefficient ($NL = 2p_{ij}/(p_i + p_j)$ most used in the literature (Gryta *et al.* 2006) is given.

Table 3 Similarity coefficients used for this study

Simple Matching Coefficient	$SMC = (p_{ij} + a_{ij})/N$
Jaccard's coefficient	$J = p_{ij}/(p_i + p_j - p_{ij})$
Nei & Li's coefficient	$NL = 2p_{ij}/(p_i + p_j)$

p_{ij}, number of bands common to individuals *i* and *j*; a_{ij}, number of bands found at either *i* or j; *N*, total number of bands.

The phylogenetic relationships between individuals from such data are generated by the neighbor-joining method (NJ) (Saitou & Nei 1987), because it takes into account the differences in the rate of evolution between branches of the phylogenetic tree. Our analysis of the specimens with no out-group provides an un-rooted tree. For both indices SMC and J, phylogenetic reconstruction was obtained by using software PAUP * 4.0b10 (Swofford 2003). The tree was built using NL index with the software Treecon 1.3b (Van de Peer & De Watcher 1993), and 1000 replicates of bootstrap were performed. A nonparametric analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) was carried out using the ARLEQUIN 3 software (Excoffier *et al.* 2005) to test for the distribution of genetic variability among and within populations.

Existence of potential reproductive barriers between populations: crosses between geographical strains

For this study, individuals from the two most geographically distant populations that we had access to, the French and Japanese populations, were chosen.

Biological material. The individuals from France were collected in February 2008 in the Toulouse region and each ladybird was kept in a 50-mm diameter Petri dish at 20 ± 1 °C, LD 16 : 8 until the beginning of the experiment. Three times a week, ladybirds were fed an excess of pea aphids, *Acyrthosiphon pisum* Harris. The individuals from Japan were originally collected from fields around Tsuruoka and were reared in our laboratory for a year, when the experiment started. Adults used in the experiment, were isolated at emergence in Petri dishes 50 mm diameter at 20 ± 1 °C, LD 16 : 8, and similarly fed an excess of pea aphids three times a week.

Crossing experiment. *(a) P Generation* Three kinds of crosses were conducted, two intra-population crosses (C1:

♀ Japan × ♂ Japan and C2: ♀ France × ♂ France) and one inter-population cross (C3: ♀ Japan × ♂ France) with 13, 9 and 8 couples, respectively. The reciprocal cross ♀ France × ♂ Japan was not performed because it was not possible to determine whether the females collected in the field in February were not fertilized the previous autumn (Hodek & Ceryngier 2000 show this is the case for 50% of the females). For each cross, the couples were isolated in 90 mm Petri dishes with a piece of corrugated filter paper, on which the females laid eggs. Every day, the couples were transferred to clean Petri dishes and fed an excess of pea aphids. One stem of broad bean, *Vicia faba* L. (variety 'Primabel'), was added to each dish to improve the survival of the aphids. The couples were kept at 20 ± 1 °C, LD 16 : 8. Daily fecundity (number of eggs lay per day and per female) and fertility (proportion of fertile eggs) were recorded for each couple from the seventh day after the beetles were first placed together and continued for 20 days.

A mean of 450 larvae resulting from each of the three crossings were reared to adult stage. They were kept in 175 cm^3 plastic boxes (12–16 larvae per box) and maintained at the same temperature and light conditions as indicated above. Three times a week, the boxes were cleaned and a piece of corrugated paper, a stem of broad bean and pea aphids were added to the box. Once the adults emerged, each individual was isolated in a 50 mm Petri dish and treated as described before.

(b) F1 Generation. New couples were formed using adults that were produced by each of the P generations crosses. These couples were reared individually like the P couples and again their daily fecundity and fertility were recorded for 20 days (see above). Finally, females of those couples were dissected and the ovarioles were checked to see if the females were in diapause (presence of a large amount of fat and ovaries consisting only of germaria, as described by Hodek 1996). The number of ovarioles in the individuals resulting from the inter-population crosses and the surface of the right elytra (an indication of adult size), were recorded. A nonparametric ANOVA (Kruskal–Wallis test) was conducted in R 2.4.0 on the fecundity and fertility data for each generation (fertility data was first arcsine transformed; Zar 1996). These analyses were followed by a *post hoc* Dunn test.

Two components of fitness, adult size (represented by the surface area of the right elytra) and number of ovarioles, were measured for F1 generation hybrids and compared with those of the individuals from the French and Japanese parental populations, by means of an ANOVA and a Tukey *post hoc* test.

Results

Variation in relative surface area of the spots on the elytra

The PCA of the relative surface area of the spots (*macula lateralis*, *discalis*, *marginalis* and *scutellaris*), indicates that their size is correlated (Fig. 2a). This analysis also indicates that the Japanese population and one of the individuals from the Indian Lucknow city population (IL1) were significantly different from the other individuals in having relatively large spots (Fig. 2b). The other populations cluster in a homogeneous group with relatively small spots, with the Algerian population having slightly larger spots.

Genetic structure of this species complex

The four ribosomal genes (two mitochondrial, 12 and 16S and two nuclear genes, 18 and 28S), with a total of ~1920 bp, are monomorphic for all the specimens studied, with the exception of one individual from Japan that has two substitutions in the 12S sequence (mitochondrial gene). As these markers are uninformative for the intra-species relationships, these results were not included in this study.

Polymorphism of the cytochrome oxidase subunit I gene. This marker amplified a 487 bp sequence in 11 populations over 12 (42 individuals), individuals from the Iran population did not amplified. A total of 14 haplotypes with 47 variable sites (9.6%), 40 parsimony-informative sites and four non-synonymous substitutions, were identified. The Tamura & Nei genetic distances were calculated after adding four species of the *Coccinella* genus as an out-group. ML and Bayesian analyses of the dataset yielded a similar topology (Supporting information, Fig. S1). All the populations cluster in a highly robust monophyletic group (bootstrap >90%), well differentiated from the other *Coccinella* species. The base of the tree is an extended polytomy, including all the populations, which indicates this marker has little resolving power and the populations are all paraphyletic. Intra-population distances are low (between 0% and 0.7%), except for the Japanese population (4%) which is highly polymorphic (37 substitutions over 45 variable sites). The inter-population variability is about 3.0% between the Japanese and other populations, and between 0% and 0.8% between the Algerian and other populations. However, this range in nucleotide diversity is much lower than that observed between seven spot ladybirds and the four other *Coccinella* species (between 12.6% and 17.2%).

A minimum spanning network further highlighted the relationships among the haplotypes. Most haplotypes differ by only a single mutational step from one another. However, haplotypes h6 and h8 (from Japan) are clearly distant from the others, with 18 and 20 mutations respectively

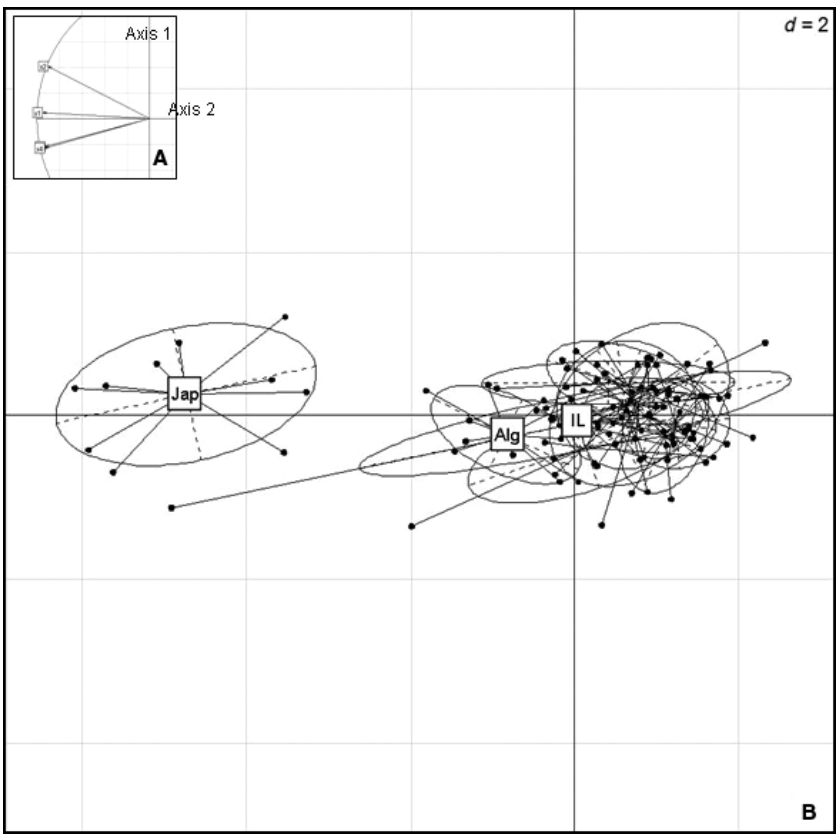

Fig. 2 Principal component analysis applied to the relative surface of the right elytron spots of individuals belonging to 11 populations of the seven spot ladybird. —A. Axis 1 represents 86.3% of the total information; Axis 2 represents 6.8%. x1 = *macula lateralis*, x2 = *m. discalis*, x3 = *m. scutellaris* and x4 = *m. marginalis*. —B. Each ellipse represents 75% of the data for each population. Jap, Japan; Alg, Algeria; IL, India (Lucknow city); IL1, Individual 1 of the Indian Lucknow city population.

(Fig. 3). The major haplotype was found in 23 individuals (54.8%), including specimens from all the populations except Algeria. The haplotype h7, which is distant by one mutation from the major haplotype h5, had a central position in the network and several haplotypes or clades could be derived from it. The haplotypes can not be grouped based on their geographical origin. In the Japanese population four haplotypes were identified (eight individuals sequenced), differing by up to 37 substitutions of which three were non-synonymous (h6 and h8 are the most divergent).

The results indicate that the seven spot ladybird group is monophyletic, with a low level of structure at the intraspecific level and a high degree of divergence within the Japanese population.

Polymorphism of the Inter Simple Sequence Repeat. Of the six non-anchored ISSR primers tested on the 12 populations, only four provide clear and reproducible banding patterns (CA+, +GACA, ACA+, +CA) (Table 2). The other two primers (GACA+ and +ACA) give amplification for few individuals but never those extracted from individuals from the Japanese or Algerian populations, even in less stringent conditions. These two primers were therefore excluded

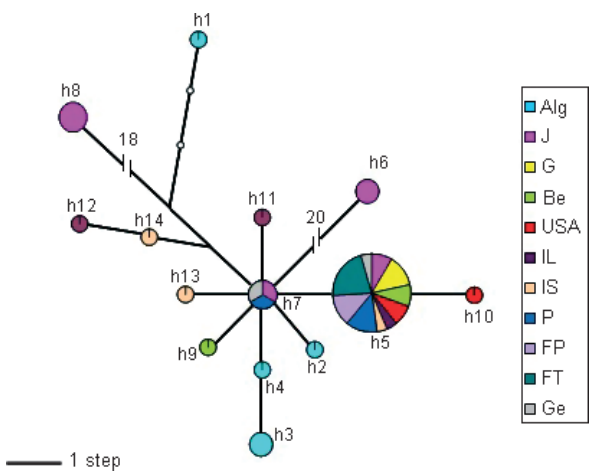

Fig. 3 Minimum spanning network among COI haplotypes. Branches connecting circles are mutation steps and the small open circles indicate missing haplotypes. The area of each circle is representative of the frequency with which the haplotypes occurred in the total sample.

from the phylogenetic analyses. By combining the four remaining ISSR markers, a total of 223 clear and reproducible polymorphic bands, present in at least two individ-

uals, were generated (Table 2) ranging in size from 200 to 1600 bp (from 12 to 60 individuals per marker).

Based on a matrix of dissimilarity indices (1-SMC, 1-J, 1-NL), a tree was constructed including all the individuals analysed, even those for which one or more primers did not amplify the sequences (Table 2). Each population is clearly monophyletic but the topology of trees depends on the dissimilarity index used. Indeed, a greater branch length is observed for individuals with missing data; this bias is observed especially for the SMC index, which gives more importance to missing data than other indices. To avoid this bias, individuals with missing data were deleted from the dataset. In this case, topologies are similar whatever the index and the most used index, the Nei & Li's index, was chosen (Wolfe & Randle 2001; Gryta *et al.* 2006).

Based on the combined data matrix obtained using four primers, a high level of genetic diversity was detected in the different populations of seven spot ladybirds. Given the specificity of the primers and the problem resulting from cross species amplification, the trees are represented un-rooted (Fig. 4). Each population is monophyletic and robust, supported by strong bootstraps (>80% in ~67% of cases). The most genetically distant individuals are those from Iran, France (Toulouse) and Algeria. The intra-population polymorphism observed in the Japanese population, when the COI data is used, is not apparent when these highly polymorphic markers are used. The AMOVA indicates that the genetic variance within populations is 57.2% and between populations 42.8%, which indicates a high level of population differentiation. The pairwise population comparisons show that the most divergent populations are those from India (Shimla) and Georgia (60.2%).

Reproductive barriers between populations

(a) P Generation. The fecundity and fertility values for the 3 types of couples are presented in Table 4. For C1 cross (♀ Japan × ♂ Japan), 46.2% of the couples did not lay any fertile eggs while all those couples used in the C2 (♀ France × ♂ France) and C3 crosses (♀ Japan × ♂ France) were fecund and fertile. The Kruskal–Wallis test shows a highly significant difference between the three crosses, both in fecundity (Kruskal–Wallis chi-squared = 17.79, d.f. = 2, *P*-value = 0.00014) and fertility (Kruskal–Wallis chi-squared = 16.62, d.f. = 2, *P*-value = 0.00025). The

Table 4 Fecundity and fertility results for couples issued from C1: ♀ Japan × ♂ Japan, C2: ♀ France × ♂ France and C3: ♀ Japan × ♂ France crosses (P generation)

	C1	C2	C3
Total number of couples	13	9	8
Number of fecund couples	13	9	8
Mean fecundity	9.43 (5.69) *a*	29.45 (7.09) *b*	20.83 (10.33) *a,b*
Number of fertile couples	7	9	8
Mean fertility	0.09 (0.06) *a*	0.55 (0.16) *b*	0.39 (0.30) *a,b*

Values between brackets = SD. *a* and *b* correspond to the results of the Dunn *post hoc* test ($\alpha = 0.05$).

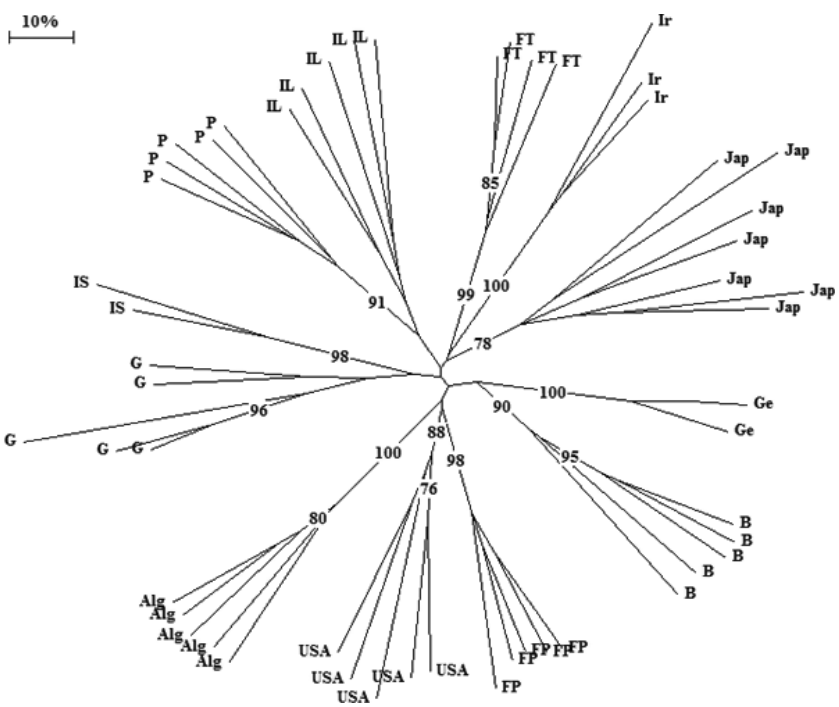

Fig. 4 Tree topology yielded by Neighbor-Joining method using the Nei & Li (1979) distances between the populations based on ISSR data. Samples are labelled according to codes listed in Table 1. Bootstrap values are given in the nodes. The scale indicates 10% of divergence and branch lengths are proportional.

Dunn *post hoc* test (Table 4) only reveals a significant difference between treatments C1 and C2 ($\alpha = 0.05\%$). Fecundity and percentage fertility of the crosses indicate there are no reproductive barriers between the populations at this stage. The fecundity and percentage fertility of the cross C1 (♀ Japan × ♂ Japan) was low, which probably indicates an inbreeding problem as these beetles had been bred under controlled conditions in the laboratory for a year.

(b) F1 Generation. The mortality of the larvae was very high (95.27%) in the C2 treatment, which greatly reduced the number of F1 couples that could be studied (Table 5). Of those, only a few were fecund and fertile which in the case of C2 and C3 is mainly due to the fact that females were in diapause (Table 5). Therefore, the fecundity and fertility results for the second generation are merely indicative. However, it is worth noticing that there were two fertile couples of hybrids

The results for measure of fitness, adult size and ovariole number, are presented in Table 6. Although individuals from the three strains do not differ in terms of adult size, the hybrid individuals have significantly more ovarioles than those from the Japanese population and the same number as those from French population.

Discussion

The seven spot ladybird is one of the most often studied coccinellids with a great number of publications on its biology and ecology (review in Omkar & Pervez 2002). Generally abundant, this eurytopic species is mainly a generalist aphid predator. Its wide distribution raises the possibility of different selective pressures which, together with the existence of geographical barriers and low gene flow between populations, could lead to phenotypic variation. Bodenheimer (1943) describes variation in some life-history parameters and ecology of the seven spot ladybirds based on a study of populations from four different geographical regions and more recently Rieder *et al.* (2008) the variation in the toxic effect of eggs from different populations of *C. septempunctata* on *H. axyridis*. In terms of morphological variation, Iablokoff-Khnzorian (1982), in a review of the systematics of *C. septempunctata*, indicates that certain populations are considered to be separate species or subspecies.

In this study, the status of seven spot ladybird populations is considered. For this, morphometric data (measures of the spots on the elytra) and molecular data (487 bp of the COI gene and the 223 polymorphic bands from ISSR) were used to study the relationships between 12 populations covering most of the seven spots' geographical range. Furthermore, as information on the mating potential of isolated populations is essential in the demarcation of species taxa (Mayr 1996), the existence of reproductive barriers to inter-population crosses between the French population and the distant and isolated Japanese population were evaluated.

Size of the elytral spots is a conspicuous morphological trait used by several taxonomists to characterize the Algerian and Japanese populations (Mulsant 1866; Kovář 1977; Bensusan *et al.* 2006). The results presented here on the relative size of the elytral spots indicate that they are larger for both the Japanese and the Algerian populations than in the other populations. However, the results do not confirm the suggestion that the proportion of pigmented forms (proportion of darker individuals) increases radially from Central Asia (Honěk 1996). Such observation is strengthened by the fact that among our Indian sample, there is a highly pigmented individual, almost black in appearance and Iablokoff-Khnzorian (1982) and Dobzhansky (1933) confirm the existence of highly pigmented individuals in the Himalayas.

The large size of the elytra spots in the Japanese population might be a result of genetic drift or local adaptation. A first study by Dobzhansky & Sivertzev-Dobzhansky (1927), show a consistent variability in the proportion of individuals with more or less pigmentation according to geographical location, a tendency also observed in other Coccinellidae (Honěk 1996). It has been pointed out that these variations could be related to humidity and temperature (Rhamhalinghan 1989; Stewart & Dixon 1989; Honěk 1996). Thus, selection rather than drift could have played a role in the evolution of this morphological trait.

Table 5 Fecundity and fertility results for the F1 generation issued from couples of C1: ♀ Japan × ♂ Japan, C2: ♀ France × ♂ France and C3: ♀ Japan × ♂ France crosses. Values between brackets = SD

	C1	C2	C3
Total number of couples	12	3	16
Diapausing females	0	2	8
Number of fecund couples	12	1	8
Mean fecundity	9.46 (3.76)	0	10.92 (5.54)
Number of fertile couples	5	0	2
Mean fertility	0.14 (0.08)	0	0.06 (0.08)

Table 6 Results on the mean right elytron surface and ovariole number of hybrids issued from crosses ♀ Japan × ♂ France (F1) and individuals from the parental strains (P). *a* and *b* correspond to the results of the Tukey *post hoc* test ($\alpha = 0.05$), $n = 10$

Strains	Right elytra size	Number of ovarioles
F1 hybrids	19.76 (SD 0.95) *a*	94.5 (SD 7.14) *a*
P France	22.02 (SD 2.26) *a*	92.63 (SD 6.07) *a,b*
P Japan	21.38 (SD 2.56) *a*	83.1 (SD 10.17) *b*

Molecular data, unlike the morphometric data for the seven spot ladybirds, is homogeneous. The low genetic differentiation of the COI sequences, the paraphyly of nearly all populations and the presence of one dominant haplotype in all the populations indicates they do not differ genetically. The genetic divergence between populations is of the same order (0–3.1%) as that recorded between populations of a closely related species [*Adalia bipunctata* (L)] using two mitochondrial genes, COI and NADH dehydrogenase subunit 5 (0.09% and 4.94%, respectively, Schulenburg *et al.* 2002). The divergence between seven spot ladybirds and the four other *Coccinella* species included in this study (*C. quinquepunctata*, *C. undecimpunctata*, *C. magnifica* and *C. miranda*) is higher (12.6–17.2%). It is worth noting, that the genetic divergence among Japanese specimens indicates a high intra-population polymorphism (up to 37 substitutions, 3.1%). The great divergence among mitochondrial haplotypes might indicate the presence of either nuclear mitochondrial DNA (Numt; Lopez *et al.* 1994) or a microorganism inferring cytoplasmic incompatibility. Because non-synonymous mutations, prematuring stop codons and/or indels, all consequences of relaxed selection, were not recorded in this study the first hypothesis is unlikely (Strugnell & Lindgren 2007). Cytoplasmic incompatibility can generate either a homogenization or an increase in the heterogeneity of mitochondrial DNA between populations. Two microorganisms, *Cardinium* and *Wolbachia*, are known to induce this phenomenon (Breeuwer *et al.* 1992; Hunter *et al.* 2003). Heterogeneity among parasites, and lack of migration between populations, increase their genetic differentiation (Hurst & Jiggins 2005). Recently, *Rickettsia* bacteria, two strains of *Wolbachia* (α-proteobacteria) and the *Mollicute* Spiroplasma were found in a species of Coccinellidae (*A. bipunctata*) (Schulenburg *et al.* 2002). This last study indicates that particular mitochondrial haplotypes are not correlated with geographic origin but rather with the presence or absence of bacteria. Detection of microorganisms such as *Wolbachia* and *Cardinium* and their linkage disequilibrium with mitochondrial haplotype in a large sample of seven spot ladybirds from Japan would support this hypothesis. Moreover, intra-Japanese population differentiation of COI is not revealed by the ISSR polymorphism. It is, however, consistent with the hypothesis of cytoplasmic incompatibility, since this phenomenon is visible only at the level of mitochondrial DNA, while polymorphism in the ISSR reflects variability in the nuclear genome. However, as no sex ratio bias was observed in the laboratory population of the Japanese beetles this hypothesis could be rejected.

Although the COI marker is one of the DNA-barcodes most extensively used to identify specimens and quantify global diversity, it is not on its own suitable for studying evolutionary history and determining systematic relationships between populations. In contrast, ISSR markers have different evolutionary histories and can be used to quickly screen a large part of the genome. This provided in our case additional, but not contradictory information, for both inter- and intra-population variation. Intra-population polymorphism was recorded in all populations and is higher (57.2% of the molecular variance) than between-population polymorphism (42.8%). Furthermore, all populations appear to be monophyletic. There were also no geographically based clusters or clines in genetic variability. This may result from either gene flow between populations, or long-distance dispersal throughout the range and/or incomplete lineage sorting linked to the colonization of a geographical area that has occurred over a very short period of time (radiation). ISSR polymorphisms cannot be used to identify alleles or determine evolutionary history in particular regions. A study of population genetics, using microsatellite or other polymorphic markers, could be used to compare the predictions of gene flow vs. ancestral gene polymorphism hypotheses and for reconstructing the demographic history of the populations and identifying their geographical origin (single or multiple).

According to the biological species concept, 'a species is an aggregation of populations with the qualifications for free interbreeding' (Mayr 2002). Traditionally, when species are crossed, it is expected that the hybrids will be unviable (Mayr 1970; Brideau *et al.* 2006), sterile (Mayr 1970; Masly *et al.* 2006) or have reduced fitness (Peterson *et al.* 2005). However, in our case, the crosses between individuals from the French and Japanese populations resulted in the production of both viable and interfertile hybrids. Therefore, the Japanese population, from a biological point of view, is similar to the French population. However, as many of the F1 individuals entered diapause it was not possible to produce an F2 generation. This is important because hybrids often express phenotypic breakdown after the F1 generation as a result of underdominance, chromosomal rearrangements or deleterious epistatic interactions (Edmands 2002).

The hybrids resulting from crosses between individuals from the French and Japanese populations seem to have mainly inherited the French strategy of having only one generation per year, with adults born in spring entering diapause in summer. High temperatures can reduce the time spent in diapause but are unable to prevent them entering diapause (Hodek 1996). In contrast, in the warm southern parts of the Japanese archipelago, bi- or multivoltine life cycles are possible although often interrupted by aestivation (Hodek 1996; Ohashi *et al.* 2003). The Japanese individuals used in our study came from the region of

Tsuruoka and were reared in the laboratory continuously without showing any sign of dormancy.

This study of two components of fitness, i.e. adult size and ovariole number, indicates that hybrids are not less fit than individuals from the parent populations.

As individuals from the Algerian population were not included in the crossing experiment their inter-fertility with the European populations remains unknown. However, molecular data clearly indicates that the Algerian population is not a distinct species.

In conclusion, individuals from the various populations analysed all belong to the species *C. septempunctata* and not a complex of species. This is consistent with Iablokoff-Khnzorian's (1982) opinion that all the forms of seven spot ladybirds described as separate species, do not deserve that taxonomic rank. As for the status of subspecies as defined by Avise & Ball (1990) and O'Brien & Mayr (1991), all members of a subspecies must share a geographical location or unique habitat and a phylogenetically consistent group of phenotypic characters. In addition, they must have an evolutionary history distinct from the other subdivisions of the species. The Japanese population is traditionally considered as belonging to the subspecies *C. septempunctata brucki.* The populations of *C. s. brucki* are geographically isolated (Iablokoff-Khnzorian 1982) and on the basis of the size of their elytral spots are well differentiated from *C. s. septempunctata*. However, morphometric characteristics are not consistent with the evolutionary history inferred from molecular markers (COI sequence and ISSR bands) and therefore the Japanese population should not be considered as a subspecies. As for the Japanese population, the North African one cannot be considered as a subspecies. That population does not share a consistent group of phenotypic characters and some individuals were found in the Iberian Peninsula (Bensusan *et al.* 2006). Therefore, the Strait of Gibraltar does not represent a physical barrier to gene flow between the Algerian and European populations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Bayesian inference topology for the COI gene fragment based on the unique haplotypes find in each population, labelled according to codes listed in Table 1. Numbers under branches are posterior probabilities values >0.50.

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