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Population history of *Eresus cinnaberinus* (Araneae: Eresidae) colour variants at a putative species transition

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Comparative population genetic and phylogenetic analyses were used to study historical and recent gene flow between two colour variants of the spider *Eresus cinnaberinus*, in order to explain variant distributions in Northern and Central Europe. Recently, the colour variants have been assigned to two species, *E. cinnaberinus* and *E. sandaliatus*, the latter found isolated in Denmark and in Bavaria. Explaining *Eresus*'s distributions thus poses a twofold problem: (i) clarifying species limits at a population–species transition and (ii) explaining noncontinuous distributions in a postglacially colonized area. Combined allozyme and mtDNA data suggest that disjunct distributions of *E. sandaliatus* in Bavaria and Denmark were caused by introgression of *E. cinnaberinus* into a *E. sandaliatus* background, giving rise to *E. cinnaberinus* phenotypes, rather than competitive exclusion of a genetically independent species by the other. Introgression caused mtDNA paraphyly of the derived *E. sandaliatus* whereas paraphyly of *E. cinnaberinus* outside the putative introgression zone may be associated with lineage sorting. Allozymes reveal local and extant gene flow processes better than mtDNA, but, because of the divided population structure, allozymes have limited power in making inferences about historical gene flow and the speed of postglacial colonization. Mitochondrial DNA distributions indicate that postglacial colonization of Northern Europe occurred rapidly and in several waves from different source populations.

Keywords: allozymes, genetic structure, introgression, mitochondrial DNA, morphological variation, phylogeography.

Introduction

In boreal regions, repeated cycles of range expansion and contraction are thought to play a major role in the evolution of subspecificity in animals and plants (e.g. Hewitt, 1996; Comes & Kadereit, 1998; Taberlet *et al*., 1998). If intraspecific variation is common, allopatric morphological and genetic differences may reflect drift or local adaptation rather than true species. Renewed gene flow among formerly geographically isolated populations may lead to hybrid zones (Hewitt, 1989). Such secondary contact may result in rapid prezygotic isolation among subspecies (Coyne & Orr, 1997). Sympatry is therefore one 'measure' of species specificity. Insights into the extent of variation and character evolution may be gained from spatial population structure analyses (Hedin, 1997a; Thompson, 1999) and phylogeographical patterns (Avise, 2000).

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The lady-bird spider *Eresus cinnaberinus* (formerly *niger*) (Olivier, 1798) exhibits two disjunctly distributed colour and phenological variants. Recently, *Eresus cinnaberinus* was split into two presumptive species: *E. cinnaberinus* and *E. sandaliatus* (Ratschker & Bellmann, 1995). *Eresus sandaliatus* occurs isolated in Northern Europe (Denmark, Sweden, northern Germany and England), in southern Germany (Danube region), and in pockets in the Alps and Pyrenees (Ratschker, 1995). *Eresus cinnaberinus* are found in the rest of Europe, thereby isolating Danish and northern German populations from those along the Danube.

Because of their mosaic distribution and lack of sympatry we shall henceforth refer to the two presumptive species as 'variants'. *Eresus cinnaberinus* is characterized by highly structured populations indicating limited dispersal power (Johannesen *et al*., 1998). The objective of this study is to explain discrete variant distributions in Central and Northern Europe, relating historical and recent gene flow to an uncertain population–species status in an area of postglacial

re-colonization. First, we investigate whether gene flow and population substructuring within and among *E. sandaliatus* and *E. cinnaberinus* are similar. The analysis was carried out in order to compare gene flow and genetic drift in structured populations with respect to interpreting phylogenetic and phylogeographical patterns among variants (Porter, 1990). One possible outcome is that one variant has highly structured populations locally, whereas the other does not. This could affect the distribution of alleles that are being used to infer historical patterns. At the population–species transition, inferring phylogenetic relationships faces the problem of the shallow time divergence (Crandall & Templeton, 1996).

Secondly, having gained knowledge of the population structures, we study the phylogenetic state of the variants. We ask whether variants are independent evolutionary lineages with present-day distributions determined by colonization history, or whether they represent convergent fixation of traits as a result of genetic drift in subdivided populations, e.g. a single gene often determines colour in spiders (Oxford & Gillespie, 1998). A possible scenario to explain the discrete occurrences of *E. sandaliatus* in Denmark and along the Danube could be that *E. sandaliatus* has been outcompeted by *E. cinnaberinus* in central Germany.

The variants

Eresus cinnaberinus males have small, elongated pedipalp conductors; they seek mates in autumn and have red hairs on hind leg pairs; the females are characterized by red to orange colouration of the sternum. In contrast, *E. sandaliatus* males seek mates in spring and have pronounced white leg hairs (Ratschker & Bellmann, 1994); females always have black sternums. Males and females of both variants become sexually mature in autumn. However, it seems that *E. sandaliatus* wait until spring before mating, whereas *E. cinnaberinus* mate in autumn after the final moult (Nørgaard, 1941; Baumann, 1997).

Despite these differences, many traits do not fit the division into two distinct groups. A reported male between-variant size difference (Ratschker, 1992) did not include a correction for effects of locality. Both variants fall within the size range of eastern German *E. cinnaberinus*, $N = 703$ (Baumann, 1997). At least two *E. cinnaberinus* populations have males that migrate in spring (Leist, 1994; Ratschker, 1995). Variation within variants in pedipalp morphology and abdominal colour (Ratschker, 1995; Bellmann, 1997) and the timing of sexual maturity have been reported. In Wallis (Switzerland), both sexes apparently have a synchronized maturity cycle, becoming adult only every third year (Walter, 1999).

Materials and methods

Sampling

Eresus variants for population genetic analyses were compared in a North–South (*E. sandaliatus*) vs. East–West (*E. cinnaberinus*) pattern (Fig. 1) comprising individuals from variant typical localities (Nørgaard, 1941; Nørgaard, 1988; Ratschker & Bellmann, 1995). *Eresus cinnaberinus* are based on samples from Johannesen *et al.* (1998). *Eresus sandaliatus* were collected in Denmark and in the west Danube drainage system (Fig. 1). The average distance between the three Danish localities (Table 1) was 7 km. To analyse within-patch structure, the patch Gammel Rye was sampled along a hill-transect (300 m) and divided into four subsamples, each separated by 50–100 m. Collections from the Danube took place at 5 locations (Table 1). At one location, Altmühltal, animals were sampled at 4 sites

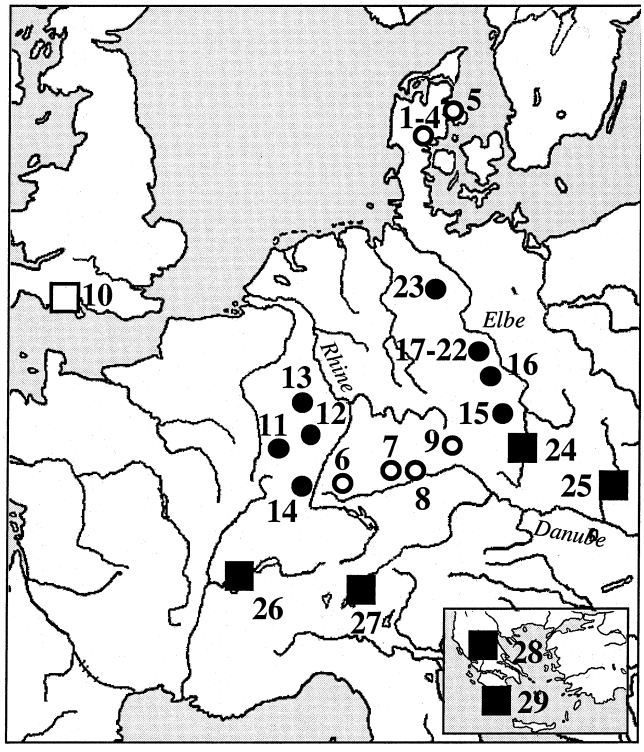

Fig. 1 Sampling locations used for mtDNA analysis, and for population genetic structure (allozymes) of *E. sandaliatus*. *E. cinnaberinus* (closed signs), *E. sandaliatus* (open signs). Circles represent samples used in population structure and phylogenetic analyses. Populations with squares were included in phylogenetic analyses only. Locality names are presented in Table 3. All *Eresus cinnaberinus* sampling locations included in population genetic analyses are given in Johannesen *et al.* (1998). Danish and Danube *E. sandaliatus* are isolated into these regions by continuous *E. cinnaberinus* distributions.

	N	Alleles per locus	H_e
E. sandaliatus			
Denmark			
Allinge	18	1.43	0.116
Gammel Rye	72 (9–12)[1]	1.67 (1.33–1.42)[1]	0.117 (0.083–0.137)[1]
Addit	33	1.54	0.116
Mean (±SD)		1.54 ± 0.12	0.116 ± 0.001
Danube			
Kallmünz	18	1.38	0.074
Kinding	4	1.24	0.094
Altmühltal	16	1.57	0.089
Fridingen	10	1.29	0.058
Mean (±SD)		1.37 ± 0.15	0.079 ± 0.016
E. cinnaberinus[2]			
East of Rhine	10.3	1.50 ± 0.18	0.125 ± 0.041
(range)	(8–24)	(1.14–2.05)	(0.020–0.224)
West of Rhine	10	1.21 ± 0.11	0.064 ± 0.037
(range)	(6–14)	(1.10–1.38)	(0.013–0.126)

Table 1 Genetic variability estimates for *E. sandaliatus* populations, and a comparison with *E. cinnaberinus*: sample size per population *N*, average number of alleles per locus, and expected heterozygosity H_e. The *E. cinnaberinus* estimates are sample averages

[1] Subsample values generated for among and within variant comparisons;
[2] Data from Johannesen *et al.* (1998).

(Finstereckfelsen 1 and 2, Schernfeld and Obereichstätt) with an average distance between sites of 2 km. In order to boost the sample size at Altmühltal, animals from here were pooled into one sample (collecting permission was granted for only four spiders per site). At Fridingen, spiders were collected at two sites 300 m apart.

Samples for phylogenetic and phylogeographical analyses were obtained from surrounding countries. Adult female *E. cinnaberinus* were collected at Pesina, Italy, $N = 10$. Swiss *E. cinnaberinus* from Wallis, $N = 3$, belong to a population showing three-year maturity cycles (Walter, 1999). Czech *E. cinnaberinus*, $N = 7$, come from the Elbe drainage system, and Slovakian, $N = 3$, come from the Tatra region (Danube drainage). Three Greek male *E. cinnaberinus* were sampled in May–June. Leg material from two English spiders was included in the mtDNA analysis. Only one English population is known. Morphologically, the English spiders resemble Danish spiders (Wisniewski & Hughes, 1998), i.e. *E. sandaliatus*.

Allozyme electrophoresis and mtDNA isolation

Eighteen enzyme systems were investigated representing 24 loci (Johannesen *et al.*, 1998). Staining procedures and running conditions were identical to Johannesen *et al.* (1998) except for *Hbdh* (EC 1.1.1.30) which was run for 45 min at 250 V. *Hbdh* was identified as polymorphic only after *E. sandaliatus* was included in analysis. All individuals investigated in Johannesen *et al.* (1998) were reanalysed for *Hbdh*. Turkish *E. walckenaerius*, $N = 8$, and the eresid *Stegodyphus lineatus* (Eresidae), from Israel, $N = 23$ (Johannesen & Lubin, 1999), were collected for phylogenetic outgroup comparisons. Six *S. lineatus* loci did not stain using the running conditions for *Eresus*. These loci were assigned as monomorphic and different from *Eresus* alleles.

DNA extraction was performed from frozen leg or cephalothorax tissue using the Boeringer-Mannheim High Pure PCR Purification Kit, with a final extraction volume of 300 μL. A double-stranded DNA template (548 bases) including a partial NADH dehydrogenase subunit I (ND1) (≈375 bases) and 16S ribosomal RNA (16S) and tRNA (≈173 bases) was amplified for 83 *Eresus* individuals via PCR using the primers LR-N-12945: 5′-CGA-CCT-CGA-TGT-TGA-ATT-AA-3′, and N1-J-12261: 5′-TCG-TAA-GAA-ATT-ATT-TGA-GC-3′ (Hedin, 1997b; Croucher, 1998). In order to test a *post hoc* phylogeographic inquiry within one sublineage, an additional 382 16S bases were sequenced for 23 individuals, including 2–3 animals of each haplotype found in the preceding analysis. If a haplotype occurred at more than one locality, we investigated spiders from different sites. Only a single Greek spider was re-investigated. The 16S primers were as follows, LR-J-12887: 5′-CCG GTT TGA ACT CAA ATC ATG T-3′ and LR-N-13398: 5′-CGC-CTG-TTT-AAC-AAA-AAC-AT-3′. PCR conditions were identical for both sequences. They were performed in an end volume of 25μl, consisting of 1 μL forward and backward primer (10 pmol μL^{-1}), 1 μL DNA extraction and 22 μL LiCrosolv® water. PCR reactions were performed with Ready.to.Go™ beads (0.5 mL tubes; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The PCR

reaction was started by denaturing at 95°C for 2 min, followed by 35 cycles of: annealing 47°C/1 min, extension 72°C/1.5 min, and denaturation 95°C/30 s. The PCR was terminated with a final extension at 72°C for 10 min. Single-stranded PCR for sequence analyses were performed in 20 μL end-volume, with 1 μL purified DNA, 1 μL primer, 7 μL premix, and 13 μL water. PCR products were sequenced in both directions using an ABI-377 (Perkin Elmer) automatic sequencer. Sequences were aligned using the program SEQUENCE NAVIGATOR (ABI). Subsequently, all aligned sequences were checked manually. Sequences have the Genbank numbers AF174174-174185.

Genetic analyses

Only Danish and German animals were used in the population genetic analyses. These were performed using allozyme data. A two- and three-level hierarchical analysis of variance was calculated using unbiased *F*-statistics, F_{IS}, F_{IT} and F_{ST}, of Weir & Cockerham (1984) using the program GDA (Lewis & Zaykin, 1999). Ninety-five percent confidence intervals were obtained by bootstrapping loci. The three-level hierarchical analysis was implemented to investigate variant-specific differences between *E. sandaliatus* and *E. cinnaberinus*. First, we estimated the between-variant variance, F_{VT}, and secondly, we estimated the variance among regions within each variant. Here, F_{RT} is the amount of genetic variance at the regional level relative to the total level of the respective variants. Regions were Denmark and Danube in *E. sandaliatus*, and western and eastern Germany in *E. cinnaberinus*.

For allozymes, population relationships were investigated using maximum likelihood estimates in the program CONTML from the PHYLIP program package (Felsenstein, 1993). All *Eresus* populations, except that from England, were included in this analysis.

Phylogeny of mtDNA haplotypes was analysed with a full heuristic maximum parsimony bootstrap search (1000) using the Beta version of PAUP 4.0 (Swofford, 1999). Gaps were treated as a fifth base. Branch-swapping was computed with the tree–bisection–reconnection algorithm. Clock-like behaviour of branch lengths was tested with PUZZLE (Strimmer & von Haeseler, 1996) using the HKY85 model of substitution, assuming a gamma distribution of rate heterogeneity, $\alpha = 0.5$.

Results

Allozyme variation

Danish and Danube *E. sandaliatus* populations were characterized by high frequencies of the *Pep-B1* allele 2, *Hbdh* allele 1, and *Aat-2* allele 3. The Denmark, Danube and eastern Germany populations shared the *Gpd* allele 3 as the dominant allele. In western Germany, *Pep-B1* and *Gpd* were monomorphic for other alleles. Furthermore, *Hbdh* allele 1 was absent while *Aat-2* allele 3 was nearly absent ($P < 0.01$) in western German populations. All *E. sandaliatus* and western German *E. cinnaberinus* characteristic alleles were observed in eastern Germany. Italian *E. cinnaberinus* possessed the unique alleles 1 and 2 for *Apk-2*. In all other populations, including the outgroup *S. lineatus*, *Apk-2* was monomorphic for allele 3. All Slovakian spiders were monomorphic for a unique *Acon* allele and possessed otherwise rare *Hk* and *Fum* alleles. (An allele frequency table is available on request.)

We tested for regional differences in expected heterozygosity per sample, averaged over all loci (Table 1). Two possible biases should be noted. First, the disjunct sampling at Altmühltal and at Fridingen led to enhanced heterozygosity estimates, $H_e > H_o$, i.e. a Wahlund effect. The results reported are of H_e, which was higher than the observed number of heterozygotes. Second, the sample sizes of two populations in Denmark were much larger than in the other three regions. We corrected this by dividing each Danish population into a set of randomly generated samples of 9–12 individuals comprising all individuals [Denmark 12 samples, mean sample size 10.25, $H_e = 0.101 \pm 0.041$ (range: 0.063–0.137)]. In this manner, the *E. sandaliatus* sample sizes correspond to level-4 sample sizes ($N \sim 10$) analysed for *E. cinnaberinus* (Table 1) (Johannesen *et al.*, 1998). Significant differences (*t*-tests) in expected sample heterozygosity were found between eastern and western Germany ($P < 0.01$), eastern Germany and Danube ($P < 0.05$), Denmark and western Germany ($P < 0.05$) and between Danish and Danube populations ($P < 0.05$). The difference between eastern Germany and Denmark was nearly significant, $P = 0.06$.

Genetic differentiation in E. sandaliatus

The hill-transect sample at Gammel Rye did not show intrapatch structure (Table 2) and thus did not differ from *E. cinnaberinus* (Johannesen *et al.*, 1998). No *E. sandaliatus* samples deviated significantly from Hardy–Weinberg proportions.

Within both *E. sandaliatus* regions, allele frequencies deviated highly among populations, e.g. in Denmark the *Pep-B2* allele 5 (30%) and *Mdh-2* allele 2 (18%) were found only at Addit, and *Pep-B1* allele 1 was found at 39% in Allinge, but not in the large Gammel Rye sample. Genetic differentiation among the four Danube populations, $F_{ST} = 0.28$, was higher than among the three Danish populations, $F_{ST} = 0.13$. The high variance in the Danube region was caused primarily by the

Table 2 Three- and two-level hierarchical *F*-statistics (Lewis & Zaykin, 1999) for *E. cinnaberinus* and *E. sandaliatus* populations. F_{VT} represents for 'all populations' the amount of genetic variance found between the variants. F_{RT} represents for 'all *E. cinnaberinus*'and ''all *E. sandaliatus*'' the amount of genetic variance at the regional level relative to the total level within each variant, respectively. Regional levels were Denmark and Danube in *E. sandaliatus*, and western and eastern Germany in *E. cinnaberinus*. The hill transect of the patch 'Gammel Rye' consisted of four subsamples, $N = 15$–20. Gammel Rye was treated as one sample in the 'Denmark' analysis. All *F* estimators are given with 95% confidence intervals calculated by 1000 bootstraps over loci

Level	*N*	F_{IS}	95% CI	F_{IT}	95% CI	F_{ST}	95% CI	F_{VT}	95% CI
All **Populations**	34	0.04	*0.01–0.06*	0.59	*0.30–0.73*	0.56	*0.05–0.64*	0.43	*0.28–0.64*
								F_{RT}	
All ***E. cinnaberinus***[1]	27	0.05	*0.00–0.09*	0.40	*0.22–0.57*	0.37	*0.05–0.39*	0.25	*0.19–0.55*
Western Germany	6	−0.02	*−0.15–0.09*	0.18	*0.10–0.26*	0.19	*0.14–0.25*		
Eastern Germany	21	0.06	*0.01–0.10*	0.21	*0.15–0.26*	0.16	*0.12–0.20*		
All ***E. sandaliatus***	7	0.02	*−0.02–0.10*	0.17	*0.10–0.25*	0.15	*0.10–0.19*	−0.02	*−0.05–0.02*
Hill transect	4	−0.01	*−0.09–0.10*	0.01	*−0.08–0.13*	0.02	*−0.00–0.04*		
Denmark	3	0.02	*−0.04–0.10*	0.14	*0.05–0.25*	0.13	*0.07–0.19*		
Danube	4	0.04	*−0.09–0.18*	0.31	*0.06–0.51*	0.28	*0.08–0.43*		

[1]Data reanalysed from Johannesen *et al.* (1998).

population Kallmünz where the locus *Aat-2* was nearly fixed (84%) for the regionally less common allele 3. F_{ST} declined to 0.16 if *Aat-2* was omitted, while omitting Kallmünz gave $F_{ST} = 0.09$. Thus, the Danube and Danish estimates were comparable to the differentiation indices found among populations in eastern and western German *E. cinnaberinus*: $F_{ST} \sim 0.16$–0.19 (reanalysed data) and $F_{ST} \sim 0.12$–0.18 (depending on sample sizes of either ≈30–40 or ≈10, respectively, see Johannesen *et al.*, 1998).

Hierarchical comparison of E. cinnaberinus *and* E. sandaliatus *genetic structure*

A three-level hierarchical analysis revealed high differentiation among all *Eresus* populations, $F_{ST} = 0.56$ (Weir & Cockerham, 1984) (Table 2). The regional hierarchical analysis showed that the high F_{ST} for *E. cinnaberinus* was caused by genetic variance between the two regions, $F_{RT} = 0.25$. In contrast, no among-region variance was observed in *E. sandaliatus*, $F_{RT} = -0.02$. This finding implies that, for *E. sandaliatus*, isolation among populations within the two regions was greater than the averaged regional differences. Differentiation among all seven *E. sandaliatus* populations, $F_{ST} = 0.15$, differed significantly from the estimate for all *E. cinnaberinus* populations, $F_{ST} = 0.37$. However, large and skewed confidence intervals, caused by several allele differences that were almost *fixed* between the two regions in *E. cinnaberinus*, meant that the difference was only just significant. Such fixed differences should actually indicate high levels of isolation. Calculating F_{ST} in a two-level analysis (Weir & Cockerham, 1984) using the same data and program resulted in significant within-variant differences: *E. cinnaberinus*, $F_{ST} = 0.26$ (95% CI, 0.16–0.39); *E. sandaliatus* $F_{ST} = 0.15$ (0.10–0.19). The reason for *E. sandaliatus* F_{ST} not changing is the lack of among-region variance.

Phylogeny based on allozymes

The maximum likelihood tree is shown in Fig. 2(a). There were three major findings. (i) *E. walckenaerius* did not form a significant outgroup to the *E. cinnaberinus*/*sandaliatus* group (it was, however, genetically more diverged). (ii) *E. sandaliatus* and western German *E. cinnaberinus* (including the Wallis population) each formed a group significantly different group from all others. The Italian, Greek and Slovakian samples were differentiated by equally long branches as the three groups above. (iii) Eastern German and Czech *E. cinnaberinus* did not occupy an independent branch but constituted an intermediate group between western German *E. cinnaberinus* and *E. sandaliatus*.

Variation and Phylogeny of mtDNA haplotypes

Twelve *E. cinnaberinus*/*sandaliatus* haplotypes were found for the 548 base pair ND1/16S sequence. No additional haplotypes were found by examination of the 930 base pair sequence for a reduced set of specimens. No haplotypes were shared among regions (Table 3). Within the regions of western Germany and of the Danube only a single haplotype were observed in each

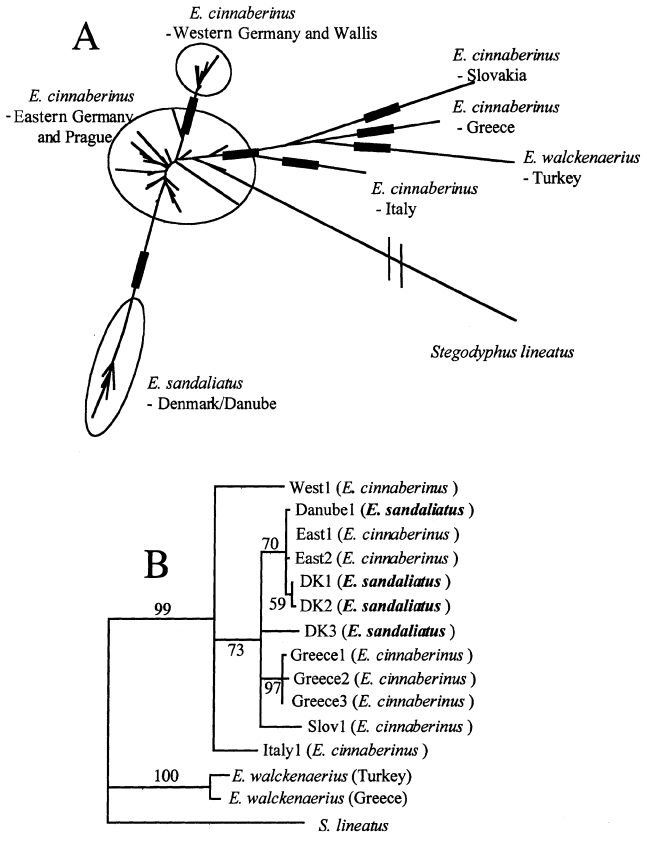

Fig. 2 (a) Maximum likelihood tree based on allozyme frequencies. Boxes indicate significant branch lengths corresponding to species and regional populations. The branch lengths are based on genetic drift only. (b) Parsimony consensus tree, 1000 bootstraps, based on *E. cinnaberinus* and *E. sandaliatus* mtDNA haplotypes, and outgroups.

case, and in the Elbe drainage (eastern Germany and the Czech Republic) a single haplotype dominated. In Denmark (*E. sandaliatus*) three haplotypes were found: two differing from the eastern German haplotypes by 4–5 base pairs and a third haplotype differing from the eastern German and from the other Danish haplotypes by about 20 bases (Table 4). Each Greek spider had a different haplotype, showing pairwise differences of 1, 2 and 3 base pairs, respectively.

Monophyly was established for the species group *Eresus cinnaberinus/sandaliatus* (Fig. 2b). Branch lengths were in accordance with a molecular clock (Likelihood ratio test statistic delta: 15.7, d.f. 13, $P > 0.28$). The geographical distributions of the three basal *E. cinnaberinus* lineages with 37–40 base differences between lineages correspond to lineage origins in Western Europe, Italy south of the Alps, and the Balkans. Incongruence between morphology (colour) and mtDNA lineages occurred within the 'Balkan' clade that included both colour variants. The Balkan clade included four sublineages differing by 15–20 base pairs. Within the Balkan sublineage 'Denmark–eastern Germany–west Danube', the geographically centrally distributed eastern German animals are *E. cinnaberinus* variants whereas the peripheral Danube and Danish animals are *E. sandaliatus* variants.

The phylogeny did not change using the 930 base pair ND1/16S sequence. From here on we will therefore discuss the larger 548 base pair dataset. However, as part of a *post hoc* inquiry for postglacial colonization by the 'Denmark–eastern Germany–west Danube' sublineage (see discussion), Danish animals could be distinguished by two additional mutations relative to Danube and eastern German spiders.

Discussion

Origin of variants

It was the aim of this study to investigate the nonoverlapping distributions of two *Eresus* variants in relation to their population histories and to their phylogenetic assignments. Because variant-distributions are nonoverlapping, phenotypic variation may be influenced by geographical separation rather than by reproductive isolation (see, e.g., Wishart & Rowell, 1997).

The phylogeny indicated that *E. sandaliatus* is a derived evolutionary lineage, and that explanations for its isolated distributions are to be sought in postglacial population history. The alternative explanation, that a divided population structure led to convergent evolution of *E. sandaliatus* traits in Denmark and along the Danube, seems unlikely because the variants are not found locally in a mosaic fashion where fragmented, highly structured populations could have produced variation (Oxford, 1989; Oxford & Reillo, 1993). Instead, variant distributions are nonoverlapping and regional.

However, there was no clear variant-specific delimitation. For allozymes, *E. sandaliatus* populations clustered as a monophyletic group within the paraphyletic *E. cinnaberinus*. On the other hand, the mtDNA genealogy indicated paraphyly of the derived *E. sandaliatus*, where eastern German haplotypes of *E. cinnaberinus* were almost identical to Danube *E. sandaliatus*. In both the allozyme and mtDNA analyses, eastern German *E. cinnaberinus* populations were intermediate (Fig. 2).

Paraphyly of both genetic markers in *Eresus* may suggest ongoing gene flow among the variants in an area of re-colonization. Hybridization at species interfaces have been observed in a number of species (e.g. Barton & Hewitt, 1985; Arnold, 1997; Clark *et al.*, 1998). However, the population structuring of *Eresus* lends some support to stochastic fixation of allozyme alleles in ancestral

Table 3 Distribution of mtDNA variation. N_{548} is the number of haplotypes sequenced for the 548 base pair 16S/ND1. N_{930} designates the number of additional sequenced spiders for 382 base pairs of the 16S gene, for investigation of alternative colonization scenarios. From Greece, only a single haplotype was sequenced. Locality numbers refer to geographical locations presented in Fig. 1. Locality abbreviations for *E. cinnaberinus* refer to sample sites in Johannesen *et al.* (1998)

Region	Locality	N_{548}	Haplotypes	N_{930}
E. sandaliatus				
Denmark	1. Allinge	3	DK3	1
	2. Gammel Rye	12	DK1 (11), DK3 (1)	2
	3. Addit	3	DK2	2
	4. Sepstrup	1	DK3	1
	5. Mols	1	DK3	
Danube	6. Fridingen	4	Danube1	2
	7. Altmühltal (3 sites)	6	Danube1	1
	8. Kindingen	3	Danube1	
	9. Kallmünz	3	Danube1	
England	10. Dorset	2	DK1	1
E. cinnaberinus				
Western Germany	11. Idar-Oberstein (Nahe: RP-1.3)	2	West1	
	12. Rotenfels (Nahe: RP-1.4)	3	West1	1
	13. Pommern (Mosel: RP-1.5)	3	West1	1
	14. Battenberg (Pfalz: RP-1.6)	3	West1	1
Eastern Germany	15. Kyffhäuser (SA-2.1)	3	East1	1
	16. Glücksburger Heide (SA-2.2)	3	East1	1
	17. Saalehang (SA-4.5)	1	East1	
	18. Lüneburger Heide	1	East1	
	19. Franzigmark-B (SA-3.5)	3	East2	2
	20. Lunzberge (SA-3.1)	3	East1	
	21. Brandberge (SA-3.2)	3	East1	1
	22. Teichgrund (SA-4.1)	2	East1	
	23. Unterlüß	1	East1	
Czech Republic	24. Podbaba (Prague)	3	East1	1
Slovakia	25. Bojnice	3	Slov1	1
Switzerland	26. Wallis	3	West1	
Italy	27. Pesina (Lago di Garda)	4	Italy1	2
Greece	28. Miliotades (Mainland)	1	Greece1	1
	29. Pelepones	2	Greece2 and 3	
N_{ind}		83		23

populations. This could lead to incongruent phylogenies by lineage sorting of ancestral polymorphisms.

Gene flow and extant distributions in Central Europe

Several lines of evidence suggest introgression rather than lineage sorting as the reason for the intermediate position of eastern German *E. cinnaberinus*. (i) The mtDNA dataset revealed that a diverged *sandaliatus* haplotype occurs in the taxon *cinnaberinus*. Given the large dissimilarity among haplotypes (for rough coalescence time see Table 4) it appears unlikely that lineage sorting is responsible for this observation. (ii) Because of the fourfold larger N_es for allozymes than for mtDNA, one would expect stronger lineage sorting for mtDNA compared to allozymes. (There will be higher likelihood of reciprocal monophyly of relevant taxa for mtDNA.) The allozyme phylogeny produced a clear monophyly of the derived *sandaliatus* making it unlikely that the character conflict in the mtDNA tree results from the survival of ancestral haplotypes across speciation/taxa diversification events. (iii) All 'pure' populations occurred on independent branches in the maximum likelihood tree. Eastern German populations did not group as an independent branch but were scattered along the *E. sandaliatus*–western German *E. cinnaberinus* branch. We do not think that tree topology is a sample size artefact — *E. sandaliatus* and all regional *E. cinnaberinus* populations except eastern German/Czech

Table 4 ND1/16S mtDNA sequence differences for *Eresus cinnaberinus* (*Ec*) and *E. sandaliatus* (*Es*) and outgroup taxa (548 base pairs). The number of substitutions is shown below the diagonal, and the transition/transversion ratio above the diagonal. × – no transversions. Mean differences to the three Greek *E. cinnaberinus* sequences, and to Greek and Turkish *E. walckenaerius* (*Ew*) (9 substitutions) are shown

Haplotype	1	2	3	4	5	6	7	8	9	10	11	12
1 *Ec* East1	—	x	x	x	x	17.0	14.7	9.5	5.3	5.6	3.7	1.4
2 *Ec* East2	1	—	x	x	x	18.0	15.7	10.0	5.5	5.8	3.8	1.5
3 *Es* Danube1	1	2	—	x	x	18.0	15.7	10.0	5.2	5.8	3.6	1.4
4 *Es* DK1	3	4	4	—	x	19.0	16.7	10.5	5.7	6.0	3.5	1.4
5 *Es* DK2	4	5	5	1	—	18.0	16.0	10.0	5.5	5.8	3.6	1.4
6 *Es* DK3	18	19	19	21	20	—	x	22.0	7.0	7.8	3.7	1.4
7 *Ec* Greece (mean)	15	16	16	18	19	19	—	18.0	7.5	7.3	3.8	1.5
8 *Ec* Slov1	21	22	22	23	22	23	20	—	6.5	6.2	3.6	1.5
9 *Ec* West1	38	39	37	41	40	40	43	45	—	6.2	3.7	1.4
10 *Ec* Italy1	33	34	34	36	35	35	34	36	36	—	3.8	1.6
11 *Ew* (mean)	63	64	62	62	63	68	68	70	70	67	—	1.2
12 *S. lineatus*	92	93	92	91	92	95	95	100	94	101	95	—

possessed regionally characteristic sets of allozyme alleles and each set is correlated with private mtDNA haplotypes. This suggests that the *E. sandaliatus* and regional *E. cinnaberinus* population assemblages, other than that in eastern Germany, are old enough to have acquired regional multilocus character states. [An exception is found in postglacially colonized Denmark where two sympatric mtDNA lineages (DK1 and DK3, both *E. sandaliatus*) shared multilocus allozyme genotypes — this may suggest an additional introgression event).] (iv) Multilocus sets of alleles characteristic of *E. sandaliatus* or of western German *E. cinnaberinus*, respectively, were both found in eastern Germany. If propagules of *E. sandaliatus* and western German *E. cinnaberinus* were to merge and reproduce one would expect increased heterozygosity relative to the parental populations and the presence of allele-sets characteristic of both parental populations. This was observed.

If introgression has happened, it is more likely to have taken place between *E. sandaliatus* and western *E. cinnaberinus* than with eastern Slovakian *E. cinnaberinus*, because no Slovakian *Acon* alleles were found in neighbouring Czech or eastern German populations. The introgression hypothesis implies that the isolated distributions of *E. sandaliatus* in Denmark and along the Danube are caused by introgression of genes responsible for diagnostic *E. cinnaberinus* traits into an *E. sandaliatus* background, rather than competitive exclusion of one genetically independent species by the other.

Hedin (1997b) found evidence for peripatric speciation as a cause for polyphyly in allopatric *Nesticus* cave spiders. This scenario is also unlikely in our case as an explanation for allopatric *E. sandaliatus* distributions, because the central eastern German *E. cinnaberinus* are surrounded by derived Danube and Danish *E. sandaliatus*, both geographically and in terms of the mtDNA phylogeny.

In an area of recent range expansion, introgression would readily explain the different among-regional genetic variances within each variant (Table 2). It is unlikely that the among-regional allozyme frequency differences were biased by variant peculiarities because the within-region population structures of each variant are similar.

Morphological origin of eastern German E. cinnaberinus

The presence of several ancient regional lineages suggests that regional populations may be in the process of acquiring specific characters, but cohesive factors such as postglacial secondary contacts are preventing the budding off of new fully independent species. Males and females of both variants mature sexually in autumn but only *E. cinnaberinus* males, according to literature, disperse and mate in autumn (Nørgaard, 1941; Ratschker & Bellmann, 1995). If secondary contact between western *E. cinnaberinus* and *E. sandaliatus* has taken place, the male phenology and autumn mating behaviour could rapidly replace the white colour variant simply on a first come-first served basis. Regarding male *E. cinnaberinus* autumn phenology, it may be a derived trait of the western haploline that has been wrongly ascribed to the *E. cinnaberinus* morphotype in general.

Recently, evidence has accumulated for the positive effect of introgressive hybridization and its potential

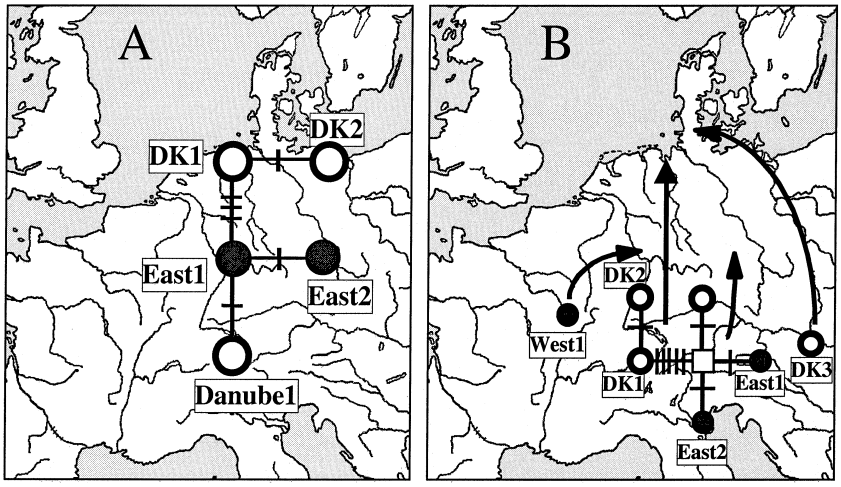

Fig. 3 Two postglacial colonization scenarios of the west Danube–eastern Germany–Danish mtDNA haplotype lineage. Bars represent one mutational step. (a) Colonization explained as a postglacial north-bound diffusion process. Three mutations suggest that Danish DK1 and DK2 haplotypes may not have arisen during postglacial range expansion. (b) Colonization by the DK, East and Danube animals explained by a rapid range expansion of two preglacially diverged lineages based on the 930 16S/ND1 sequence. Included in this scenario are the inferred colonizations of the DK3 lineage as well as a secondary immigration of western spiders into eastern Germany (based also on allozymes). This scenario suggests several waves of colonization.

for facilitating habitat invasions (reviews in Arnold, 1997; Arnold & Emms, 1998). In the jumping spider *Habronattus pugillis*, apparent character convergence among geographically proximate populations is most likely explained by introgression (Maddison & McMahon, 2000). Unfortunately, we cannot discriminate between introgression during or shortly before re-colonization, on the one hand, and introgression after arrival in eastern Germany, on the other. If introgression took place during re-colonization one might expect extremely narrow trait clines, or none at all. This question remains open for further investigations, as does whether the acquisition of male phenotypes is associated with the hypothesized male-mediated introgression.

Colonization history of Northern Europe

The homogenous mtDNA haplotype distributions within regions are a sign of recent and rapid colonization (Avise, 2000) by single ancestral stocks (except in Denmark). Unfortunately, the high level of population subdivision prohibits an extrapolation backward in time for inferences of historical colonization routes and impedes precise assertions of the preglacial geographical origin of the (Balkan) lineages.

The gene-tree of the 548 base pair sequence suggests a split (significant bootstrap scores) between the Danish and German lineages but this could also have evolved during a postglacial diffusion migration from South to North (Fig. 3a). In order to investigate this scenario, we sequenced an extra 382 bases of the 16S gene from 23 individuals (total sequence length: 930 base pairs). Now the Danish/English animals are distinguished by two additional mutations, relative to the Danube and eastern German spiders (Fig. 3b). The extra substitutions suggest that the haplotypes did not evolve in a stepwise fashion during re-colonization of Northern Europe. Rather, the DK1 *E. sandaliatus* haplotype originated before the postglacial range expansion. The latter scenario agrees with discrete regional mtDNA distributions and suggests that postglacial colonization occurred quickly from a single (set of) discrete ancestral population(s), and in several waves.

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