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Phylogeography of the burnet moth *Zygaena transalpina* complex: molecular and morphometric differentiation suggests glacial refugia in Southern France, Western France and micro-refugia within the Alps

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Abstract

Patterns of common recolonization routes from glacial refugia to Central Europe during the Pleistocene are generalized to paradigms of postglacial recolonization in Europe. Recent studies indicate, however, that the actual phylogeographic history of many species might be more complex and cannot be simplified to generalized patterns. Burnet moths of the *Zygaena transalpina* complex represent a group of closely related taxa, which are considered as a typical example for these generalized patterns. At present, three groups are recognized that are assumed to have spread from three classical refugia in Western Europe, Italy and the Balkans to Central Europe. Here, we re-investigate their phylogeography using a combined molecular and morphometric approach. Phylogenetic and nested clade phylogeographic analyses of 476 samples from 55 localities taken from Southern and Central Europe reveal that the *Zygaena transalpina* complex consists of three distinct haplotype clusters, which geographically roughly correspond to possible refugia in Western Europe, Italy and the Balkans. A synthesis of the data with a geometric morphometry dataset of 425 specimens from 46 localities corroborates this molecular result but differs in several aspects. Important new aspects are multiple refugia of the western '*hippocrepidis*' branch and micro-habitats within the Alps of the central '*transalpina*' branch. Further, our results display a more complex phylogeographic pattern for this species complex, which is not tractable with a rigid, generalized pattern.

Key words: Phylogeography – morphometric – Pleistocene – glacial refugia – micro-habitats – *Zygaena* – burnet moths – Alpine refugia

Introduction

Several, recent reviews describe general aspects of phylogeography and its contribution to evolutionary biology and other fields (Hewitt 2004b; Emerson and Hewitt 2005; Beheregaray 2008; Hickerson et al. 2010). In particular, a vast majority of phylogeographic articles focus on the role of ice ages affecting speciation of organisms (Avise et al. 1998; Taberlet et al. 1998; Hewitt 2000, 2001; Taberlet and Cheddadi 2002; Schmitt 2007). The Pleistocene is assumed to have played a veritable role in speciation processes of arthropods and any other organisms in the Palaearctic (Avise et al. 1998; Pfenninger et al. 2003; Knowles 2004; Schmitt 2007). Fragmentation of populations into subpopulations during a glacial period has been assumed, to evoke allopatric differentiation (Avise and Walker 1998; Hewitt 1999; Schmitt and Hewitt 2004) with zones of secondary contact after recolonization from glacial refugia (Hewitt 2004a). Central to this view of geographic separation and secondary contact is the idea of concomitant climate and range oscillations. More data are, however, needed to draw general conclusions on postglacial dispersal patterns of winged insects.

In this study, we apply a molecular and morphometric approach to investigate differentiation between populations of the two formally recognized burnet moth species, *Zygaena transalpina* Esper, 1780 and *Zygaena angelicae* Ochsenheimer, 1808, and their described numerous subspecies (Hofmann and Tremewan 1996; Nauman et al. 1999). Burnet moths of the genus *Zygaena* Fabricius, 1775, form a monophyletic clade within the subfamily Zygaeninae, which is most likely a monophyletic group within the Zygaenidae (Niehuis et al. 2006). Species of this genus are restricted to the palaearctic region (Hofmann and Tremewan 1996; Nauman et al. 1999). *Zygaena transalpina* and its subspecies (including *Z. transalpina hippocrepidis*, Hübner, 1799) are distributed in Western Europe and Italy, representatives of *Zygaena angelicae* and its subspecies in Eastern Europe. More specifically, Hofmann (1994) recognizes three branches, the ponto-mediterranean '*angelicae*' group, the adriato-mediterranean '*transalpina*' group and the atlanto-mediterranean '*hippocrepidis*' group. Some authors (Naumann and Tremewan 1984; Nauman et al. 1999) consider the three taxa (*Z. angelicae*, *Z. transalpina and Z. hippocrepidis*) and their described subspecies as semispecies, forming a 'super' species complex with extensive geographical variation throughout the distributional range. Based on extensive molecular data and a phylogenetic approach, Niehuis et al. (2007) show that *Z. transalpina* and *Z. angelicae* are indeed sister taxa. However, the grade of differentiation between both species and associated populations is not addressed. The present differentiation and distribution of both species are assumed to have resulted from a Pleistocene allopatric habitat fragmentation into a Western European, Italian and eastern refugium and subsequent postglacial recolonization. However, the data are still too scattered to fully support this conclusion. Our goal was, therefore, to complement and extend the previous studies with a phylogeographic analysis (Templeton 1998, 2004) using mitochondrial data of 476 specimens. Recently, this method, now called nested clade phylogeographic analysis (NCPA), is critically discussed assuming a possible sampling error in the results (Panchal and Beaumont 2007; Petit 2008; Beaumont et al. 2010). Yet, this controversy is still persisting, while alternative methods are criticized either (Garrick et al. 2008; Nielsen and

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Beaumont 2009; Templeton 2009b), see also methods section. We combined the NCPA with AMOVA and *F*-statistics. In addition, a geometric morphometry analysis (Bookstein 1997; Zelditch et al. 2004; Klingenberg 2009) was performed of wing-spot patterns and wing shape, (White and Winokur 2003; Prieto et al. 2009) based on 15 landmarks of 425 specimens.

In summary, previous molecular phylogenetic, allozyme data and taxonomic work suggest (1) that a differentiation of the *Zygaena* taxa contributing to the postulated 'three branches' is unclear (2) that based on morphological data genetic introgression is present between the *Z.* '*hippocrepidis*' and the '*angelicae*' group and (3) that founded on morphological descriptions geographic differentiation is extensive with many locally restricted forms. The goals of the present study are the following: (1) to reveal whether the glacial refugia pattern matches one of the classical described recolonization paradigms, (2) to reveal the glacial history of this species complex inferring subsequently the postglacial recolonization of the different groups and (3) to draw a more detailed picture of the present separation of the three subgroups.

Material and methods

Taxon sampling

In total, 101 European localities in Spain, France, Italy, Greece and Germany with 901 specimens were sampled (Fig. 1). If possible, a minimum number of 10 specimens were collected in each of the 55 localities for molecular work (Supporting information Table S1), and tissue preserved in 94% Ethanol. Each of the 476 sequenced specimens was assigned to nominal species or subspecies. In some cases, however, species assignment was ambiguous following the determination of Nauman et al. (1999) including forewing spot pattern, 'Nebelstreif' (connection between spots) of the forewing underside, antennae form and genital morphology (characters see: Von Reumont 2005). We consequently added the abbreviation cf. to indicate uncertainty for 18 specimens. Additionally, to the 472 specimens of the *Zygaena transalpina* complex, four specimens of two outgroup taxa (Niehuis et al. 2006), *Zygaena ephialtes* and *Zygaena dorycnii*, were sequenced. For the morphometric analysis, 425 specimens of 46 localities were analysed (Supporting information Table S2), which correspond as close as possible to the localities for molecular sampling. All specimens used in the morphometric analysis are part of the collection of the ZFMK (Bonn) and identified by specialists on that group. Analyses of specimens from identical localities for both approaches were often impossible because of nature conservation regulations, limiting either the numbers of collected specimens or restricting the collection to one leg. However, the museum material (partly 100 years old) could fill some locality gaps of the recent collection for molecular work and *vice versa*.

Molecular work

DNA was extracted from either thoracic muscle or leg tissue. DNA extraction was performed using the standard NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). DNA fragments were amplified using following primers: 5′ AAC TCT ATA AAC CCC TAA TC 3′ (forward) and 5′ ACA TGA TCT GAG TTC AAA CC 3′ (reverse). The amplified fragment (650 bp) encodes the 3′ end of the 16S rRNA (227 bp), the tRNA Leucine gene (93 bp) and the 5′-end ND1 (330 bp). Previous tests with

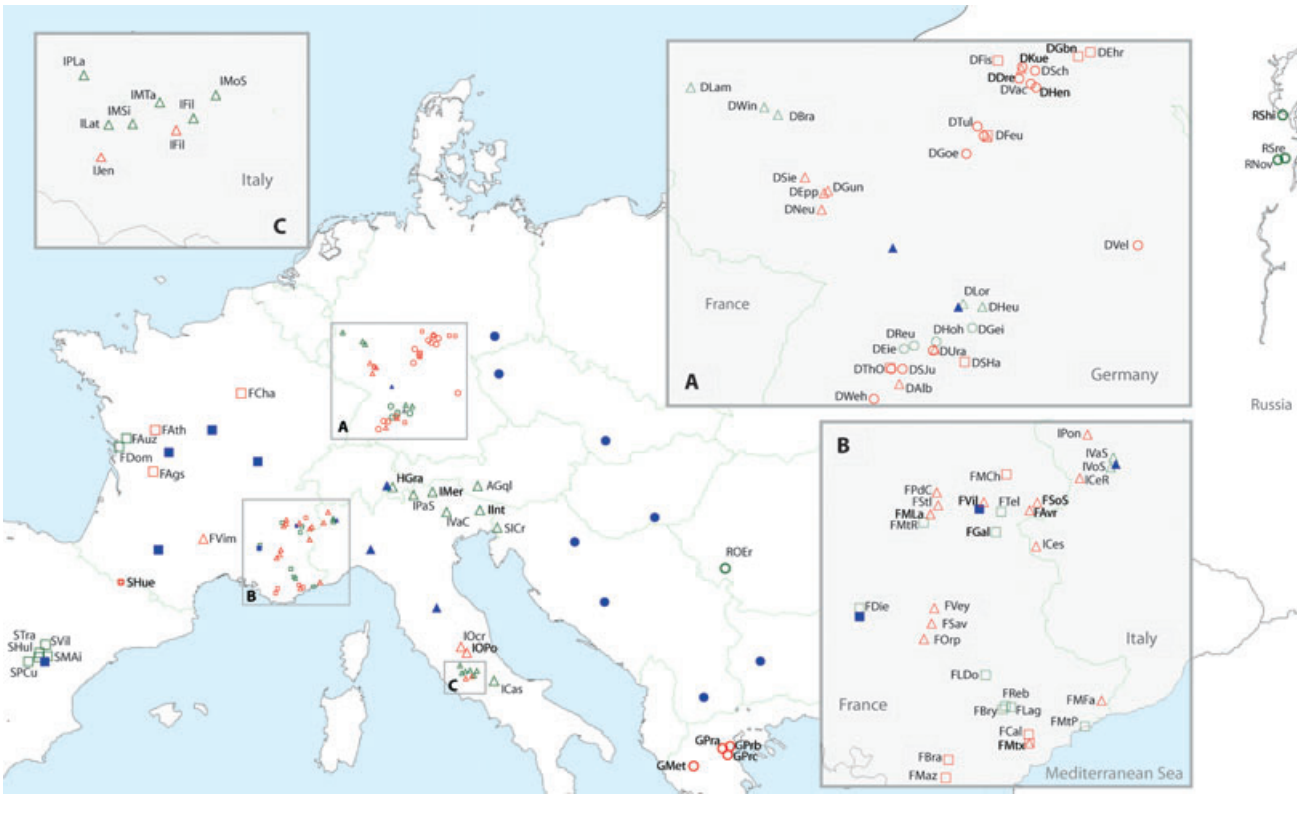

Fig. 1. Sample localities. Green symbols display localities for the morphometric, red for the molecular analysis. Specimens of *Zygaena angelicae* are represented by circles, *Zygaena transalpina* by triangles and *Zygaena hippocrepidis* by squares. The abbreviations for the localities are given in supporting Information Table S1. The first letter indicates its country: D = Germany, I = Italy, F = France, S = Spain/Slovenia, G = Greece, A = Austria, R = Russia/Romania, H = Hungaria, the following three letters code the sample area. Blue symbols illustrate not sampled localities in which zygaenids occur, and these were implemented into the nested clade phylogeographic analysis analysis for a correct phylogeographic inference

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different fragments revealed that it harbours sufficient information to address our specific questions within the *Z. transalpina* complex. PCR was carried out in GeneAmp® PCR Systems (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). The temperature profile started with a denaturation step of 3 min (94°C), 15 cycles of 35 s (94°C), 30 s (55–40°C) and 90 s (72°C), followed by 25 annealing cycles (50°C). The total reaction volume of 25 µl contained 0.075 µl Taq-polymerase (5 U/µl; Sigma-Aldrich, St. Louis, MO, USA), 2 µl dNTP (2 mM; Sigma-Aldrich, St. Louis, MO, USA), 0.25 µl primer (10 p mol^{-1}), 2.5 µl $MgCl_2$ (Sigma-Aldrich), 3.5 µl PCR buffer (Sigma-Aldrich, St. Louis, MO, USA), 1.5 µl DNA and 14.1 µl ddH_2O. For cycle sequencing, we applied an internal sequencing primer: 5′ TGA AAC CGG TGT AAG CCA GG 3′. Products were separated on an ABI PRISM 377 XL (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Sequences [GenBank accession numbers: DQ832767 – DQ833242] were aligned in Clustal × 1.8 (Thompson et al. 1997) and adjusted by eye using BioEdit 7.0 (Hall 1999).

Phylogeographic analyses (NCPA) and phylogeographic statistical analyses

A haplotype network was reconstructed in ANECA (Panchal 2007; Panchal and Beaumont 2007). This software automates the nesting procedure and subsequent geographical analysis implementing the programs TCS (Clement et al. 2000) and GEODIS (Posada et al. 2000). Before the automated nesting algorithm was run for each of the reconstructed and clearly separated subnetworks (Figs 2 and 3), network ambiguities were solved by hand following Templeton et al. (1993, 1995). For TCS, default settings were chosen with a 95% confidence interval, 5th character state = gap. In GEODIS, 1 000 000 million iterations were chosen. We are aware that the NCPA is critically discussed as a method that is biased by sampling errors and its assumptions testing against the null hypothesis (Panchal and Beaumont 2007; Petit 2008; Beaumont et al. 2010). Some arguments against NCPA are obviously based on outdated versions (Templeton 2009b). However, possible alternative methods like Bayesian statistics are criticized for their complex model assumptions (Templeton 2009a,b). Yet, the automatized NCPA using ANECA takes possible sampling errors into account by the inclusion of localities that are known but not sampled for the analysis (see Fig. 1) and that reduces the bias, while applying the inference key. A major reason to apply the NCPA is that to date it represents the best method to illustrate the network and nested clade structure (TCS), which is finally interpreted within a statistical framework. We did not design this study to compare different methods, but we performed complementary analyses to compensate eventual shortcomings and to account for the critics. We applied geometric morphometry relying on independent characters, complementary to the analyses based on the molecular data. Additionally, to the NCPA amova and *F*-statistics were computed for the molecular data to test and interpret independently the results from the NCPA (Table 1). The sequences were collapsed to haplotypes (Supporting information Table S3) in the software DNASP (Rozas et al. 2003). amova and *F*-statistics (F_{SC}, F_{ST}, F_{CT}) were calculated with Arlequin 3.5 (Excoffier et al. 2005) to infer the haplotype structure, among group variation and separation between and within clades inferred by the NCPA. Standard settings with 10 000 generations and a significance level of 0.05 for the discussed clades were chosen.

Corresponding additional phylogenetic analysis

A neighbour-joining network (default) of the sequence alignment was constructed in Splitstree 4.10 (Huson 1998; Huson and Bryant 2006) to compare the resulting network with the haplotype network and results from the NCPA. A classical tree was reconstructed with the MPI version of MrBayes 3.0 (Ronquist and Huelsenbeck 2003) using uniform priors for model and tree parameters. A suitable substitution model was chosen applying a Bayes factor test (BFT), (Kass and Raftery 1995; Nylander et al. 2004; Posada and Buckley 2004). Nine different models were compared, the HKY, JC and REV, each with the implementation of rate heterogeneity and without. Model testing revealed that only JC and HKY showed parameter convergence, and the BFT strongly favoured the HKY + G model with a calculated value of 2 ln(B10) = 355.24. The final topology derived from 20 000 000 generations and a sample frequency of 1000. A burnin of three million iterations was discarded.

Geometric morphometry analyses (GM)

Landmark-based geometric morphometry (Bookstein 1997; Zelditch et al. 2004; Klingenberg 2009) was applied to analyse morphometric changes in wing-shape and wing-spot pattern, e.g. (Prieto et al. 2009) between *Zygaena angelicae*, *Z. angelicae elegans*, *Z. transalpina hippocrepidis* and *Z. transalpina transalpina*. A standardized picture of the dorsal view of each first left wing was taken, including a size standard (1 cm) using a digital camera (35 mm lens; Panasonic DMC-FZ5EG, Panasonic, Hamburg, Germany). Pictures were taken with a standardized distance between wing and camera lens of 31 cm. Morphological changes in wing shape, venation and position, and size of the spots between each individual of the sampled localities were analysed. Landmarks were set using the TPS software package (TpsDig1, TpsUtil), resulting in a dataset of 15 homologous landmarks (see Supporting information Figures S1, S2 and S3). Subsequently, PVA and CVA analyses of the morphometric data were conducted using the IMP software package (Sheets 2002). Effects of size and position were removed using the 'General Procrustes Analysis' procedure (Zelditch et al. 2004) implemented in the IMP software package. Principal component analyses, analysing variation within the groups, followed by Canonical variates analyses (CVA), describing the relative positioning of the groups to each other, were conducted based on the Procrustes residuals. Assignment tests, included in the CVAGen6o program, were performed to test for the stability of groups on an individual level. The degree of differentiation between the tested groups was exposed by determining the Mahalanobis distance of each specimen from the mean value of the CVA scores for each group, and assigning each specimen to its closest group. To estimate the robustness of the CV axes and assignments of the groups, jackknife assignment tests with 10%, 30%, 50% and 60% of random specimen exclusion were conducted.

Results

Phylogeography and general structure of the species complex

The NCPA grouped the 472 sequences (outgroups were omitted) into three unconnected subnetworks, with 95% confidence limit (see Figs 2 and 3). To estimate the number of steps between the subnetworks, a connection limit of 40 steps was necessary to generate a single network (not shown) that was composed of 168 haplotypes. The three subnetworks match with the '*transalpina*', '*hippocrepidis*' and '*angelicae*' branches suggested by Nauman et al. (1999) for this species complex. The '*transalpina*' subnetwork (Fig. 2) is clearly separated from the '*hippocrepidis*' subnetwork by 21 unobserved haplotypes and the '*hippocrepidis*' network (Fig. 3) from the '*angelicae*' subnetwork by 31 unobserved haplotypes. The '*angelicae*' subnetwork (Fig. 3) shows a larger distance and separation compared to the remaining two subnetworks, an outcome that differs from the assumption by Nauman et al. (1999) assuming a closer relation between the '*hippocrepidis*' and '*angelicae*' branch. This separation of these three haplotype networks is also corroborated by the results of the amova (among group variation = 82.51) and a F_{ST} value of 0.937 (Table 1, amova 1) and the independent morphometric data, which supports this result with high reassignment rates to respective groups (Table 2a, see Supporting information Figures S1, S2, S3 and S4). The additional neighbour network

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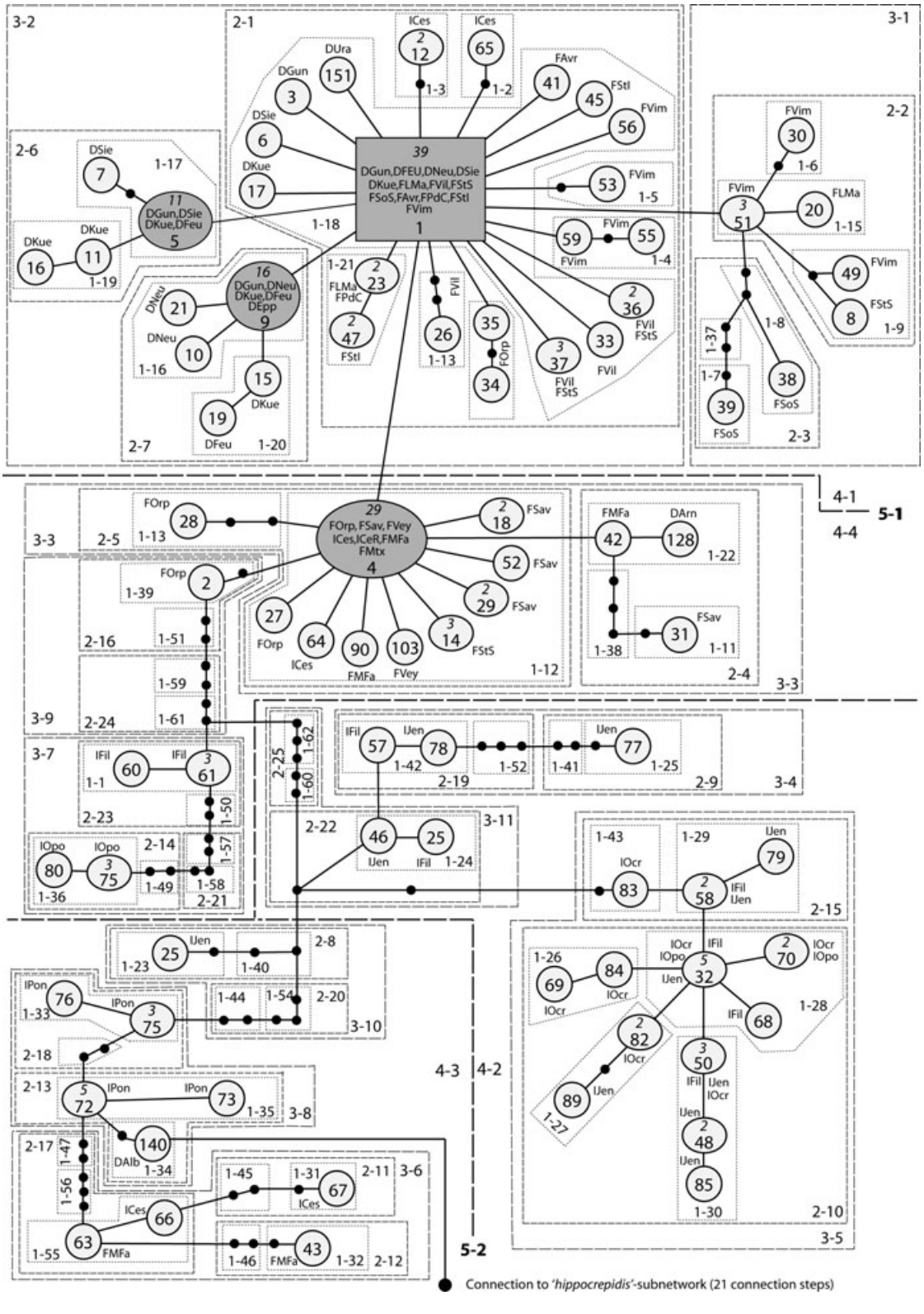

Fig. 2. '*Transalpina*' subnetwork reconstructed with TCS 2.1 implemented in ANECA. The larger circles with centred numbers represent the haplotypes. The size of the haplotype ovals/rectangles corresponds to the haplotype frequency. Numbers written in italics (positioned above) give the haplotype frequency (number of individuals that share the same haplotype), if it is >1. For each haplotype, the locality abbreviations are shown for an easier understanding of the network structure (further information on haplotypes is given in Supporting information Table S3). The four largest frequencies are shaded in grey. Each connection between the haplotypes represents one mutational step. The small black dots illustrate unsampled haplotypes. The squares represent the assumed 'root' of the network estimated in TCS. Level 1 clades have dotted borders and level two clades dashed lines. Clades of level 3, 4 and 5 are marked by increasing lengths of the border dashes. The clade numbers are given within the clades

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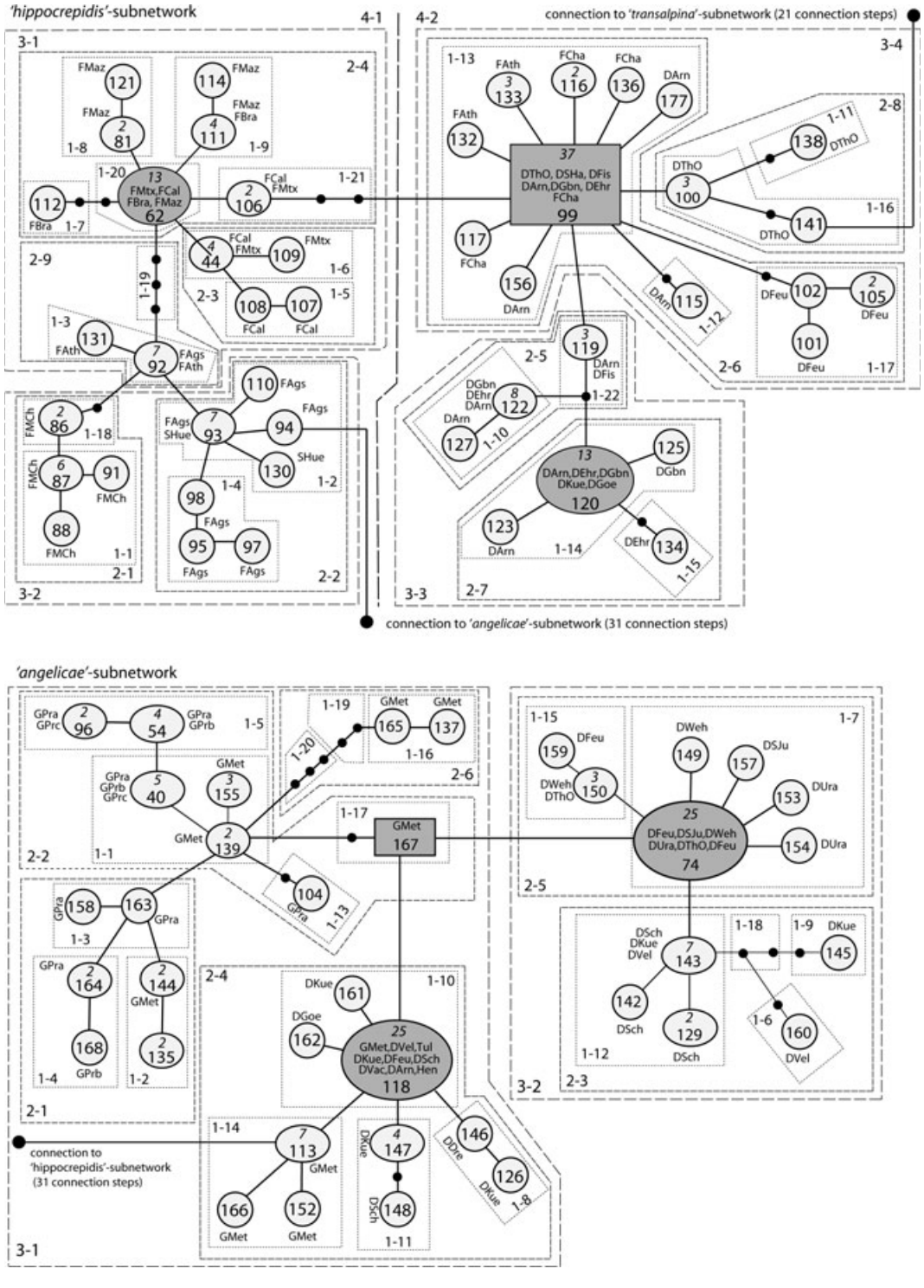

Fig. 3. '*Hippocrepidis*' subnetwork and '*angelicae*' subnetwork. Description see Fig. 2

of the 476 sequences reconstructed in SPLITSTREE 4.10 (Huson 1998) and the Bayesian consensus tree also depict the same, clearly separated clades (Fig. 4).

Phylogeographic patterns of the three subnetworks

Haplotypes of the '*transalpina*' subnetwork (Supporting information Figure S1) comprise localities from Italy, eastern and southern France, and Southern, Western and Central Germany. A 'Western' group comprises the most south-western population in France (FVim), the populations in the western Alpine ridge in France, localities in Western and Central Germany and the Italian population ICes within the Alps (clade 1-18 and 2-1). The highest clade level (3-2) for this group extends from the western Alpine ridge to localities in Central Germany including all localities in the river Rhine valley. NCPA suggests contiguous range expansion for this group (Fig. 3). Moreover, NCPA suggests allopatric fragmentation of French populations expanding from localities within the Alps to Western France (clade 3-1, FVim, FMLa, FStS

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Table 1. AMOVA results calculated in Arlequin 3.5. For each run, 10 000 numbers of permutation were performed with standard settings. F_{SC} = variation within groups, F_{ST} = variation between groups, F_{CT} = variation among populations but within groups

AMOVA	Source of variation	Percentage of variation	F_{SC}	F_{ST}	F_{CT}
1	3 subnetworks : 'transalpina' versus 'hippocrepidis' versus 'angelicae'		0.638 p = 0.000	0.937 p = 0.000	0.825 p = 0.000
	Among groups	82.51			
	Among populations within groups	11.16			
	Within populations	6.33			
2	'*Transalpina*' subnetwork: populations Central Italy versus All 'transalpina'		0.446 p = 0.000	0.856 p = 0.000	0.740 p = 0.000
	Among groups	74.05			
	Among populations within groups	11.58			
	Within populations	14.37			
3	'*Transalpina*' subnetwork: populations Central Italy versus Italian 'transalpina'		0.673 p = 0.000	0.783 p = 0.000	0.335 p = 0.154
	Among groups	33.54			
	Among populations within groups	44.73			
	Within populations	21.72			
4a	'*Transalpina*' subnetwork: populations clade 3-2 versus 3-3, 4-4 (without ICes)		0.516 p = 0.000	0.643 p = 0.000	0.265 p = 0.001
	Among groups	26.53			
	Among populations within groups	37.8			
	Within populations	35.67			
4b	ICes in clade 3-2		0.477 p = 0.000	0.601 p = 0.000	0.237 p = 0.001
	Among groups	23.73			
	Among populations within groups	36.36			
	Within populations	39.91			
4c	ICes in clade 3-3,4-4		0.474 p = 0.000	0.566 p = 0.000	0.232 p = 0.000
	Among groups	23.16			
	Among populations within groups	36.41			
	Within populations	40.43			
5	'*Transalpina*' subnetwork: clade 3-1 (Fvim, FMLa, FStS, FSoS)		–	0.140 p = 0.001	–
	Among populations within groups	14.04			
	Within populations	85.96			
6	'*Transalpina*' subnetwork: population IPon versus Italian and French populations		0.673 p = 0.000	0.783 p = 0.000	0.335 p = 0.154
	Among groups	33.54			
	Among populations within groups	44.73			
	Within populations	21.72			
7	'*Transalpina*' subnetwork: population IPon versus all 'transalpina' populations		0.673 p = 0.000	0.822 p = 0.000	0.455 p = 0.138
	Among groups	45.49			
	Among populations within groups	36.67			
	Within populations	17.84			
8	'*Hippocrepidis*' subnetwork: 3 major clades: 2-4; 3-2; 2-6,3-4,4-2		0.36797 p = 0.000	0.73979 p = 0.000	0.58830
	Among groups	58.83			
	Among populations within groups	15.15			
	Within populations	26.02			
9	'*Hippocrepidis*' subnetwork: Southern provence versus All 'hippocrepidis'		0.598 p = 0.006	0.728 p = 0.000	0.323 p = 0.000
	Among groups	32.26			
	Among populations within groups	40.53			
	Within populations	27.21			
10	'*Angelicae*' subnetwork: DSJu,Dura, DThO, DWeh versus remaining German populations		0.24116 p = 0.074	0.391 p = 0.021	0.19730 p = 0.015
	Among groups	19.73			
	Among populations within groups	19.36			
	Within populations	60.91			
11	'*Angelicae*' subnetwork: populations of Greek versus Central Germany versus Southern Germany		0.21860 p = 0.000	0.424153 p = 0.000	0.25970 p = 0.000
	Among groups	25.97			
	Among populations within groups	16.18			
	Within populations	57.85			

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Table 1. (Continued)

AMOVA	Source of variation	Percentage of variation	F_{SC}	F_{ST}	F_{CT}
12	'*Angelicae*' subnetwork: populations of Greek versus Central Germany versus Southern Germany		0.18785 p = 0.051	0.45675 p = 0.000	0.33126 p = 0.000
	Among groups	33.13	Remark: GMet included to populations in Central Germany		
	Among populations within groups				
	Within populations	12.55			
		54.32			
13	'*Angelicae*' subnetwork: populations of Gmet versus Gpra,GPrb,GPrc			0.3 p = 0.000	
	Among groups	29.2			
	Within populations	70.8			

and FSoS, see Fig. 3). The AMOVA of four populations shows low F_{ST} values between populations (Table 1, AMOVA 5), high percentages of variation within populations (85.96%) and low variation among populations (14.04%). Morphometric comparison of '*transalpina*' populations showed significant separation on the first three CV axes (CV axis 1-3, Lambda 1 = 0.1501, p > 0.001, Lambda 2 = 0.4185, p < 0.001, Lambda 3 = 0.6781, p = 0.048) with reassignment rates between 37% and 80% (Table 2). Populations of eastern and western Italy are separated on the second CV axis but show clear overlap. Populations of the 'Rheinland' (Germany) show the strongest separation of populations compared with localities in Central Italy (CV1). These separations were, however, not strongly supported in jackknife replicates with correct

Table 2. CVA assignment test for the different zygaenid groups. CVA assignments of the *Zygaena* species complex (a) and different population within species (b–d). Total values and the percentage of assignment are given in brackets

a) *Zygaena* species

	CVA assignments			Jackknife assignments				
	Z. transalpina	*Z. hippocrepidis*	*Z. angelicae*	% correct	% correct ns	% false	% false ns	% left out
Z. transalpina	150 (81.1)	27 (14.6)	8 (4.3)	74.1	0.3	25.6	0	10
Z. hippocrepidis	37 (24.3)	110 (72.4)	5 (3.3)	73.2	0.3	26.5	0	30
Z. angelicae	5 (5.7)	7 (8.0)	76 (86.4)	72.0	0.4	27.5	0	50
				70.7	0.5	28.7	0.1	60

b) *Z. transalpina*

	CVA assignment					Jackknife assignments				
	1/NWI	2/NCI	3/NEI	4/CI	5/R	% correct	% correct ns	% false	% false ns	% left out
1/NWI	14 (73.7)	1 (5.3)	1 (5.3)	1 (5.3)	2 (10.5)	53.9	0	46.1	0	10
2/NCI	2 (4.9)	29 (70.7)	5 (12.2)	1 (2.4)	4 (9.8)	51.1	0.2	48.7	0	30
3/NEI	3 (10.0)	7 (23.3)	11 (36.7)	5 (16.7)	4 (13.3)	45.3	0.5	54	0.2	50
4/CI	0	2 (4.3)	6 (13.0)	37 (80.4)	1 (2.2)	41	1.1	57.3	0.6	60
5/R	3 (6.1)	4 (8.2)	5 (10.2)	1 (2.0)	36 (73.5)					

c) *Z. hippocrepidis*

	CVA assignment			Jackknife assignments				
	1/S	2/WF	3/EF-SA	% correct	% correct ns	% false	% false ns	% left out
1/S	50 (100)	0	0	91.9	0.5	7.6	0	10
2/WF	3 (7.5)	37 (92.5)	0	90.6	1.3	8.1	0	30
3/EF-SA	1 (1.6)	0	61 (98.4)	87.1	2.9	9.7	0.3	50
				81.0	6.7	11.2	1.1	60

d) *Z. angelicae*

	CVA assignment			Jackknife assignments				
	Rom-Bul	Rus	SwA	% correct	% correct ns	% false	% false ns	% left out
Rom-Bul	14 (70)	5 (25)	1 (5)	62.8	2.2	34.8	0.1	10
Rus	5 (17)	25 (83)	0	59.8	3.1	36.4	0.7	30
SwA	2 (5)	0	36 (95)	49.3	8.3	39.1	3.3	50
				32.7	18.6	35.6	13	60

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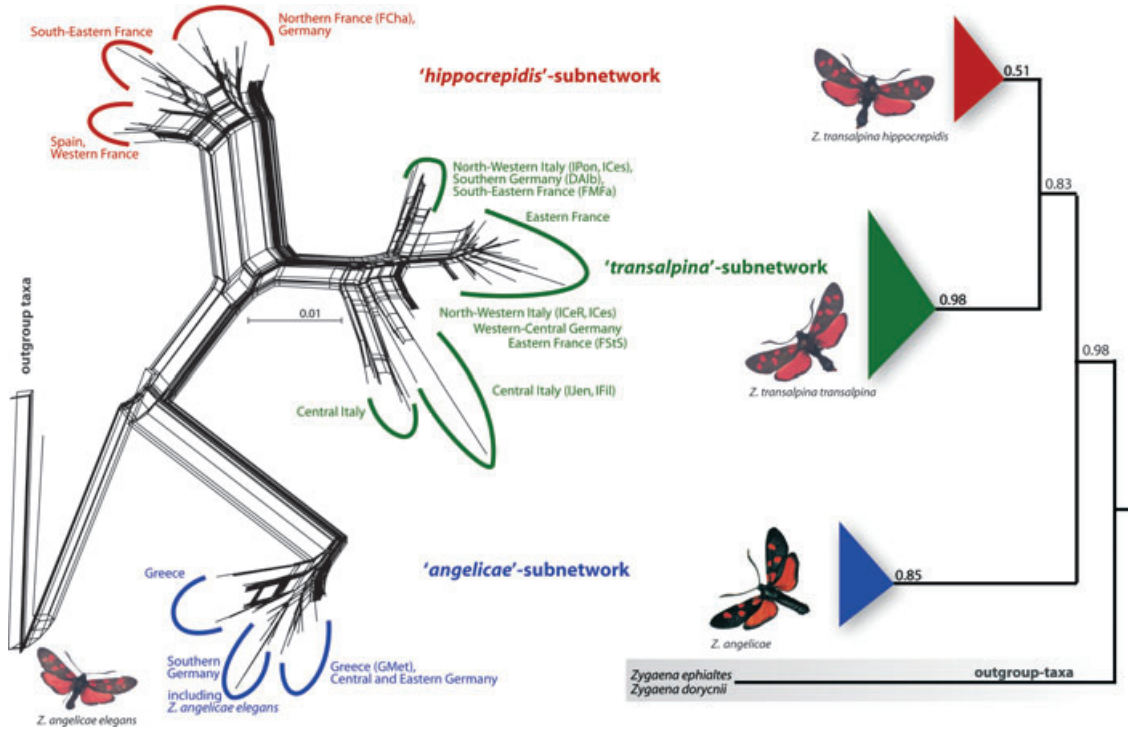

Fig. 4. Neighbour-joining net and Bayesian consensus tree of the dataset. On the left, the network reconstructed in Splitstree 4.10 (default settings) is shown including *Zygaena dorycnii* and *Zygaena ephialtes* as outgroups. The areas of the sample localities from included species are given for the subnetworks. On the right the consensus tree computed in MRBAYES 3.1 is pictured, the given node values represent posterior probabilities. The colour code is blue for the '*Z. angelicae*' branch, green for the '*Z. transalpina*' branch and red for the '*Zygaena angelicae*' branch

assignments around 50% when only 10% of data are excluded (Table 2b).

Haplotypes of the '*hippocrepidis*' subnetwork are found in north-western Italy, eastern and southern France and also Southern and Central Germany. Three major clades were present within this group (Fig. 4) corroborated by an AMOVA (Table 1, AMOVA 8). In all three subgroups, NCPA suggests restricted gene flow with isolation by distance: (1) Clades 2-6, 3-4 and 4-2 show restricted gene flow with isolation by distance for the two north-western populations in France (FAth, FCha) and the German populations. Within these clades, a substructure (clade 2-5) is present for a distinct group of the Central German populations (DFis, DArn, DEhr, DGbn) with restricted gene flow with isolation by distance, (2) The second clade (3-2) comprises the Spanish (SHue) and western and eastern populations in France (FAgs, FMCh) (3) in the area of the Provence in southern France restricted gene flow with isolation by distance is inferred for a separated clade (clade 2-4, FBra, FCal, FMaz, FMtx). The results of the morphometric analysis corroborate those from the NCPA (see Fig. 6). All '*hippocrepidis*' populations are clearly separated along the first two CV axes (Lambda 1 = 0.0421, $p < 0.001$, Lambda 2 = 0.4018, $p < 0.001$). Populations from Spain, Western and south-eastern France are clearly separated, forming distinct clusters on the first CV axis. On the second CV axis, French is separated against Spanish populations (see Supporting information Fig. S2). Reassignment rates of '*hippocrepidis*' populations were high with values between 93% and 100%. These results remain robust under various jackknife regimes (Table 2c).

Haplotypes of the '*angelicae*' subnetwork are found in Greece, Central and Southern Germany ('Schwäbische Alb'). Sampling within this haplotype group is not sufficient to infer phylogeographic processes. We, therefore, refrain from a detailed presentation of the results. The morphometric analysis includes more localities that separate the German populations from the Russian and Rumanian along the first CV axis (lamda = 0.1188, $p > 0.001$). On the second CV axis, a separation between Russia and Romania is present (Supporting information Figure S6). CVA reassignment rates were between 74% and 95% (Table 2d). However, reassignment rates start to decrease below 60% in jackknife trials when more than 30% of data were excluded. Regarding the endemic *Zygaena angelicae elegans* form found in the 'Schwäbische Alb', our sampling was very dense and the AMOVA results (F_{ST} = 0.391, p = 0.021, Table 1: AMOVA 10) indicate that the Southern German populations of the 'Schwäbische Alb' (DWeh, DThO, DSJu, DUra) are separated from the remaining German *Z. angelicae* populations.

Discussion

The general phylogeographic pattern of the *Zygaena transalpina* complex

Recently published studies draw a detailed picture of the role of the Pleistocene and its impact on speciation of insects and lepidopterans in the Palearctic (Habel et al. 2005; Vila et al. 2005; Wahlberg and Saccheri 2007; Gratton et al. 2008). Habel et al. (2005) describe a 'fourth European paradigm of post-glacial range expansion' for lepidopterans. This hypothesis postulates a recolonization route around the western Alps, which is frequently used by lepidopteran species (Satyrinae). A second hypothesis is the 'hedgehog' paradigm

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sensu Hewitt (Hewitt 2000), which postulates three classical refugia on the Iberian Peninsula, in Italy and the Balkans. Specific for this model is a migration route right through the Alpine ridge.

Addressing the first goal of our analyses, if (1) the glacial refugia pattern of the *Zygaena transalpina* complex matches one of the classical described recolonization paradigms, we draw following general conclusions: Parts of the described patterns of postglacial recolonization for both, previously named paradigms are indeed realized within the studied species. We also identified two classical Mediterranean refugia, namely the Appenin ('*transalpina*' subnetwork) and presumably the Balkan region ('*angelicae*' subnetwork), which is roughly in line with previous studies (Sternberg 1998; Hewitt 2000, 2001; Habel et al. 2005; Schmitt 2007). However, a shortcoming in our data is that we have only indications (based mainly on the morphometric analyses) for the Balkan refugium, because of a scattered sampling, respectively, for molecular data.

Yet, some aspects of our inferences are new for winged insects and differ from previous studies. Differentiating the subnetwork structure, we find strong indications for a microrefugium of populations from the '*transalpina*' subnetwork within the Southern Alps. Furthermore, the '*hippocrepidis*' subnetwork displays a novel pattern with areas in Western France and south-eastern France as possible glacial refugia (Fig. 7) additional to the generally assumed 'Iberian' refugium for zygaenid moths (Alberti 1958; Hofmann 1994; Nauman et al. 1999).

Complex phylogeographic patterns of the subnetworks

Micro-refugia and Alpine penetration of the 'transalpina' haplotypes

Our results indicate a spreading towards the Alps from Central Italy for clade 3-3 and 4-4 (see Figs 3 and 5). Data support that some zygaenids split off from this common route in Italy and probably penetrated the eastern Alps towards Central Germany, while others migrated north-west towards/around the Alpine ridge. This spreading and subsequent Alpine Penetration (AP) is supported mainly by localities used for the morphometric analysis, but molecular data corroborate the following pattern: from the western Alps two major groups inferred by the NCPA (3-3/4-4 and 3-2) spread to Central Germany showing restricted gene flow with isolation by distance. Only the western group (clade 3-2) of the '*transalpina*' branch links the populations of Central Italy with those in the western Alps and Central Germany. The eastern group (clades 3-3/4-4) comprises localities in the Provence, the Italian, western Alps (ICer) and Central Germany (DArn), indicating a migration route through the Alps towards Germany. Interestingly, this scenario of AP was already assumed by Alberti (1958). A possible scenario and explanation is that the western-passage of the Alps was blocked by '*hippocrepidis*' haplotypes spreading from their glacial refugia in the southern Provence hence forcing other specimens of the '*transalpina*' group to Alpine Penetration (see Figs 6 and 7). However, our sampling in the central Alps might be insufficient to further

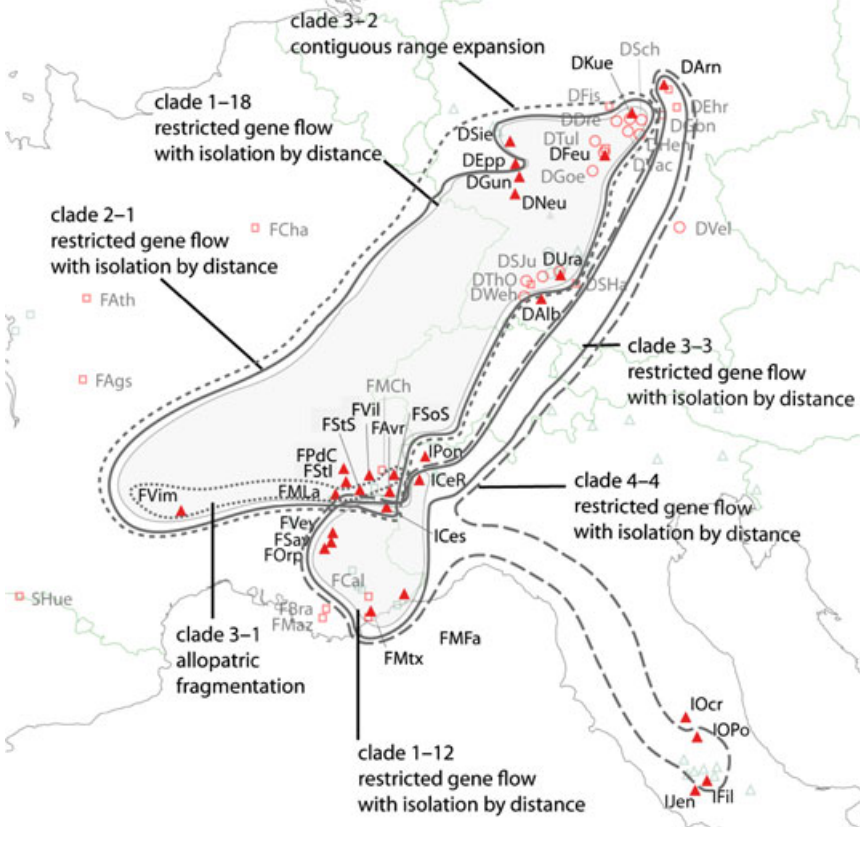

Fig. 5. Nested clade phylogeographic analysis results for the '*transalpina*' subnetwork. Clade levels and inference are given only for significant results. Specimens of *Zygaena transalpina* are represented by filled triangles. Localities of the '*angelicae*' (circles) and '*hippocrepidis*' branch (squares) are included, but shaded

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underpin this scenario with localities between the western Alps and Germany.

The pattern of the Western branch implies that the French populations invaded Southern and Central Germany from a glacial refugium in the Alps: the two groups (clades 3-3/4-4 and 3-2) overlap and come into contact namely in the area of the western and Italian Alps (ICes). This supports the hypothesis that this area might have been a sheltered glacial refugium of these haplotypes. Another good example for the occurrence of a sheltered micro-refugium is the closely located population in the western, Italian Alps (IPon), which shows a strong distinction to all other '*transalpina*' populations (see Table 1). Possibly, this population was trapped and endured oscillations of glacials and interstadials under relative stable climatic conditions. Sheltered differentiation despite harsh climatic oscillation is a plausible explanation for the observed picture (see Fig. 5). Interestingly, this locality was excluded from the NCPA showing no significant nesting result. The AMOVA and F_{ST} results demonstrate this clear distinction, which is also illustrated in the NCPA network (Fig. 2). This scenario of micro-refugia is supported only by few studies on plants (Tzedakis 2002; Schönswetter et al. 2005), snails (Haase et al. 2003; Pinceel et al. 2005; Benke et al. 2009; Haase and Misof 2009) and insects (Pauls et al. 2006; Previsić et al. 2009). A similar pattern is found for lepidopterans by Gratton et al. (2008) but indicating an eastern alpine refugia for *Parnassius mnemosyne*. Our results contribute to the hypothesis that micro-refugia played in general a more important role for postglacial expansion of insects and, respectively, lepidopterans as previously appreciated.

New refugium of and blocked passage to Central Europe for the 'hippocrepidis' subnetwork

The results of the NCPA and statistical testing imply that multiple refugia existed for the '*hippocrepidis*' group (Fig. 7). The molecular data support a refugial area in Western France and a subsequent spreading to Southern and Central Germany. Our molecular data are insufficient to indicate a classical Iberian refugial area (which might have existed) as the only sampled Spanish population is located in the Pyrenean Mountains (SHue, Fig. 1). The morphometric analysis (based on a more extensive sampling) supports a differentiation of populations in eastern France, Western France and in Spain. Spanish populations are not strongly separated from populations in France, which can be expected in case of a Spanish refugium. However, for a clearer identification, more data are needed. We conclude that our combined results corroborate a recolonization pattern from Western France through the Burgundian gate to Southern Germany and further to Central Germany. This recolonization route has been assumed for insects like Odonata (Sternberg 1998) and Lepidoptera (Schmitt et al. 2002).

A second potential refuguim in the Southern Provence in France (see Fig. 7) is underpinned by molecular and morphometric data and inhabits at present rather isolated zygaenid populations (Table 1) with variable haplotypes (Supporting information Tables S1 and S3). This result is congruent with a 'classical' supposed migration route of insects that originates from this area through the Burgundian gate to Central Europe. Schmitt et al. (2002) and Schmitt and Krauss (2004) reconstruct this migration route for the butterfly *Polyommatus coridon* using allozyme data. Our sampling in the Provence and

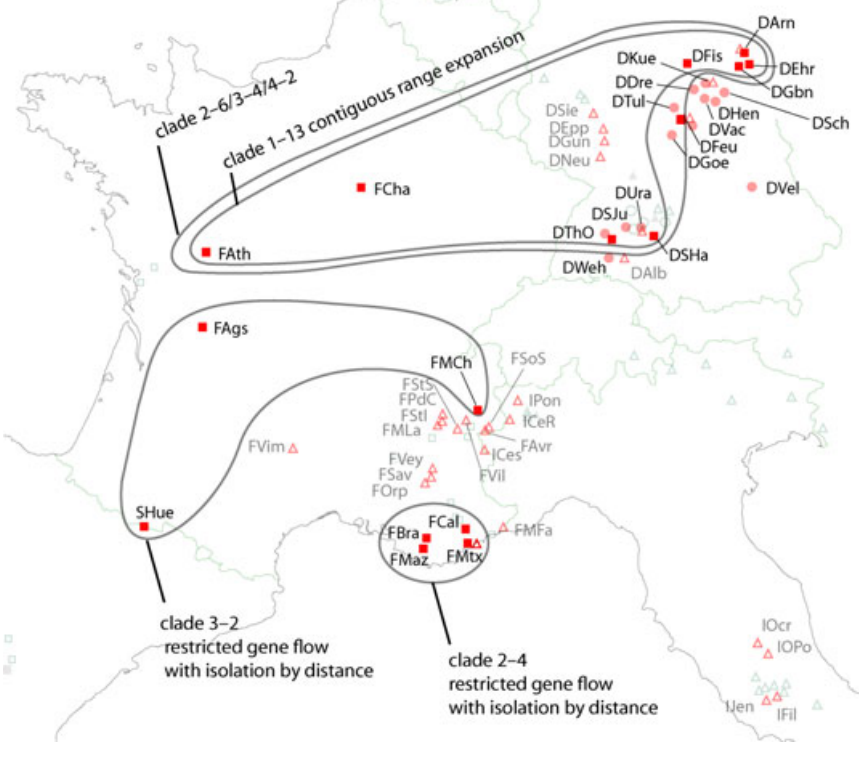

Fig. 6. Nested clade phylogeographic analysis results for the '*hippocrepidis*' subnetwork. Clade levels and inference are given only for significant results. Specimens of *Zygaena hippocrepidis* are represented by filled squares. Localities of the *angelicae* (circles) and *transalpina*-branch (triangles) are included, but shaded

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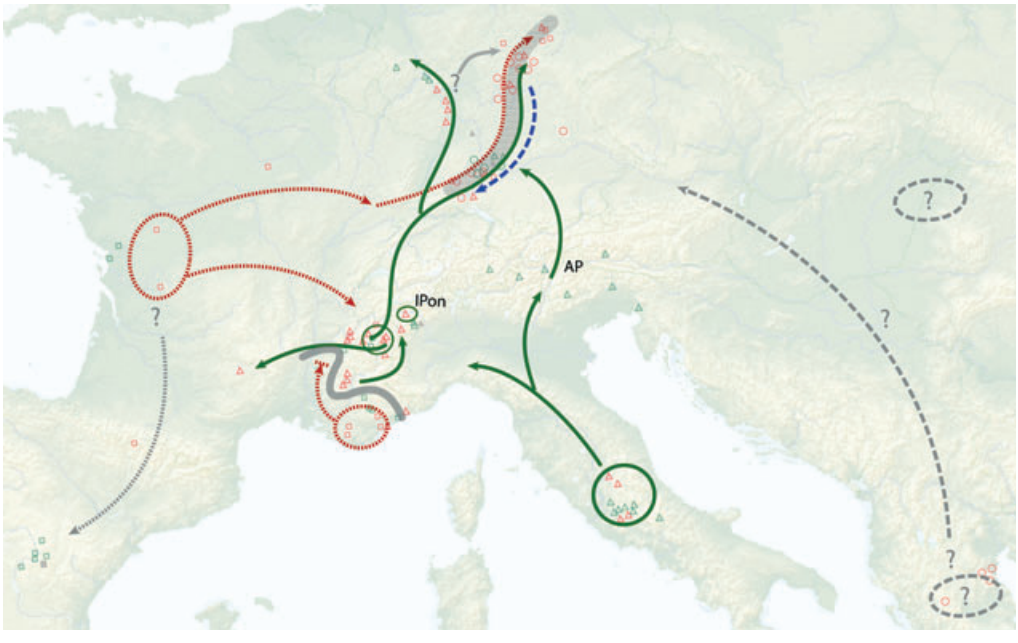

Fig. 7. Phylogeographic pattern based on molecular and morphometric data. The tree 'classical' branches of zygaenids are corroborated. The '*hippocrepidis*' branch (red dotted lines) spreads from Western Europe, and the '*transalpina*' branch (green bold lines) moves in two separated flanks from South Italy to Central Germany. Haplotypes from the '*angelicae*' branch migrated very likely from Eastern Germany to Southern Germany representing now the distinct *Zygaena angelicae elegans* form. Localities are encodes as in Fig. 1. Grey lines and question marks indicate routes or refugia assumed on scattered samples, this was, respectively, the case for the '*angelicae*' branch. The inferred glacial refugia are marked by circles. The grey, broad area in Central to Southern Germany displays the zone of secondary contact for all three branches. New patterns to existing hypotheses are (1) the multiple refugia for the '*hippocrepidis*' branch (Western and Southern France), (2) the distinct spreading of the '*transalpina*' branch, one eastern group expanded through the Alps (AP = alpine penetration) and (3) the inferred refugium within the western Alps for specimens of the 'transalpina' branch (IPon). The grey borderline in the south-western Alps depicts the possibly blocked passages for *Z. transalpina and Z. hippocrepidis*

south-eastern Rhone valley was dense, consequently such a migration pattern, if present, should have been detected for the zygaenids, which was not the case. We assume that possibly, the Rhone valley was already inhabited by zygaenids of the '*transalpina*' subnetwork and therefore blocked as an expansion area for haplotypes of the '*hippocrepidis*' subnetwork (Fig. 7).

Open questions remain regarding the '*angelicae*' *subnetwork*

Sample localities between Greece and Europe are rather scattered (except for Germany); consequently, a precise colonization route can not be inferred, and results of the NCPA and morphometric analyses should be interpreted with caution suggesting a Balkan refugium.

Network structure, Bayesian tree (Fig. 4) and statistical tests (Table 1) suggest that the endemic *Zygaena angelicae elegans* found in the 'Schwäbische Alb' is very likely a local form of the *Zygaena* '*angelicae*' branch contrary to Nauman et al. (1999) assuming a hybrid between *Z. hippocrepidis* and *Z. angelicae*. However, we are aware that this statement is only based on mitochondrial data from the maternal lineage.

Conclusions

In general, the data reveals a complex pattern of glacial refugia, a micro-habitat within the Alps and postglacial expansion routes for the *Zygaena transalpina* complex. Much like it has been revealed for the survival of plant species (Tzedakis 2002; Schönswetter et al. 2005) and few studies on invertebrates (Haase et al. 2003; Pinceel et al. 2005; Pauls et al. 2006; Gratton et al. 2008; Benke et al. 2009; Haase and Misof 2009; Previsić et al. 2009). It thus becomes apparent that the complex topography of the Southern Alps enabled the survival of populations throughout the Quaternary in micro-refugia. Our results support the hypothesis that micro-refugia in the Alps possibly played an essential and more important role for recolonization of Central Europe during the ice ages than previously assumed. Also the 'classical' refugia represent probably a rather rigid grid from which patterns inferred for zygaenids deviate. The consequence of these results should be that more data and model organisms are needed to develop more realistic generalized patterns of postglacial expansion and glacial refugia for insects.

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Zusammenfassung

Phylogeographie des Zygaena transalpina*–Artenkomplexes: molekulare und morphometrische Differenzierung weist auf glaziale Refugien in Südfrankreich, Westfrankeich und Mikrorefugien innerhalb der Alpen hin*

Einige der rekonstruierten Rekolonisierungsrouten für verschiedene Arten aus den glazialen Refugien nach Zentraleuropa werden als klassische Muster der Rekolonisierung während des Pleistozäns betrachtet. Neuere Studien zeigen jedoch, dass die tatsächliche Phylogeographie oder biogeographische Vergangenheit einiger Arten viel komplexer gewesen ist und nicht durch vereinfachte Muster wiedergegeben werden kann. Die Schmetterlinge des *Zygaena transalpina*–Artenkomplexes repräsentieren eng verwandte Arten, die oft als typische Vertreter einer durch das Pleistozän geprägten Gruppe genannt werden. Es werden zur Zeit drei Gruppen unterschieden, die aus den drei klassischen glazialen Refugien in Westeuropa, Italien und dem Balkanareal nach Europa einwanderten. Wir untersuchen in dieser Studie die Biogeographie dieses Artenkomplexes, indem morphometrische und molekulare Daten kombiniert werden. Die phylogenetische und phylogeographische Analyse von 476 Tieren aus 55 Fundorten in Europa zeigen, dass der *Zygaena transalpina*–Komplex aus drei verschiedenen Haplotypengruppen besteht, die grob mit den angenommenen Refugialräumen übereinstimmen. Eine Synthese mit den morphometrischen Daten von 425 Tieren aus 46 Standorten bestätigt dieses Ergebnis. Es wird jedoch auch unterstrichen, dass für diese Gruppe deutliche Abweichungen zu den bisher allgemeinen Mustern bestehen. Wichtige neue Aspekte, die auch für die Ableitung allgemeiner Muster eine Bedeutung haben, sind: Multiple Refugien des, '*hippocrepidis*' Zweigs in Westfrankreich und die offensichtliche Existenz von Mikrohabitaten für den zentralen, '*transalpina*'-Zweig in den Alpen.

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Supporting Information

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

Figure S1. Morphometric differentiation of the '*transalpina*'-group.

Figure S2. Morphometric differentiation of the '*hippocrepidis*'-group.

Figure S3. Morphometric differentiation of the '*angelicae*'-group.

Figure S4. Morphometric differentiation of the three subgroups.

Table S1. List of the sample localities, number of sampled individuals, haplotypes and the haplotype frequency for the molecular data.

Table S2. List of sampled localities and numbers of sampled individuals for the morphometric analyses.

Table S3. Detailed list of haplotypes, haplotype frequency, number of localities and individuals carrying the haplotype.

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