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Deep phylogeographic divergence and cytonuclear discordance in the grasshopper *Oedaleus decorus*

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ABSTRACT

The grasshopper *Oedaleus decorus* is a thermophilic insect with a large, mostly south-Palaearctic distribution range, stretching from the Mediterranean regions in Europe to Central-Asia and China. In this study, we analyzed the extent of phylogenetic divergence and the recent evolutionary history of the species based on 274 specimens from 26 localities across the distribution range in Europe. Phylogenetic relationships were determined using sequences of two mitochondrial loci (ctr, *ND2*) with neighbour-joining and Bayesian methods. Additionally, genetic differentiation was analyzed based on mitochondrial DNA and 11 microsatellite markers using F-statistics, model-free multivariate and model-based Bayesian clustering approaches. Phylogenetic analyses detected consistently two highly divergent, allopatrically distributed lineages within *O. decorus*. The divergence among these Western and Eastern lineages meeting in the region of the Alps was similar to the divergence of each lineage to the sister species *O. asiaticus*. Genetic differentiation for ctr was extremely high between Western and Eastern grasshopper populations ($F_{ct} = 0.95$). Microsatellite markers detected much lower but nevertheless very significant genetic structure among population samples. The nuclear data also demonstrated a case of cytonuclear discordance because the affiliation with mitochondrial lineages was incongruent in Northern Italy. Taken together these results provide evidence of an ancient separation within *Oedaleus* and either historical introgression of mtDNA among lineages and/or ongoing sex-specific gene flow in this grasshopper. Our study stresses the importance of multilocus approaches for unravelling the history and status of taxa of uncertain evolutionary divergence.

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1. Introduction

The distribution and genetic variation of present day organisms is influenced by both recent and historic evolutionary processes. In many regions, the last glacial maximum (LGM) in the Pleistocene (15,000–18,000 years ago) had dramatic consequences for the genetic diversity and distribution of various taxa (Hewitt, 2000). Climatic changes and ice cover led to the extinction of some cold-intolerant species and to latitudinal and altitudinal shifts into more favourable habitats. In Europe, many studies focus on the impact of the LGM on temperate taxa, which have often been pushed by changes associated with colder conditions into refugia in the South (Hewitt, 2004). The Iberian Peninsula, Italy and the Balkans are widely accepted as the major southern refugia and long-term isolation of populations in these regions has often led to the formation of distinct genetic lineages or even species (Hewitt, 2000). As the

climate warmed after the LGM, mountains such as the Alps and the Pyrenees, partially blocking northward recolonization routes from the southern regions, had a strong effect on whether or when post-glacial expansion could occur (reviewed in Taberlet et al., 1998; Hewitt, 2004). As these mountain chains were the last regions to be freed from ice, they represent often important suture zones of secondary contact between previously isolated genetic lineages (Taberlet et al., 1998; Ruedi et al., 2008; Braaker and Heckel, 2009).

This general framework of post-glacial histories valid in many temperate European organisms may however need to be adapted for the particular temperature and habitat requirements of some species. For example, cold-tolerant organisms may have persisted during the LGM in additional northern or southern refugial areas (e.g. Heckel et al., 2005; Kotlik et al., 2006; Krystufek et al., 2009). Furthermore, steppe-living species have likely inhabited different refugial regions than forest-dwelling ones, and species requiring particularly warm temperatures may not have been present in Europe during the LGM as conditions in southern refugia were not favourable enough.

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In this study we analyzed genetic divergence, population structure and biogeographic patterns in the band-winged grasshopper *Oedaleus decorus* (Acrididae, Germar 1826) in Europe, in particular with respect to the importance of the region of the Alps as a suture zone. The species is found throughout most of southern Europe and adapted to dry and warm conditions (xero-thermophilic) with a narrow temperature range (stenothermic), inhabiting steppe-like areas at sunny sites with a high amount of bare ground (Schmidt and Lilge, 1996; Ingrisch and Köhler, 1998). *O. decorus* is rapidly declining in Switzerland where it is constricted to two isolated sites in the South (Monnerat et al., 2007) and it is endangered in Slovenia (Gomboc and Trontelj, 2001). It is rarely found in Slovakia (Kočárek et al., 1999) and became extinct in the 1940/1950s in Austria (Berg and Zuna-Kratky, 1997) and in the Czech Republic (Holuša and Kočárek, 2005), where it reached its northern distribution limit (Kaltenbach, 1970). In France, the species is absent north of the river Seine (Bellmann and Luquet, 2009) and a single animal was recently recorded for the first time in Germany (Boczki, 2007). Long distance flights in this grasshopper (Schmidt and Lilge, 1996; Boczki, 2007) may have contributed to achieve its wide distribution range extending apparently into large parts of Africa and Central-Asia (Kaltenbach, 1970; Fet, 2007). Ritchie (1981) suggested that *O. decorus* is replaced by its sister species *O. asiaticus* (Fries et al., 2007; Ma et al., 2009) in the steppes of the Mongolian Plateau and the Transbaikal region of southern Russia.

The goals of this study were (i) to examine the level of evolutionary divergence in *O. decorus* across its distribution range in Europe (ii) to test for the relevance of the suture zone in the region of the Alps for this thermophilic grasshopper and (iii) to further investigate genetic diversity in populations from previously glaciated areas and populations from localities which were not affected by the ice-cover during the LGM. Due to the xero-thermophilic biology of the species, its evolutionary history in Europe may significantly differ from the refugial and post-glacial recolonization patterns proposed for northern temperate organisms. Our study is based on phylogenetic and population genetic analyses of mitochondrial DNA (mtDNA) sequence loci together with information from highly polymorphic nuclear microsatellite markers (nucDNA). The combination of molecular data with different evolutionary speed and effective population sizes serves to provide information over different time scales, as e.g. mtDNA may detect events further back in time whereas the high resolution of microsatellites may rather reflect more recent patterns of gene flow in a species (e.g. Canestrelli et al., 2007; Braaker and Heckel, 2009). The comparison of results from different data sources may further be useful to evaluate natural factors such as selection or sex-biased dispersal, or some technical issues like pseudogenes (Bensasson et al., 2001; Song et al., 2008) or null alleles (Ustinova et al., 2006; Chapuis and Estoup, 2007) and assess their potential confounding influence on evolutionary reconstructions.

2. Materials and methods

2.1. Samples and DNA extraction

We analyzed 274 fresh or dry *O. decorus* samples and two dry *O. asiaticus* specimens from 26 different localities with sample sizes varying between one and 37 individuals (Fig. 1, Table 1). Additional dry museum material from 150 *O. decorus* and 68 *O. asiaticus* from further 82 localities in Europe, Asia and Africa could not be included in the dataset as either DNA extraction or subsequent amplification failed, even with internal primers and adjusted PCR protocols. Fresh *O. decorus* samples (n = 267) were stored in absolute ethanol at 4 °C, and genomic DNA was extracted exclusively from muscle tissue from hind legs following a standard phenol–

chloroform protocol (see Braaker and Heckel, 2009). Genomic DNA from seven dry *O. decorus* and two dry *O. asiaticus* specimens was extracted according to an adapted phenol–chloroform protocol: hind legs were ground and digested with 30 μL proteinase K (10 mg/mL) at 37 °C overnight. Then 20 μL proteinase K was added and digestion was continued at 55 °C for 2 h. The ethanol precipitation step of the extraction procedure was carried out overnight at −20 °C to improve precipitation. To prevent contamination, extraction of DNA from museum material was carried out in a sterile and closed environment and subsequent PCR amplification and sequencing was performed in two spatially separated rooms and with sterile material.

2.2. DNA sequencing

We analyzed a 546 base-pair (bp) DNA fragment, furthermore referred to as ctr, covering parts of the highly variable mitochondrial control region (426 bp) and of the 12S rRNA gene (120 bp). The newly developed primers Odec-dloop-F1 (5′-TGGCACGAAA TATGCCAATA-3′) and Odec-dloop-R3 (5′-CATTTAACTGAATGTAA GACCCATAC-3′) were used for amplification in a reaction volume of 25 μL in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The Polymerase Chain Reaction (PCR) mix contained 12.5 μL dH$_2$O, 1 μL MgCl$_2$ (25 mM), 1 μL of each primer (10 pmol/μL), 4.8 μL dNTPs (2.5 mM), 2.5 μL 10× buffer (containing 1.5 mM MgCl$_2$), 0.2 μL *Taq* (5 unit/μL; QIAGEN, Valencia, CA, USA) and 2 μL template DNA (100 ng/μL). The PCR conditions were the following: initial denaturation at 95 °C for 2 min, subsequent 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. The PCR products were checked for correct size on a 1.5% agarose gel by comparing them to a 100 bp ladder (Invitrogen). Products were cleaned using the GenElute PCR Clean-up kit (Sigma, Switzerland) following the manufacturer's protocol. Sequencing reactions using the forward primer Odec-dloop-F1 were carried out with the Terminator Ready Reaction Mix "Big Dye" (v 3.1, Applied Biosystems) in a reaction volume of 10 μL, containing 4 μL dH$_2$O, 1 μL primer (10 pmol/μL), 2.5 μL Big Dye and 2.5 μL template DNA. The reaction conditions were as follows: initial denaturation at 96 °C for 50 s, 35 cycles with denaturation at 96 °C for 10 s, annealing at 50 °C for 10 s and extension at 60 °C for 4 min and 30 s. The PCR products were purified according to a sodium-acetate precipitation protocol and then separated and detected on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

We used internal primers and nested PCR to amplify ctr from an additional five dry museum specimens as the amplification from museum material with the standard primers failed, likely because of DNA fragmentation and low concentration. The resulting concatenated sequence was a shorter portion (352 bp) of the original fragment (546 bp). The amplification of the 5′ fragment was carried out using Odec-dloop-F1 and the newly designed internal reverse primer Odec-dloop-int-R (5′-GGTTTGCGGGATATGTGTTC-3′), and the downstream segment was amplified using the forward internal primer Odec-dloop-int-F (5′-GAACACATATCCCGCAAACC-3′) and Odec-dloop-R3. A nested PCR protocol was used to increase the amount of the target sequence: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. In the successive amplification run, 1 μL of the PCR product was used as template with the reaction conditions as mentioned above. Direct sequencing was carried out with each of the four primers and the reaction was adjusted by increasing the amount of template DNA to 4 μL.

Thirty-five *O. decorus* with wide geographical coverage and one *O. asiaticus* were additionally analyzed for the mitochondrial gene ND2 (NADH dehydrogenase subunit 2). The newly designed primer
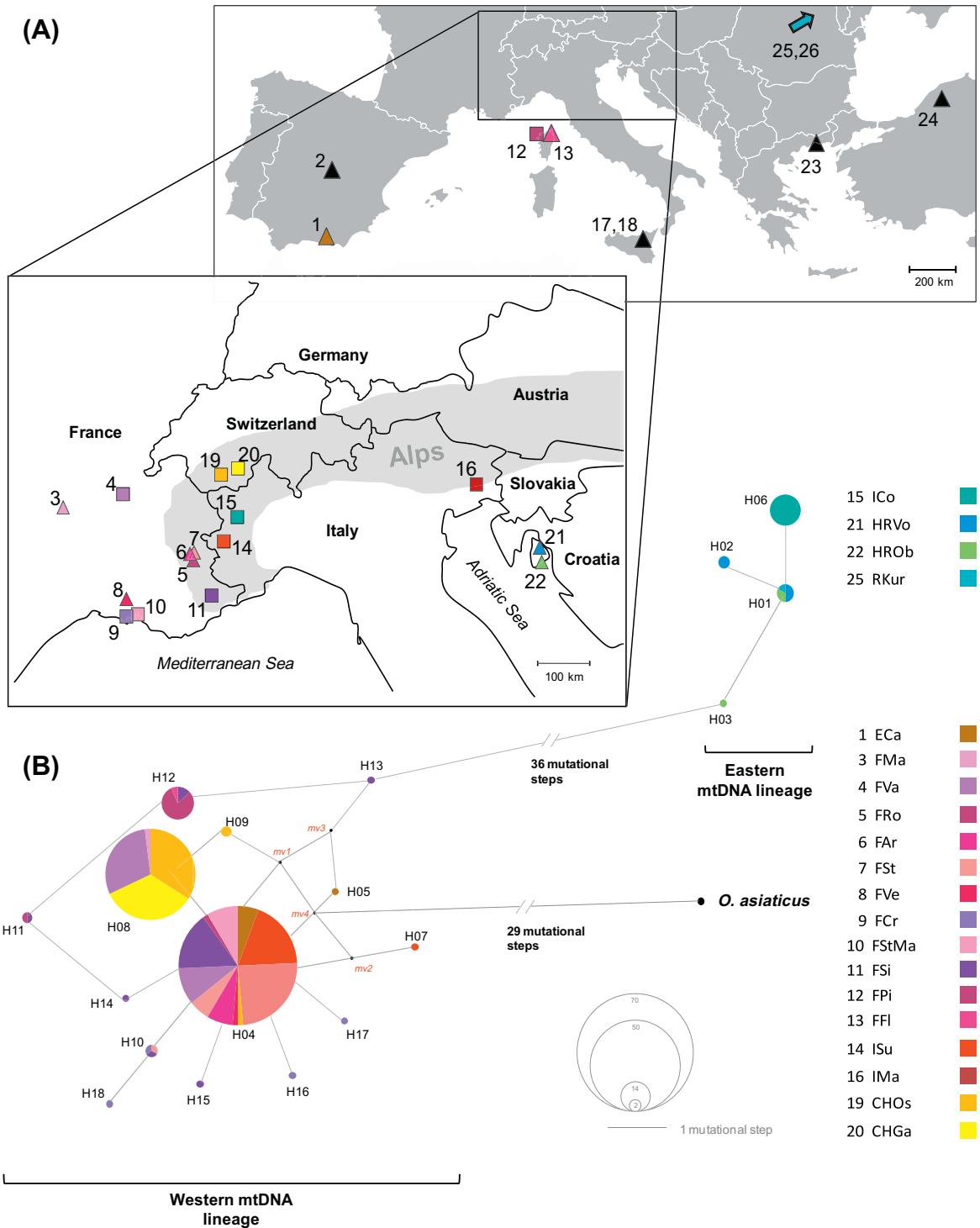
Fig. 1. (A) Sampling sites of *Oedaleus decorus* across its distribution range in Europe. Triangles indicate sampling positions for phylogeographic analyses only. Squares represent sampling locations additionally covered in population genetic analyses. Numbers and sample sizes are displayed in Table 1. The Alps are highlighted in grey. The arrow points toward the direction of the sampling locality in Russia and the sampling site of *Oedaleus asiaticus* in China. (B) Unrooted median joining haplotype network based on 546 bp ctr for 173 *O. decorus* individuals, including *O. asiaticus* as outgroup. Haplotypes from the Western mtDNA lineage are coloured in yellow-purple and haplotypes from the Eastern mtDNA lineage in blue-green, respectively. The size of the circles is proportional to the number of individuals with a particular haplotype and the distance between two haplotypes is proportional to the mutational steps separating them. Haplotype abbreviations are displayed in *Supplementary* Table S1 and sampling localities are described in Table 1.

pair Odec-nd2-F (5′-GAGATGCCTGAATAAAGGGTTA-3′) and Odec-nd2-R (5′-AAGGTGCCAATGTCCTTATG-3′) was used to amplify the first 439 bp of ND2. The PCR mix and the cycling conditions were identical to ctr, but the annealing temperature was increased to 56 °C. Sequencing was carried out as for ctr.

2.3. Precautions against pseudogenes

The relatively high frequency of pseudogenes (Numts) in some acridid grasshoppers may lead to incorrect phylogenetic inferences if their presence remains unrecognised (Bensasson et al., 2000). In

Table 1
Sampling sites of *Oedaleus decorus* and *Oedaleus asiaticus* with reference numbers from Fig. 1. Given are sampling sites (label, country, location, latitude and longitude) and the number of samples analyzed for mtDNA (*n ctr*, *n ND2*) and for microsatellite loci (*n nucDNA*), respectively. Localities from where population samples were available are underlined. * refers to dry museum material, ( ) indicates samples not included in the 546 bp mtDNA alignment, ** specifies *Oedaleus asiaticus*.

Map ref.	Label	Country	Location	Latitude	Longitude	*n ctr*	*n ND2*	*n nucDNA*
1	ECa	Spain	Capileira	36°57′N	03°21′W	5	3	
(2*)	EBe	Spain	Brunete	40°24′N	03°59′E	1		
3*	FMa	France	Saint-Ilpize à Saint-Marcel	45°11′N	03°23′E	1		
4	FVa	France	Valbonne	45°52′N	05°07′E	15	3	19
5	FRo	France	La Roche-de-Rame	44°45′N	06°34′E	1		
6	FAr	France	L'Argentière-la-Bessée	44°47′N	06°33′E	5		
7	FSt	France	Saint-Martin de Queyrières	44°50′N	06°35′E	5		
8*	FVe	France	Vergières	43°34′N	04°49′E	1		
9	FCr	France	Crau	43°08′N	06°04′E	15		20
10	FStMa	France	Saint-Martin de Crau	43°34′N	04°50′E	6		34
11	FSi	France	Sisteron	44°11′N	05°56′E	14	2	19
12	FPi	France, Corsica	Piana	42°14′N	08°38′E	13	3	24
13*	FFl	France, Corsica	Saint-Florent	42°40′N	09°18′E	1		
14	ISu	Italy	Susa	45°08′N	07°02′E	14	2	27
15	ICo	Italy	Cogne	45°36′N	07°22′E	14	1	20
16	IMa	Italy	Magredi	46°04′N	12°44′E	17	2	37
(17*)	IRN	Italy	Rocca di Novara	38°02′N	15°05′E	1		
(18*)	IRa	Italy	Randazzo	37°52′N	14°57′E	1		
19	CHOs	Switzerland	Ossona	46°10′N	07°25′E	20	2	21
20	CHGa	Switzerland	Gampel	46°18′N	07°44′E	17	1	20
21	HRVo	Croatia	Voz	45°13′N	14°34′E	5	4	
22	HROb	Croatia	Obzovo	44°58′N	14°45′E	3	3	
(23*)	GRNk	Greece	Nea Karvali	40°57′N	24°30′E	1		
(24)	TCa	Turkey	Çankırı	40°50′N	32°45′E	1		
25	RKur	Russia	Kurgan	54°37′N	64°27′E	1		
26**	CNXi	China	Xilinhot	43°57′N	116°08′E	2	1	

order to minimize the accidental co-amplification of these nuclear copies, DNA was exclusively extracted from muscle tissue rich in mitochondria to ensure a relatively high amount of mitochondrial compared to nuclear DNA (Sorenson and Quinn, 1998; Bensasson et al., 2001). PCR products were carefully checked for multiple bands on agarose-gels and samples showing ambiguous sequences were not included in further analyses. Sorenson and Quinn (1998) showed that the use of internal primers and overlapping PCR products is a good precaution against pseudogenes as Numts are less likely to be amplified by two different primer pairs. In addition, concordance of phylogenetic patterns based on different gene fragments may indicate a successful elimination of pseudogenes (Sorenson and Quinn, 1998). As Ma et al. (2009) used isolated mitochondria for sequencing the complete mitochondrial genome of *O. asiaticus* and as the two *O. asiaticus* samples in our study showed the same haplotype (see results), their mitochondrial origin is likely. Furthermore, we translated *ND2* sequences into amino acids in order to check for premature stop codons which would indicate non-functional nuclear copies of mtDNA genes (Song et al., 2008).

2.4. Microsatellite genotyping

Nuclear DNA from 241 samples, originating from 10 sampling sites in the region of the Alps where population samples were obtained (Fig. 1; Table 1), was analyzed at the following 11 microsatellite loci in two separate multiplex sets: *OD2*, *OD4*, *OD6*, *OD7*, *OD9*, *OD12* and *OD3*, *OD5*, *OD8*, *OD18*, *OD31* (Berthier et al., 2008). PCR amplifications were performed using the thermal cyclers GeneAmp PCR System 9700 (Applied Biosystems) in a 10 μL reaction volume containing 5 μL PCR Multiplex-Mix (QIAGEN), 2 μL dH₂O, 1 μL primer mix (0.02 μL of each primer) and 2 μL template DNA. The amplification conditions were the following: *Taq* activation at 96 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 1 min and 30 s, extension at 72 °C for 60 s and final extension at 60 °C for 30 min. Fragments were separated by electrophoresis on an ABI 3100 Sequencer and their length was determined using GeneMapper software v.3.7 (Applied

Biosystems) against an internal size standard (GeneScan 500LIZ™ Applied Biosystems). Genotypes which failed to amplify were repeated following an adjusted amplification protocol: initial denaturation at 94 °C for 5 min, subsequent 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, extension at 72 °C for 1 min and 15 s and a final extension step at 72 °C for 10 min. Repetitions of previously analyzed individuals were used to ensure consistency of genotyping (Schweizer et al., 2007).

2.5. Phylogenetic analyses of mtDNA

DNA sequences were aligned using the ClustalW algorithm implemented in BioEdit 7.9 (Hall, 1999) and revised manually. A published sequence of *O. asiaticus* was included in the ctr data set (GenBank, Accession no: NC_011115; Ma et al., 2009) and the closely related species *Gastrimargus marmoratus* (Acrididae, GenBank Accession no: EU13373; Ma et al., 2009) was used as outgroup to root the phylogenetic trees. *ND2* sequences from 15 other Oedipodinae were included in phylogenetic analyses: *Aiolopus tumulus*, EF395804.1; *Angaracris barabensis*, EF395798.1; *Angaracris rhodopa*, EF395796.1; *Bryodema luctuosum*, EF395800.1; *Bryodema miramae*, EF395799.1; *Bryodema nigroptera*, EF395802.1; *Bryodemella holdereri*, EF395801.1; *Compsorhipis davidiana*, EF395737.1; *Gastrimargus marmoratus*, EF395793.1; *Oedaleus asiaticus*, EF395792.1; *Oedaleus infernalis*, EF395790.1; *Oedaleus manjius*, EF395794.1; *Sphingonotus tsinlingensis*, EF395794.1; *Sphingonotus yenchihensis*, EF395795.1 and *Trilophidia annulata*, EF395803.1. A sequence of *Acheta domesticus* was used as outgroup to root the phylogenetic tree based on *ND2*. The number of segregating sites as well as the identity and frequency of haplotypes was determined using DnaSP 5.0 (Librado and Rozas, 2009). Redundant haplotypes were removed from phylogenetic analyses of ctr as the large number of sequences prevents displaying such a tree efficiently. The smaller dataset of *ND2* allows the display of all sequences and thus visualizes directly the extent of haplotype sharing between sampling localities across Europe.

Phylogenetic relationships were inferred first for the shorter ctr alignment with all samples mentioned in Table 1, second for the
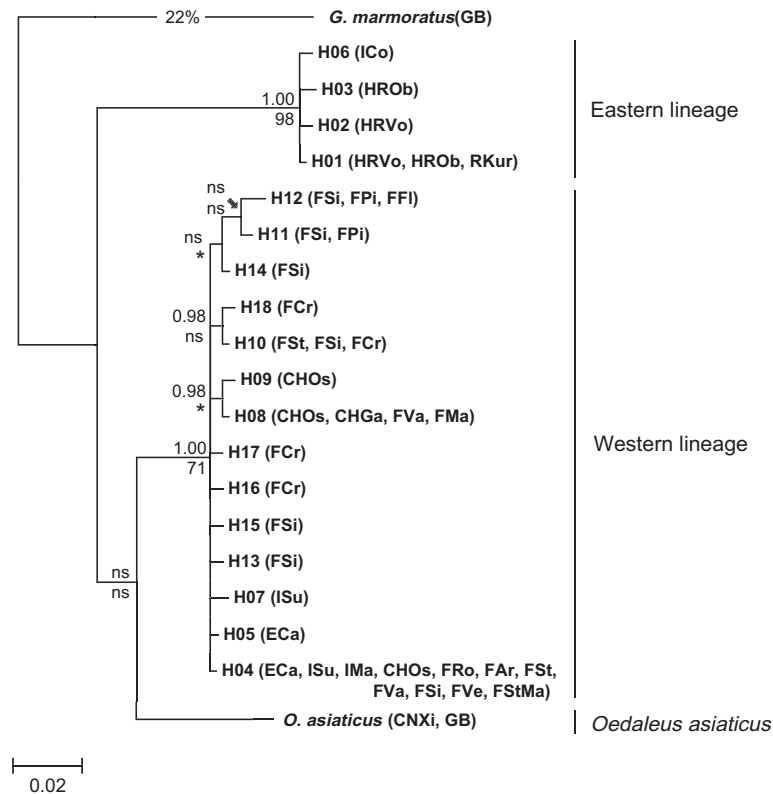
Fig. 2. Bayesian reconstruction of phylogenetic relationships based on 546 bp mtDNA (ctr) haplotypes from *Oedaleus decorus* and *Oedaleus asiaticus* with *Gastrimargus marmoratus* as outgroup. Posterior probabilities are indicated above the major branches and percentage of bootstrap support for neighbour-joining algorithms below the branches. Locality labels are shown in Table 1 and the haplotype distribution is displayed in Supplementary Table S1. GB refers to sequences from GenBank, * indicates a different topology based on NJ algorithms, ns refers to posterior probabilities <0.70 and percentages of bootstrap support <70%.

longer ctr fragment for which the five museum specimens could not be included and third for the *ND2* alignment together with the sequences from GenBank. Phylogenetic relationships were determined by Bayesian inference (BI) implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and by Neighbour-Joining algorithms (NJ) implemented in Mega 3.1 (Kumar et al., 2004). The general time-reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites was selected as the optimal nucleotide substitution model for ctr based on the Bayesian Information Criterion (BIC) implemented in jModeltest 0.1.1 (Posada, 2008). The Hasegawa–Kishino–Yano model with a proportion of invariable sites and gamma-shaped distribution was selected for *ND2*, accordingly. The neighbour-joining analyses were run with 10,000 replicates each, using the Maximum Composite Likelihood model for ctr and the Tamura-3-parameter model for *ND2*, respectively. The Bayesian analyses were run three times for 1 million generations for ctr and 2 million generations for *ND2* with every 10th generation sampled using one cold and three heated chains. The first 25% of the samples were discarded as burn-in and convergence was determined by examining the log likelihood values and the split frequencies. The trees were visualized in Mega. Net average divergences among phylogenetic lineages were computed with Mega using the mentioned nucleotide substitution models. Furthermore, a haplotype network was constructed for the longer ctr fragment based on a median joining algorithm (Bandelt et al. 1999) in Network 4.516 (http://www.fluxus-engineering.com).

2.6. Statistical analyses of population samples

Mitochondrial DNA. The number of haplotypes (546 bp ctr; A), haplotype (h) and nucleotide (π) diversity, and the average number of nucleotide differences (k_1) was estimated for the 10 different population samples (Supplementary Table S3) using DnaSP. Haplotype diversity within population samples originating from previously glaciated sites (Ch. Schluechter, Institute of Geological Sciences, Bern) was compared to the variability in populations outside the glaciers range and tested with a Kruskal–Wallis rank sum test in r 2.10.0 (R Development Core Team, 2009). Arlequin 3.5 (Excoffier and Lischer, 2010) was used to perform AMOVAs and to calculate overall and pairwise Φ_{ST}-values. Statistical significance was tested by 10,000 permutations and significance levels were adjusted using the Bonferroni correction (Rice, 1989). To check for an isolation-by-distance pattern (IBD) we assessed the correlation between matrices of geographical distances expressed in log kilometres and pairwise linearized genetic distances $\Phi_{ST}/(1 - \Phi_{ST})$ (Rousset, 1997) with Mantel tests using 999 permutations in GenAlEx 6.2 (Peakall and Smouse, 2006).

Microsatellites. The number of alleles per locus, allelic richness (k_2) and mean observed (H_O) and expected heterozygosity (H_E) per population sample was estimated using Fstat 2.9.3.2 (Goudet, 2001). As null alleles may be a potential issue of microsatellite analyses in certain Acrididae (Ustinova et al., 2006; Chapuis and Estoup, 2007), their frequency was estimated based on Expectation Maximization (EM) algorithms implemented in FreeNA (Chapuis and Estoup, 2007). Furthermore, tests for deviation from Hardy–Weinberg equilibrium (HWE) were computed for each locus and population sample using the same program. Allelic richness within population samples located at previously glaciated sites was compared to the variability at sites located outside the glaciers range and tested with a Kruskal–Wallis rank sum test in R. IBD tests and AMOVAs were performed analogous to mtDNA. In addition, F_{ST} corrected for the presence of null alleles was calculated ($F_{ST}^{(ENA)}$) with the program FreeNA. Discriminant Analysis of Princi-

pal Components (DAPC) (Jombart et al., 2010), a multivariate model-free method, was used to visualize genetic differentiation without using population information. DAPC relies on a data transformation based on Principal Component Analysis (PCA) prior to a Discriminant Analysis (DA), which then maximizes the between-group variance while minimizing the within-group variability. DAPC was performed and results were illustrated using the package "adegenet" (Jombart, 2008) implemented in r. The estimation of the optimal number of genetic clusters (K) was based on BIC values. Additionally, we used the Bayesian approach implemented in Structure 2.3.3 (Hubisz et al., 2009) to estimate the number of genetic clusters (K) and to assign individuals to them. We used the admixture model with correlated allele frequencies, 1,000,000 Markov chain Monte Carlo (MCMC) iterations and a burn-in of 1,000,000 iterations. We ran 20 independent runs each for K = 1–10 and used the method suggested by Evanno et al. (2005) to estimate K. Figures were displayed using Distruct 1.1 (Rosenberg, 2004).

3. Results

3.1. Phylogenetic patterns in mtDNA

Phylogenetic analyses revealed two highly divergent, allopatrically distributed evolutionary lineages in *O. decorus* in Europe, irrespective of the sequenced mtDNA region and well supported by Bayesian and neighbour-joining algorithms (Figs. 2 and 3, Supplementary Fig. S1). Analysis of the 546 bp mtDNA (ctr) of 173 *O. decorus* individuals revealed 50 variable sites, which defined the 18 haplotypes shown in Figs. 1 and 2 (GenBank Accession Nos. JX417452 – JX417469). The haplotype network revealed that the most widespread haplotype H04 was found in 11 out of 20 locations in Spain, France, Italy and Switzerland (Fig. 1, Supplementary Table S1). Within the 352 bp mtDNA (ctr) of 178 *O. decorus,* a total of 38 variable sites defined 18 haplotypes (Supplementary Fig. S1; GenBank Accession Nos. JX417470 – JX417487). H_d was the most common haplotype and found in 14 out of 25 locations covering Spain, France, Italy and Switzerland (Supplementary Table S2). Phylogenetic trees and the network showed that haplotype sharing occurred most often between geographically close sites (e.g. Ossona and Gampel, or Voz and Obzovo). Shared haplotypes were additionally found among Sisteron (Southern France) and Corsica.

Analysis of the 439 bp mtDNA *(ND2)* of 27 *O. decorus* individuals revealed 13 variable sites defining five haplotypes in two lineages (Fig. 3; GenBank Accession Nos. JX417488 – JX417492). Translation of these sequences showed nine variable amino acids. No premature stop codon was found, which supports the mitochondrial origin of these sequences. The trees based on ctr and *ND2* were compatible for all individuals for which both markers were sequenced.

Haplotypes found in the eastern part of the European distribution range of *O. decorus* formed a deep monophyletic lineage relative to the Western *O. decorus* lineage and to *O. asiaticus*. The net genetic distance among the two main lineages within *O. decorus* was 3.3% (ctr) and 2.6% *(ND2)*, respectively (Table 2). This differentiation was comparable to the divergence of each lineage to *O. asiaticus*. The Eastern lineage differed by 4.1% (ctr) and 3.0% *(ND2)* from *O. asiaticus*, the Western lineage by 2.5% (ctr) and 1.5% *(ND2)*, correspondingly. However, the relationship between the two *O. decorus* lineages was only weakly supported (BI: < 0.7, NJ: < 70%). The Eastern lineage within *O. decorus* includes specimens from Russia, Turkey, Greece and Croatia, but individuals from the population from North-western Italy (Cogne) were part of this lineage as well, irrespective of the sequenced region (Figs. 2 and 3; Fig. S1). The Western lineage comprised samples from Spain, France, Italy and Switzerland. Within this Western lineage, Bayesian analyses based on 546 bp ctr revealed a separate cluster consisting of haplotypes (H08, H09) from Switzerland (Ossona, Gampel) and from two northern locations in France (Valbonne, Saint-Ilpize à Saint-Marcel), but neighbour-joining algorithms did not support this substructure (Fig. 2). Haplotypes from sampling sites in Southern France (H10, H18) clustered together, as well as haplotypes from Corsica and some individuals from Sisteron in Southern France (H11, H12; Fig. 2).

3.2. Genetic variability in population samples

Polymorphism in mtDNA ranged from no variability in several population samples to haplotype diversity h = 0.76, nucleotide diversity π = 0.46 and average number of nucleotide differences k_1 = 2.54 all in Sisteron (Supplementary Table S3). Haplotype diversity was not significantly different between population samples from previously glaciated areas (h: 0–0.28) compared to samples from locations not affected by the ice-cover during the LGM (h: 0–0.76; Supplementary Table S3; X^2 = 0.173; P = 0.68).

Genetic variability at the microsatellites ranged from 25 to 59 alleles per locus with a mean of 43.92 overall. Mean observed heterozygosity per population sample ranged from 0.61 (Cogne) to 0.78 (Sisteron) and mean allelic richness (k_2) from 7.53 (Susa) to 16.81 (Saint-Martin de Crau). Allelic richness in samples from previously glaciated areas (range 7.53–10.61) was significantly lower than in samples outside the glaciated areas (range 13.90–16.81; X^2 = 14.79; P < 0.001).

Significant deviations from Hardy–Weinberg equilibrium (HWE) were detected in 36 of all 110 tests (at the Bonferroni corrected significance level of 0.45%). All populations showed at least two loci not in HWE and two loci displayed deviations from HWE in eight out of 10 populations. These deviations can be explained by a relatively high frequency of null alleles or potentially Wahlund effects. Mean estimated null allele frequency per locus across populations ranged from 1.49% to 22.34% and from 8.13% (Susa) to 13.38% (Crau) per population sample across loci. However, there were no significant differences between samples from glaciated and not glaciated regions (9.30% vs. 10.36%; t = −0.90; P = 0.39). Given these null allele estimates, all further microsatellite analyses were additionally performed with a dataset excluding the four loci with the highest null allele frequencies and with a dataset corrected for null allele presence. However, there were only slight quantitative and no qualitative differences compared to the results based on the original data set (results not shown).

3.3. Population genetic structure

For mitochondrial DNA, the overall Φ_{ST} of 0.96 (P < 0.001) suggests extremely strong differentiation among *O. decorus* population samples. Most population pairs were significantly different from each other except for some geographically close ones (Supplementary Table S4). Nonsignificant pairwise Φ_{ST}–values were found between the samples from the two Swiss locations and Valbonne in Northern France, between the three samples in Southern France, and between Susa and Magredi in Italy. The highest Φ_{ST}–values were found in all pairwise comparisons with Cogne from the Eastern lineage. An AMOVA with Cogne as one unit and the remaining population samples from Italy, Switzerland and France as a second explained a very high amount of the variation (Φ_{CT} = 0.95; P < 0.0001). The correlation between genetic and geographical distance was neither significant for the entire data set (R^2 = 0.00006; P = 0.42), nor for populations from the Western lineage only (R^2 = 0.00051; P = 0.39).

Overall differentiation among population samples in nucDNA was highly significant with F_{ST} = 0.07 (P < 0.0001). Pairwise F_{ST} ran-
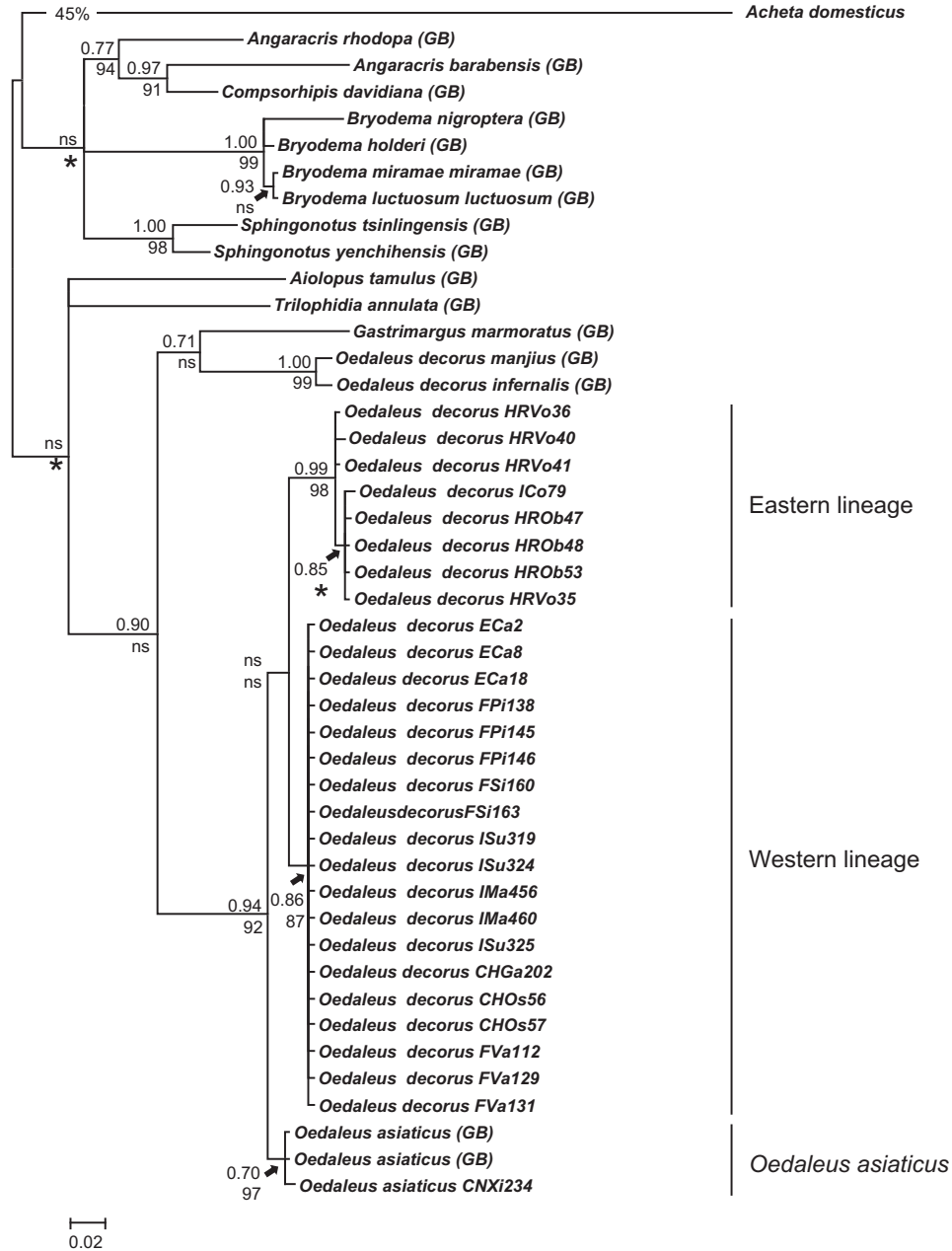
Fig. 3. Bayesian reconstruction of phylogenetic relationships based on 439 bp mtDNA (*ND2*) from 27 *Oedaleus decorus*, 3 *O. asiaticus* and 14 published sequences from other Oedipodinae. The sequence of *Acheta domesticus* was used as outgroup to root the phylogenetic tree. Posterior probabilities are indicated above the major branches and percentage of bootstrap support for neighbour-joining algorithms below the branches. Abbreviations of *O. decorus* individuals are displayed in Table 1. GB refers to sequences from GenBank, * indicates a different topology based on NJ algorithms, ns refers to posterior probabilities <0.70 and percentages of bootstrap support <70%.

Table 2

Net average divergence among the Eastern and Western lineage of *Oedaleus decorus*, *Oedaleus asiaticus* and *Gastrimargus marmoratus*. The divergence, using the nucleotide substitution models mentioned in the main text, based on 546 bp ctr is given above and based on 439 bp *ND2* below the diagonal. All values are given in percent. The divergence between the two lineages within *O. decorus* is comparable to the differentiation of both lineages to *O. asiaticus*, irrespective of the sequenced region.

	Eastern lineage	Western lineage	*O. asiaticus*	*G. marmoratus*
Eastern lineage	–	3.3	4.1	16.7
Western lineage	2.6	–	2.5	15.9
O. asiaticus	3.0	1.5	–	17.4
G. marmoratus	17.3	17.8	16.8	–

ged from 0.025 to 0.16 with highest values involving comparisons with Susa. All population samples differed significantly from each other except for the three sites located in Southern France (Supple-

mentary Table S4). F_{ST}–values corrected for null allele presence were slightly lower, but nevertheless significantly different from zero (all *P*-values < 0.0001). Differentiation between the geograph-

ically adjacent populations Gampel and Ossona (F_{ST} = 0.09; 29 km distance) or Cogne and Susa (F_{ST} = 0.15; 60 km distance) was much higher than between two distant populations e.g. Crau and Magredi (F_{ST} = 0.03; 625 km distance). Consequently, microsatellite variability did not follow a pattern of IBD for the entire dataset (R^2 = 0.021; P = 0.26) or within the Western lineage alone (R^2 = 0.00008; P = 0.48).

DAPC and Structure both supported substantial subdivision among the *O. decorus* population samples but not particularly stronger differentiation of Cogne, the sample containing the Eastern mtDNA lineage for nuclear DNA. In the initial step of DAPC, 116 principal components (PCs) of the PCA were retained, which accounted for more than 90% of the total variance. However, the first two PCs were sufficient to describe the subdivisions in the dataset. The assignment rate for each individual to the correct genetic cluster was 99%, indicating a high robustness of the analysis. The estimation of the optimal number of genetic clusters resulted in highly similar BIC-values between K = 3 and 6 with a minimum at K = 4. The scatterplots, however, clearly showed that differences between groups were maximal for K = 3 (Fig. 4). The individuals from the two samples from Switzerland (Ossona, Gampel) and from Northern Italy (Susa) were clearly separated from each other and from the rest of the individuals. Additional individual assignment (Supplementary Fig. S2) supported this subdivision and indicated limited admixture in Ossona, Gampel, Magredi and Susa. Bayesian clustering with Structure indicated that the most likely number of genetic clusters in microsatellite data according to the modal value of the distribution of ΔK was three. The separation of the two Swiss population samples and the one from Susa was confirmed by Structure in all 20 independent iterations (Supplementary Fig. S3). Cogne, the population sample containing only Eastern mtDNA clustered for nuclear markers always with samples containing the Western mtDNA lineage which is fully in line with the results from DAPC. Further evaluation of Structure results with K = 2 or K = 4 additionally corroborated the absence of a particular

Fig. 4. Discriminant Analysis of Principal Components (DAPCs) using microsatellite genotypes of individuals from 10 *Oedaleus decorus* population samples from the region of the Alps. The scatter plot shows the two first principal components of the DAPC and the data were best explained if the number of genetic clusters was set to three (K = 3). Individuals from the sample in Northern Italy (Susa) built cluster 1, the majority of individuals from the two Swiss samples were comprised in cluster 3, and cluster 2 consisted of individuals from French localities and the two Italian samples Cogne and Magredi. Individual genotype assignment is displayed in Supplementary Fig. S2.

distinctness of the Cogne population from the others for nuclear DNA.

4. Discussion

Phylogenetic analyses revealed a previously unknown and surprisingly deep genetic split in European *O. decorus*. The largely allopatric occurrence of the two lineages in southwestern and southeastern Europe and the wide distribution of haplotypes within them are consistent with a post-LGM range expansion of *Oedaleus* and secondary contact between lineages in the region of the Alps. However, low haplotype diversity within lineages prevents the reconstruction of colonization patterns based on mtDNA. Interestingly, divergence between populations at nuclear microsatellite loci did not consistently reflect mtDNA differentiation, which illustrates the importance of marker- or sex-specific processes in the biogeographic history of this thermophilic grashopper species.

4.1. Deep genetic divergence in mtDNA

The genetic split between the Western and Eastern mtDNA lineage in European *O. decorus* is similar in magnitude to the divergence to *O. asiaticus* (Fig. 2; Table 2) and may thus point towards the existence of three distinct, but closely related grasshopper species or subspecies (Fries et al., 2007). In principle, technical issues such as the accidental inclusion of Numts in a dataset together with mitochondrial sequences could result in an apparent deep divergence between closely related taxa (Bensasson et al., 2001; Song et al., 2008). However, the presence of Numts in our data seems unlikely given that the analysis of different PCR primers and mtDNA loci under rather strict experimental conditions (see methods) congruently revealed the same deep split (Figs. 2 and 3 and Supplementary Fig. S1). Interestingly, nucDNA provided no evidence of genetic divergence patterns analogous to population data of mtDNA where the population from Cogne was clearly most distinct. In principle, genetic variation and statistical power was obviously large enough to detect subdivisions based on nucDNA (Fig. 4 and Supplementary Figs. S2 and S3), which suggests region or population specific processes in the evolutionary history as the likely cause of this discrepancy (see below). At present, it remains unclear whether the Western and Eastern mtDNA lineages could represent so-called cryptic species in Europe, distinct species which had not been recognized within a taxon (e.g. Mayer and von Helversen, 2001). Given that currently there are no data available on the extent of morphological differences or the level of reproductive isolation between these lineages, molecular data alone cannot fully resolve the present taxonomic status of these two lineages.

The distribution of the Western and Eastern mtDNA lineage in Southern Europe is similar to biogeographic patterns found in a larger number of temperate European taxa, and may thus correspondingly be associated with habitat shifts and subsequent recolonization of new territories after Pleistocene glaciations (Hewitt 2000). It is unclear when divergence between mtDNA lineages in *Oedaleus* may have started as the fossil record is not informative for these grasshoppers and dating molecular divergence without reliable calibration points is highly error prone (see Ho and Phillips, 2009; Debruyne and Poinar, 2009). Previously, divergence between *O. decorus* and *O. asiaticus* was estimated around 34 million years ago (Fries et al., 2007) but divergence of a few percent at the mtDNA markers (Table 2) seems rather compatible with a split of *Oedaleus* lineages in the Pleistocene (see Papadopoulou et al. 2010). However, it is clear that the climatic and habitat changes induced by multiple ice age cycles during the Pleistocene in Europe must have had an impact on the distribution of this thermophilic
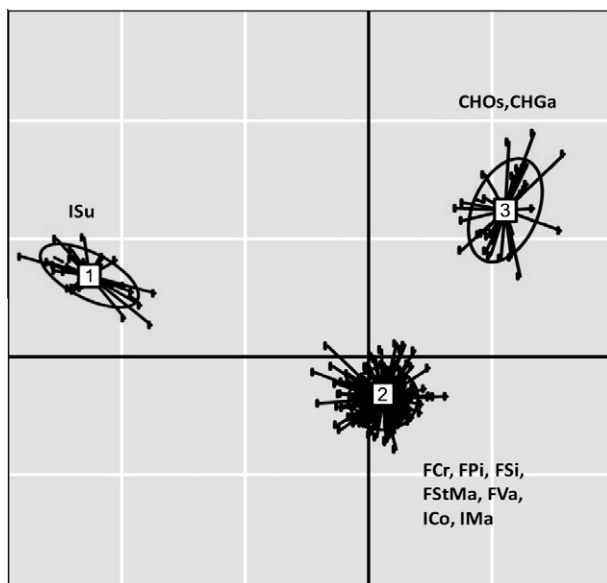
species. Allopatric distributions of the Western and Eastern lineages and relatively low genetic divergence and haplotype numbers within them are consistent with rapid post-glacial expansions from separate refugial areas. The Eastern lineage may have colonized Europe after the LGM from the classical refugial areas in the Southern Balkans or Asia, and the Western lineage from the South of Iberia or Italy. In addition, Northern Africa may represent a very plausible refugial area for the Western lineage, given its presence in Algeria (Benkenana and Harrat, 2009) and Libya (Massa 2009) and its relatively high dispersal ability (Boczki, 2007). Unfortunately, the museum material from Northern Africa collected for the present study did not allow us to genetically confirm the identity as O. decorus or test for mtDNA lineage affiliation. Thus additional geographically very wide sampling will be necessary to distinguish between the mentioned hypotheses for potential refugial areas.

4.2. A suture zone in Northern Italy?

The Eastern and Western mtDNA lineage in O. decorus come together in the region south of the Alps (Fig. 1). For a broad variety of organisms, the Alpine chain was an important barrier to post-glacial expansion and thus the region corresponds to one of the most important suture zones in Europe where different evolutionary lineages met (reviewed in Taberlet et al., 1998). Interestingly, we detected both lineages in the mountainous area in North-western Italy, but they did not co-occur in any of the O. decorus population samples. Fine-scale geographic sampling in Northern Italy will be necessary to determine where the two lineages exactly meet and which properties the contact zone shows (see below). However, this region contains such zones also in other insect species. For example, Flanagan et al. (1999) described a hybrid zone for the grasshopper Chorthippus parallelus across the Col de Larche, a mountain chain in the Italian Alps, and Dapporto (2010) characterized a suture zone for the butterfly Zerynthia polyxena in Northern Italy where the two lineages were separated by the river Po.

4.3. Cytonuclear discordance

Population-based analyses revealed a lack of congruence between mitochondrial and nuclear data in the sample in North-western Italy (Cogne). Remarkably, all individuals from Cogne were exclusively part of the Eastern mtDNA lineage, but clustered for nucDNA with individuals from samples containing only Western mtDNA rather than being most distinct from these (Fig. 4, Supplementary Figs. S2 and S3). Such cases of cytonuclear discordance have been observed in mtDNA suture zones of several organisms (e.g. Braaker and Heckel, 2009; Renoult et al., 2009; Bastos-Silveira et al., in press), and some differences among mtDNA and nuclear markers at the population level may be expected simply because of the lower effective size and thus stronger genetic drift in the former (Canestrelli et al., 2007; Bos et al., 2008).

However, we suggest that historical introgression of mtDNA among Oedaleus lineages and/or ongoing sex-specific gene flow may have contributed to cytonuclear discordance in the contact area in north-western Italy. Range expansions and secondary contact between evolutionary lineages are often associated with the introgression of gene pools, and depending on local population sizes and dispersal patterns, either genes from the residents or the invaders may introgress (see Currat et al., 2008). In the case of Oedaleus, e.g. a range expansion and invasion by the Western lineage may have led to asymmetrical introgression of Eastern mtDNA from resident individuals into the gene pool of the invaders – a situation that can lead to a very high local frequency of introgressed alien alleles (see Renoult et al., 2009). Additionally, strongly male-biased dispersal may lead to local admixture be-

tween the two lineages at microsatellite loci while female philopatry would conserve phylogeographic patterns in maternally inherited mtDNA (e.g. Braaker and Heckel, 2009). Thus, local cytonuclear discordance in Oedaleus could be explained at least in part by the predominant immigration of males from the Western mtDNA lineage and mixing with local females from the Eastern mtDNA lineage (see e.g. Alves et al., 2008). Male-biased dispersal has been suggested for some grasshoppers (e.g. Walters et al. 2006) but unfortunately data for Oedaleus are absent. In conclusion, it seems most promising for a deeper understanding of the evolutionary processes in O. decorus in the future to combine detailed population genetics analyses in the region of contact with information about sex-specific dispersal behaviour from the field and experiments investigating the extent of interbreeding among the two highly divergent mtDNA clades.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.07.025.

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