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Nuclear and mitochondrial markers reveal the existence of two parapatric scorpion species in the Alps: *Euscorpius germanus* (C. L. Koch, 1837) and *E. alpha* Caporiacco, 1950, stat. nov. (Euscorpiidae)

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Nuclear and mitochondrial markers reveal the existence of two parapatric scorpion species in the Alps: *Euscorpius germanus* (C. L. Koch, 1837) and *E. alpha* Caporiacco, 1950, stat. nov. (Euscorpiidae). - A molecular (mtDNA and allozyme) data set reveals a clear divergent phylogeny within the Alpine scorpion species *Euscorpius* (*Alpiscorpius*) *germanus* (C. L. Koch, 1837). Two distinct (ca. 7 % DNA sequence divergence), monophyletic clades exist which are geographically separated by the Adige (Etsch) River in northern Italy. At the allozyme level, these population groups are fixed for alternative alleles at eight out of 18 gene loci and correspond roughly to the morphological subspecies *E. g. germanus* and *E. g. alpha*. No evidence of introgressive hybridization between the two groups is shown by the allozyme data. The branching points of the two population groups are found at unusually high distances compared with the outgroup taxa *E. gamma* Caporiacco, 1950 and *E. flavicaudis* (De Geer, 1778). The subspecies *E. g. alpha* is therefore elevated to species level: *Euscorpius alpha* Caporiacco, 1950, stat. nov. A neotype for *E. germanus* (C. L. Koch, 1837) and lectotypes for *E. alpha* stat. nov., *E. germanus beta* syn. nov. of *E. alpha*, and *E. germanus croaticus* are designated. Phylogenetic and biogeographic implications are discussed.

Key-words: Scorpions - trichobothria - allozymes - 16S mtDNA - biogeography - parapatric species - Alps - phylogeny.

INTRODUCTION

Several species of the scorpion genus *Euscorpius* Thorell, 1876 (Euscorpiidae) are common in the circum-Mediterranean region and in southern Europe. Taxonomy of this genus is not well resolved; there are currently seven "good" species, and 40 (!) formally valid subspecies in *Euscorpius* (Fet & Sissom, 2000; Scherabon *et al.*,

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2000). Recently, we began to apply molecular data to solve complicated taxonomic problems in this genus (Gantenbein & Scholl, 1998; Gantenbein *et al.*, 1998, 1999a; Fet *et al.*, 1999; Scherabon *et al.*, in press). This paper reports new phylogenetic data, based on the analyses of mtDNA sequences and allozymes, from the Alpine populations, which have been traditionally placed in *Euscorpius germanus* (C. L. Koch, 1837).

E. germanus is a mountainous species, recorded from the western Balkans, Austria, northern Italy and southern Switzerland (Capra, 1939; Caporiacco, 1950; Valle *et al.*, 1971; Bonacina, 1980; Scherabon, 1987; Crucitti, 1993; Fet & Braunwalder, 1997; Gantenbein *et al.*, 1998). Scorpion taxa are traditionally classified using morphological characters such as variation in the trichobothrial numbers and patterns ('trichobothriotaxy') (Birula, 1900, 1917; Hadzi 1931; Vachon 1962, 1981). However, molecular markers have recently become a powerful tool for evaluating the taxonomic status of populations and subspecies/species. The combination of nuclear and mitochondrial markers has been efficiently applied to detect introgression between taxa (Barton & Hewitt, 1989; Harrison, 1990; Bernatchez *et al.*, 1995). The subspecies *Euscorpius germanus gamma* Caporiacco 1950 was recently elevated to species rank (Scherabon *et al.*, in press) after using allozyme and mtDNA data. Gantenbein *et al.* (1998) and Gantenbein & Scholl (1998) demonstrated that the Swiss *E. germanus* populations probably have originated from two different refuges during the glaciations, forming two genetically highly divergent population groups. In order to further clarify the taxonomic status of the two subspecies *E. g. germanus* and *E. g. alpha* and to confirm the hybrid zone between these taxa assumed by Bonacina (1980), we initiated a molecular survey applying previously established nuclear (allozymes) and mitochondrial (16S mtDNA sequences) gene markers.

METHODS AND MATERIALS

Specimens analysed

A map of Switzerland, northern Italy, Austria and Slovenia from where *E. germanus* samples were collected is given in Fig. 1. Two outgroup species were collected, *Euscorpius gamma* Caporiacco, 1950 (see Scherabon *et al.*, 2000) from Koschuta (Carinthia, Austria) and *Euscorpius flavicaudis* (DeGeer, 1778) from Lauris (Vaucluse, France). The animals were caught in a sampling area of about 100-300 m^2 and were brought alive to the laboratory where they were killed by deep-freezing and stored in -80°C prior to biochemical analyses. In general, only few animals were taken from one site because small sample sizes ($N < 10$) are already expected to result in relatively good estimates of gene frequences at allozyme loci. Previous studies reported a low genetic variability within *E. germanus* populations (Gantenbein *et al.*, 1998, 1999a). Sample sizes are given in appendix I. After biochemical analyses, the animals were transferred to 70 - 80% ethanol for morphological analysis.

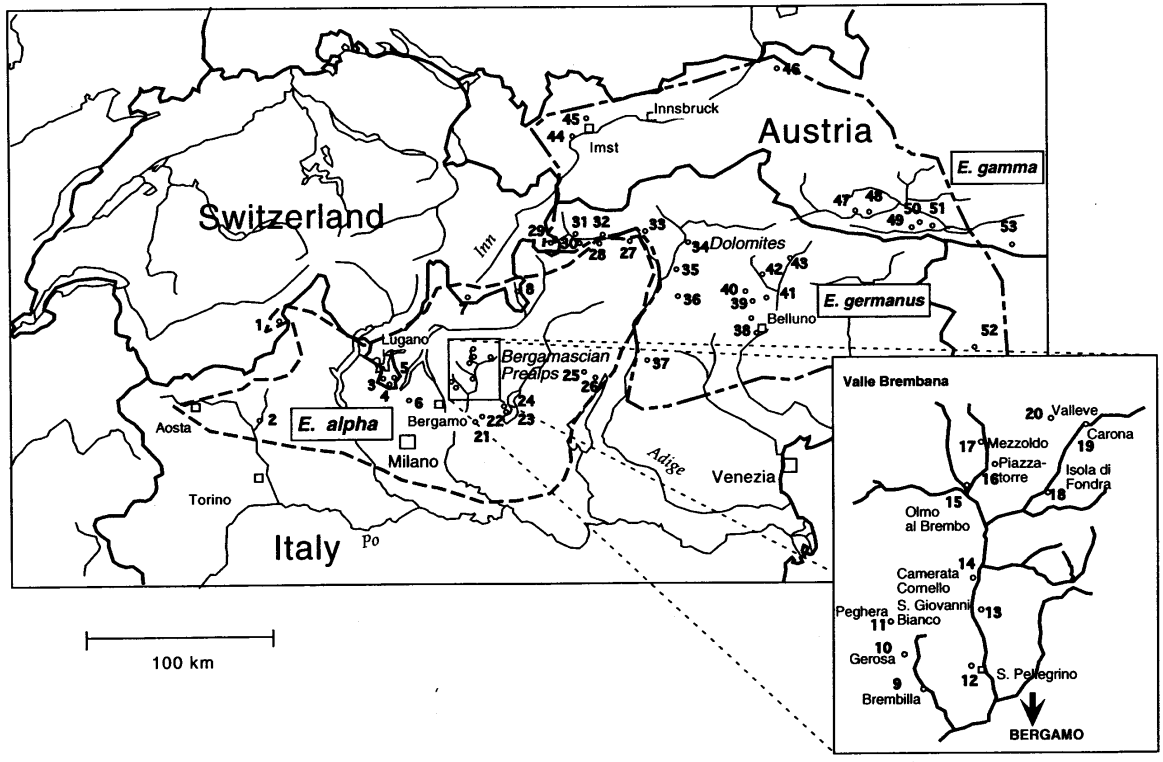

FIG. 1

Sampling sites. Western clade (*E. alpha*): samples 1-28; 1 Gondo, 2 Fontainemore, 3 Rancate, 4 Monte, 5 Fornace, 6 Pontide, 7 Sottoponte, 8 San Carlo, 9 Brembilla, 10 Gerosa, 11 Peghera, 12 San Pellegrino, 13 San Giovanni Bianco, 14 Camerata Cornello, 15 Olmo al Brembo, 16 Piazzatorre, 17 Mezzoldo, 18 Isola di Fondra, 19 Carona, 20 Valleve, 21 Selvino, 22 Nembro, 23 Tavernola, 24 Vigolo, 25 Bezzecca, 26 Molina di Ledro, 27 Marling, 28 Bad Salz. Eastern clade (*E. germanus*): samples 29-52; 29 Sta Maria, 30 Lichtenberg, 31 Schluderns, 32 Schlanderns, 33 Verdins, 34 Brixen, 35 Völs, 36 Bremer, 37 Vetriolo, 38 Belluno, 39 Voltago, 40 San Tomaso, 41 Mezzocanale, 42 Borca di Cadore, 43 Auronzo di Cadore, 44 Starkenbach, 45 Tarrenz, 46 Kranzach, 47 Dellach, 48 Oberdrauburg, 49 Dobratsch, 50 Schütt, 51 Federaun, 52 Crnice (near Nova Gorica). Outgroup *E. gamma*: 53 Koschuta.

DNA ANALYSIS

DNA extraction and sequencing techniques: A comparative analysis of the mitochondrial 16S ribosomal RNA and allozymes has been recently used for resolving species-level phylogeny of *Euscorpius* (Gantenbein *et al.*, 1999a); this contribution should be consulted for the technical details and protocols. Total DNA was extracted from fresh or preserved (95% ethanol) muscle tissue using a standard extraction method. An approximately 400 bp long fragment of the mitochondrial (mt) 16S rRNA gene was amplified by the polymerase chain reaction (PCR) using the primers 16Sbr (= LR-J-12887), (Simon *et al.* 1994; CGATTTGAACTCAGATCA; forward, 18-mer) and a scorpion-specific reverse primer (GTGCAAAGGTAGCA-TAATCA, 20-mer). A total of 25 mtDNA sequences was used for the analysis (Table 1). For further analysis, all ambiguities and indels were excluded, as suggested by Swofford *et al.* (1996), with 357 characters remaining.

Haplotype diversity: We calculated the haplotype (gene) diversity (Nei, 1987), the nucleotide diversity π (Nei & Li, 1979) and the number of segregating (polymorphic) sites (S) among sequences of *E. germanus*. Neutrality of mutations within each species was examined by using Tajima's D test (1989). The genetic variability estimates and the neutrality tests were calculated using the computer program DnaSP (Rozas & Rozas, 1999).

Phylogenetic analyses: We applied character-matrix-based methods (maximum parsimony (MP) and maximum likelihood (ML)) methods (Felsenstein, 1981a) as well as distance-based methods (neighbour-joining (NJ) cluster algorithm) (Saitou & Nei, 1987). The beta-version of the computer program PAUP* 4.0 (Swofford, 1998) was used for all phylogenetic DNA analyses. We calculated hierarchical likelihood ratio tests, in order to find the most appropriate model of DNA substitution, using the program MODELTEST 2.0 (Posada & Crandall, 1998). This program calculates the likelihood ratio statistic $\delta = -2 \log \Lambda$ where Λ is defined as

$$\Lambda = \frac{\max\,[L_0(NullModel\,|Data)]}{\max\,[L_1(AlternativeModel\,|Data)]}$$

with L_0 being the likelihood under the null hypothesis (simple model) and L_1 being the likelihood under the alternative hypothesis (more complex, parameter rich, model). When the models compared are nested (the null hypothesis is a special case of the alternative hypothesis) and the null hypothesis is correct, the δ statistic is asymptotically distributed as $\chi 2$ with q degrees of freedom (q is the difference in number of free parameters between the two models). In the next step, a test for the molecular clock hypothesis (i. e. rate constancy among lineages) was calculated ($\chi 2$ distributed, *df*= N - 2 OTUs). Details on model testing using maximum likelihood ratios are given in Huelsenbeck & Rannala (1997) and in Huelsenbeck & Crandall (1997). The likelihood ratio tests suggested the Tamura & Nei (1993) model with rate heterogeneity (TrN93 + Γ), which is a submodel of the general-time-reversible (GTR) substitution model (Rodríguez *et al.*, 1990, Yang *et al.*, 1994). All parameters (base frequencies, rate matrix) were estimated via maximum likelihood. The rate

heterogeneity among sites was assumed to follow a gamma distribution (shape parameter α was ML-estimated) with four categories, each represented by its mean (Yang, 1994). Phylogenetic analysis is facilitated when rates are equal among lineages. However, the test for the molecular clock was rejected at the 0.01 level, therefore, the tree search was carried out without enforcing the clock.

For ML analyses the tree space was explored using the heuristic search option implemented in PAUP* with random addition of sequences (100 replicates, tree bisection-reconnection (TBR) branch-swapping algorithm). For the MP analysis the transitions (ti) were weighted twice over transversions (tv) according to the ML estimated ti / tv ratio using the HKY85 (Hasegawa *et al.*, 1985) model, and the tree search was done using the branch-and-bound search option. To save computing time, identical haplotypes were eliminated. The consistency index (CI) and the retention index (RI) (Kitching *et al.*, 1998) were calculated as measures for tree stability with PAUP*.

Alternatively, pairwise ML-distances were estimated using the TrN93 + Γ model. Estimating distances via ML has the advantage of constant parameters over all pairwise comparisons and consequently the variance of distances is reduced. These distances were used as a matrix for neighbour-joining (NJ) clustering (Saitou & Nei, 1987). NJ is assumed to be a good heuristic approach for estimating the minimum evolution tree (Page & Holmes, 1998). The trees were rooted using two outgroup species: *E. gamma* and *E. flavicaudis*. The trees were bootstrapped resampling 1,000 data sets with 357 characters.

DNA SEQUENCE AVAILABILITY

All sequences were deposited in the EMBL Nucleotide Sequence Database with the following accession numbers: *E. alpha* stat. nov.: *Eal*GO (= *Eal*FO) = AJ389379; *Eal*RA = AJ271886; *Eal*SP = AJ286751; *Eal*SG = AJ286752; *Eal*CA = AJ286753; *Eal*OL = AJ286754; *Eal*SO = AJ286755; *Eal*TA = AJ286756; *Eal*MA (= *Ege*ML) = AJ286757; *E. germanus*: *Ege*VO (= *Ege*SH = *Ege*SM = *Ege*Tz = *Ege*KR = *Ege*VE) = AJ389380; *Ege*OB (= *Ege*DE = *Ege*ST) = AJ249553; *Ege*BO = AJ286758; *Ege*ME = AJ286759; *Ege*CR = AJ249552; *E. gamma*: *Ega*KO = AJ249554; *E. flavicaudis*: EflLA = AJ389381. Abbreviations for haplotypes are given in appendix I.

ALLOZYME ANALYSIS

Horizontal starch gel electrophoresis of allozymes was carried out according to the protocols described in Harris & Hopkinson (1976) and Murphy *et al.* (1996). We scored the same 18 loci as described in Gantenbein *et al.* (1998): N-(3-Aminopropyl)-morpholine-citrate (AC, pH 6.2, modified from Clayton & Tretiak 1972), Tris-citrate (TC, pH 7.3, Ayala *et al.* 1972) and Tris-borate-EDTA (TBE, pH 9.3, modified from Ayala *et al.*, 1972). The loci scored were: AAT-1 and AAT-2 (aspartate aminotransferase; EC 2.6.1.1), ALPDH (alanopine dehydrogenase; EC 1.5.1.17), ARK (arginine kinase; EC 2.7.3.3), DDH (dihydrolipoamide oxidase; EC 1.8.1.4), GAPDH (glyceraldehyde-3-phosphate dehydrogenase; EC 1.2.1.12), GTDH (glutamate dehy-

drogenase; EC 1.4.1.2), GPI (PGI) (glucose-6-phosphate isomerase; EC 5.3.1.9), HK (hexokinase; EC 2.7.1.1), IDH-1 and IDH-2 (isocitrate dehydrogenase; EC 1.1.1.42), MDH-1 and MDH-2 (malate dehydrogenase; EC 1.1.1.37), MPI (mannose-6-phophate isomerase; EC 5.3.1.8), PEP (pepdidase; EC 3.4.-.-), PGM (phosphoglucomutase; EC 5.4.2.2), 6-PGD (6-phosphogluconate dehydrogenase; EC 1.1.1.44), and PK (pyruvate kinase; EC 2.7.1.40). We refer to the observed electromorphs as alleles which are identified by their electrophoretic mobility relative to the most common mobility in the *E. flavicaudis* population from Lauris, France (assigned mobility=100) as described in Gantenbein *et al.* (1998). To assess the genetic variability within each population, the mean number of alleles per locus, the percentage of polymorphic loci and the mean heterozygosity were calculated by the direct count method and by Nei's (1978) unbiased estimate. Calculations were done using BIOSYS-1 (Swofford & Selander, 1989). Cavalli-Sforza & Edwards' (1967) chord distance was calculated from pairwise comparisons of populations using the program GENDIST from the PHYLIP 3.5 package (Felsenstein, 1995). Using Nei's pairwise distances as an input matrix, an additive tree was created by the neighbour-joining algorithm (NJ). Alternatively, an unrooted maximum likelihood tree was calculated using the computer program CONTML. This estimates phylogenies by the restricted maximum likelihood (REML) method, based on the Brownian motion model (Cavalli-Sforza & Edwards, 1967). The REML algorithm was described in Felsenstein (1973, 1981b). It uses less parameters than the full ML analysis and is therefore considered to be more consistent. Additionally, the program calculates branch lengths and rough confidence intervals for the branches. Bootstrap values were obtained from 1,000 pseudo-replicates of allele frequencies using the SEQBOOT routine in PHYLIP.

Morphological analysis

We scored the number of pectinal teeth (Dp) and the numbers of trichobothria on the ventral (Pv) and external (Pe) aspects of the palpal patella (called tibia by some authors; see Hjelle, 1990). ♂♂ have higher numbers of pectinal teeth. We tested the differences between two discovered clades using a one-sided *t*-test.

RESULTS

Molecular analyses

mtDNA data: We analysed 25 mtDNA sequences representing 17 different haplotypes. The heuristic tree search (100 replicates) using maximum likelihood (ML) revealed a single tree with a ln likelihood of –904.57 (Fig. 2A). The nucleotide frequencies within the 16S mtDNA were estimated via ML to A = 0.332, C = 0.13, G = 0.12, and T = 0.41, respectively. The substitution rate matrix (R) was estimated via ML to A <--> C = 1, A <--> G = 19.0, A <--> T = 1, C <--> G = 1, C <--> T = 3.82, and G <--> T = 1. The shape parameter α of the gamma distribution was estimated via ML to 0.085. The relatively low estimate of α indicates a high rate of heterogeneity among nucleotide sites. The tree topology of the ML tree was identical to that

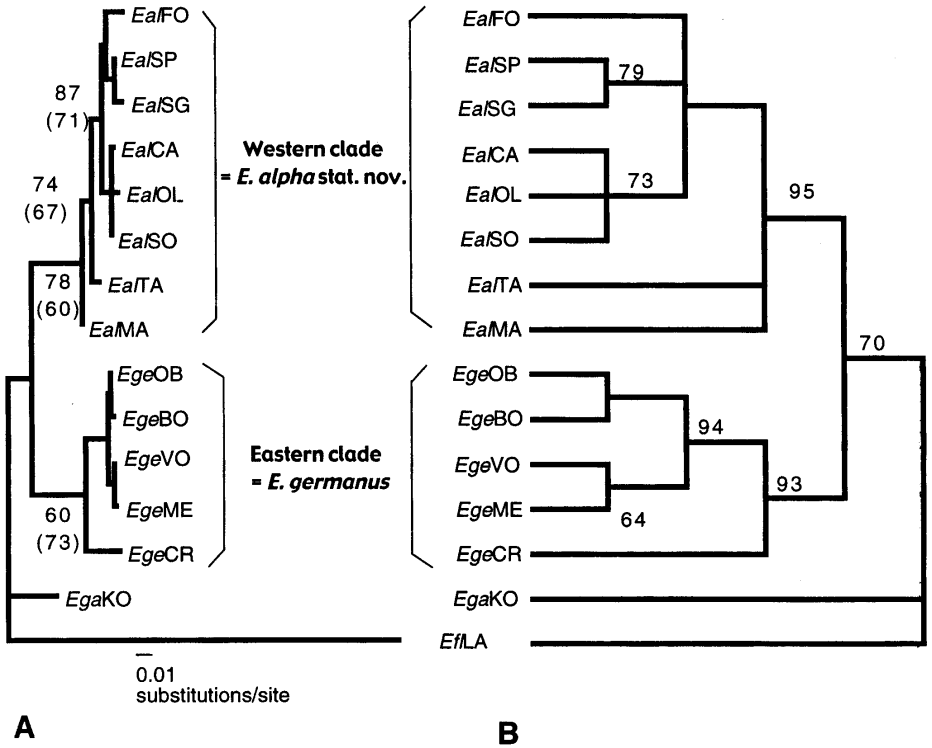

FIG. 2

(A) Maximum likelihood (ML) phylogeny based on the 16S mtDNA gene sequences in the "western" clade (*Euscorpius alpha* stat. nov., *Eal*) and in the "eastern" clade (*E. germanus*, *Ege*). The -ln Likelihood was 904.57 using the model by Tamura & Nei (1993) with rate heterogeneity (TrN93 + Γ). (B) Strict consensus tree of six equally parsimonious trees (91 steps, CI = 0.80, RI = 0.85) calculated by weighted maximum parsimony (MP). Numbers at nodes refer to bootstrap values calculated from 1,000 pseudoreplicates. Bootstrap values in parentheses (in A) are from neigbour-joining (NJ) analysis, which resulted in the same tree topology showing a deep split between both clades (species). Abbreviations for haplotypes are explained in appendix I.

of a NJ tree (tree not shown) which was built using TrN93 + Γ distances. The bootstrap values for the ML tree and the NJ analysis (in parentheses) are given in Fig. 2A. This phylogeny splits all analysed mtDNA sequences of E. germanus into two clearly distinct clades ("western" and "eastern"), both supported by relatively high bootstrap values.

The analysis using weighted maximum parsimony (MP) revealed six equally parsimonious trees with a tree score of 91 steps. 297 characters were constant, 27 characters were parsimony-informative. The consistency index (CI) for all eight trees was 0.80 and the retention index (RI) was 0.85, respectively. Both indices indicate a high tree stability. The strict consensus tree is shown in Fig. 2B. The deep splitting of

two population groups in the MP analysis is consistent with the ML tree and the NJ tree. However, the sequence from Crnice (EgeCR) showed an ambiguous grouping in ML and NJ analysis. Therefore, the bootstrap values for the two clades were moderate (about 70%) in ML and NJ analyses. This was not the case in MP analysis where both clades were supported by high values (about 90%).

The DNA polymorphism of the 16S data is listed in Table 1 and in Appendix II. The analysis of DNA variation revealed that 16 sites out of 357 characters were polymorphic (segregating) among the eleven "western clade" sequences whereas 14 sites out of 364 were polymorphic among the twelve "eastern clade" sequences. The probability that two randomly chosen haplotypes are different (= gene diversity) was 0.94 and 0.72, respectively (Table 1). Within the "western clade", the average nucleotide diversity π was 0.03 ± 0.00, whereas in the "eastern clade" it was close to zero. Tajimaís D test statistics were not significant for both species.

TABLE 1: mtDNA diversity measures within the "western" and "eastern" clades (*E. alpha* stat. nov. and *E. germanus*, respectively).

	Sample size n	Number of haplotypes	haplotype (gene) diversity	Polymorphic sites S	Total Number of sites*	Average number of nucleotide differences	Nucleotide diversity π	θ (= 2 Ne μ) per site	Tajima's D**
Western clade = *E. alpha* stat. nov.	11	8	0.95	16	357	5.78	0.0322	0.016	0.26
Eastern clade = *E. germanus*	12	5	0.72	8	364	3.12	0.009	0.008	-1.39

* excluding indels and ambiguities.
** P>0.10

Allozyme data: The NJ tree based on allozyme gene frequency data at 18 loci (appendix I) independently revealed a tree topology comparable to those obtained by 16S mtDNA sequence analysis. It splits all populations of *E. germanus* examined into two highly divergent groups, a "western" and an "eastern" one (Fig. 3). However, bootstrap values of these two groups were not as high as in DNA analysis. This is caused by the Crnice population, which in some cases exhibited ambiguous clade groupings. The ln Likelihood of the best tree (tree not shown) using the Restricted ML (REM) criterion by Felsenstein (1981b) was -3647.51970 (14,688 trees explored) and the topology was identical to that revealed by NJ analysis.

The genetic variation estimates within both clades are given for samples $N \geq 4$ in Table 2. The populations were fixed at many gene loci, therefore, the mean number of alleles was approximatively one. Low genetic variability estimates were found for the mean number of loci polymorphic (16%) and for the average heterozygosity (0.03 ± 0.02) (Table 2).

MORPHOLOGICAL ANALYSIS

We measured the number of pectinal teeths (Dp) in ♂♂ and ♀♀ from each of the two major clades recognized by molecular analyses (Figs. 2-3). ♀♀ of the "eastern clade" had significantly higher number ($t = 2.29$, $P = 0.011$) of pectinal teeth ($(Dp_{left} + Dp_{right}) / 2$) (mean = 6.05, $s^2 = 1.10$, $N = 83$) than the ♀♀ of the "western

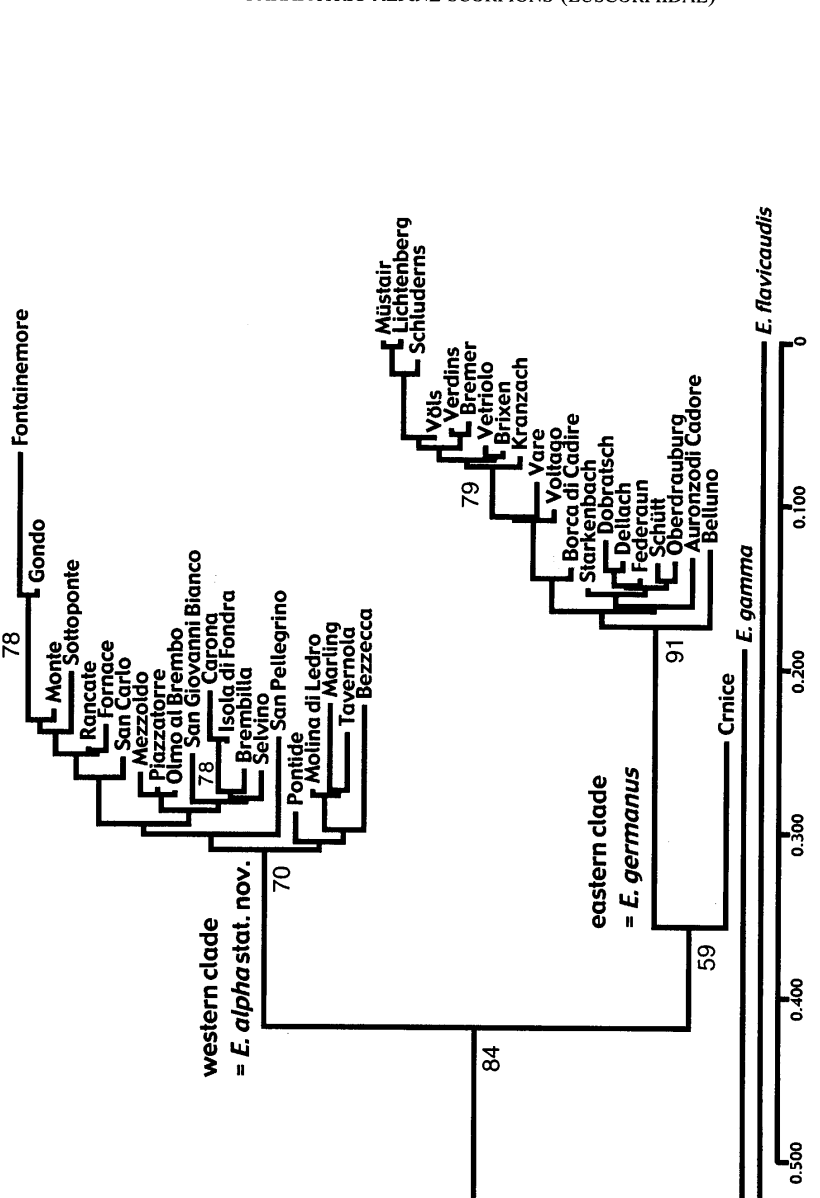

FIG. 3. Neighbour-joining (NJ) analysis of samples ($N \leq 4$) of the two clades (species) using Cavalli-Sforza chord distance (Cavalli-Sforza & Edwards, 1967) as an input matrix. Distances are based on 18 allozyme loci. Numbers at the nodes refer to bootstrap values calculated over 1,000 pseudoreplicates.

TABLE 2: Genetic variability estimates between the "western" and "eastern" clades (*E. alpha* stat. nov. and *E. germanus*, respectively) based on 18 allozyme loci (see appendix I) ($N \geq 4$).

Nr	Population	Mean no. of alleles per locus	Percentage of polymorphic loci *	Mean heterozygosity: Direct count	Mean heterozygosity: Hardy-Weinberg expected
	Western clade = *E. alpha* stat. nov.				
1	Gondo	1.2 ± 0.1	11.1	0.01 ± 0.00	0.03 ± 0.02
2	Fontainemore	1.0 ± 0.0	0	0.00 ± 0.00	0.00 ± 0.00
3	Rancate	1.2 ± 0.2	11.1	0.03 ± 0.03	0.06 ± 0.04
4	Monte	1.2 ± 0.1	11.1	0.03 ± 0.02	0.05 ± 0.03
5	Fornace	1.2 ± 0.1	16.7	0.03 ± 0.02	0.06 ± 0.04
6	Pontide	1.4 ± 0.2	33.3	0.09 ± 0.04	0.14 ± 0.06
7	Sottoponte	1.2 ± 0.1	16.7	0.04 ± 0.03	0.04 ± 0.03
8	San Carlo	1.2 ± 0.1	22.2	0.04 ± 0.02	0.07 ± 0.04
9	Brembilla	1.4 ± 0.2	27.8	0.10 ± 0.05	0.11 ± 0.06
12	San Pellegrino	1.6 ± 0.2	33.3	0.11 ± 0.05	0.19 ± 0.07
13	San Giovanni Bianco	1.6 ± 0.2	33.3	0.09 ± 0.04	0.11 ± 0.05
15	Olmo al Brembo	1.3 ± 0.2	22.2	0.04 ± 0.02	0.08 ± 0.04
16	Piazzatorre	1.4 ± 0.2	22.2	0.05 ± 0.03	0.09 ± 0.05
17	Mezzoldo	1.3 ± 0.2	16.7	0.06 ± 0.03	0.07 ± 0.04
18	Isola di Fondra	1.6 ± 0.2	38.9	0.09 ± 0.04	0.10 ± 0.04
19	Carona	1.2 ± 0.1	11.1	0.04 ± 0.03	0.04 ± 0.03
21	Selvino	1.3 ± 0.2	22.2	0.07 ± 0.04	0.09 ± 0.04
23	Tavernola	1.2 ± 0.1	16.7	0.03 ± 0.02	0.08 ± 0.05
25	Bezzecca	1.4 ± 0.2	27.8	0.08 ± 0.03	0.12 ± 0.05
26	Molina di Ledro	1.4 ± 0.2	22.2	0.05 ± 0.03	0.11 ± 0.06
27	Marling	1.4 ± 0.2	27.8	0.06 ± 0.03	0.11 ± 0.05
	Mean	1.3 ± 0.2	21.16	0.05 ± 0.03	0.08 ± 0.04
	Eastern clade = *E. germanus*				
29	Sta Maria	1.1 ± 0.1	5.6	0.01 ± 0.01	0.02 ± 0.02
30	Lichtenberg	1.1 ± 0.1	5.6	0.00 ± 0.00	0.03 ± 0.03
31	Schluderns	1.2 ± 0.1	11.1	0.02 ± 0.01	0.04 ± 0.03
33	Verdins	1.1 ± 0.1	5.6	0.00 ± 0.00	0.00 ± 0.00
34	Brixen	1.1 ± 0.1	5.6	0.01 ± 0.01	0.03 ± 0.03
35	Völs	1.2 ± 0.1	16.7	0.02 ± 0.01	0.06 ± 0.04
36	Bremer	1.1 ± 0.1	11.1	0.02 ± 0.02	0.04 ± 0.02
37	Vetriolo	1.1 ± 0.1	11.1	0.02 ± 0.01	0.02 ± 0.01
38	Belluno	1.1 ± 0.1	5.6	0.02 ± 0.02	0.03 ± 0.03
39	Voltago	1.2 ± 0.1	16.7	0.08 ± 0.04	0.09 ± 0.05
40	San Tomaso	1.1 ± 0.1	11.1	0.06 ± 0.04	0.05 ± 0.03
42	Borca di Cadore	1.3 ± 0.2	22.2	0.06 ± 0.03	0.10 ± 0.05
43	Auronzo di Cadore	1.2 ± 0.1	16.7	0.07 ± 0.04	0.05 ± 0.03
44	Starkenbach	1.1 ± 0.1	11.1	0.04 ± 0.03	0.05 ± 0.04
45	Tarrenz	1.0 ± 0.0	0	0.00 ± 0.00	0.00 ± 0.00
46	Kranzach	1.1 ± 0.1	5.6	0.00 ± 0.00	0.03 ± 0.03
47	Dellach	1.1 ± 0.1	11.1	0.01 ± 0.01	0.03 ± 0.03
48	Oberdrauburg	1.2 ± 0.2	11.1	0.01 ± 0.01	0.06 ± 0.05
49	Dobratsch	1.2 ± 0.1	16.7	0.06 ± 0.03	0.05 ± 0.03
50	Schütt	1.2 ± 0.1	11.1	0.04 ± 0.03	0.07 ± 0.05
51	Federaun	1.1 ± 0.1	11.1	0.03 ± 0.03	0.05 ± 0.03
52	Crnice	1.2 ± 0.1	16.7	0.04 ± 0.02	0.07 ± 0.04
	Mean	1.1 ± 0.1	10.87	0.03 ± 0.02	0.04 ± 0.03
	Overall Mean	1.2 ± 0.1	16.02	0.03 ± 0.02	0.06 ± 0.04

* A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.99

** Unbiased estimate (Nei, 1978)

TABLE 3: Distance matrix of the sequence divergence (uncorrected p) (upper right) and of TrN93 + Γ (lower left) calculated from pairwise comparisons of 16S mtDNA sequences.

	EalFO	EalSP	EalSC	EalCA	EalOL	EalSO	EalTA	EalMA	EgeOB	EgeBO	EgeVO	EgeME	EgeCR	EgaKO	EflLA
EalFO	-	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.06	0.06	0.06	0.06	0.07	0.06	0.10
EalSP	0.02	-	0.00	0.02	0.02	0.01	0.02	0.02	0.06	0.06	0.06	0.07	0.07	0.06	0.12
EalSC	0.03	0.00	-	0.02	0.02	0.02	0.02	0.02	0.07	0.06	0.07	0.07	0.07	0.06	0.12
EalCA	0.02	0.03	0.04	-	0.00	0.00	0.02	0.02	0.07	0.06	0.07	0.07	0.07	0.06	0.11
EalOL	0.03	0.02	0.03	0.00	-	0.00	0.02	0.02	0.07	0.07	0.07	0.07	0.07	0.06	0.12
EalSO	0.03	0.02	0.03	0.00	0.00	-	0.02	0.02	0.06	0.06	0.06	0.07	0.07	0.06	0.11
EalTA	0.05	0.05	0.05	0.05	0.03	0.03	-	0.01	0.06	0.06	0.06	0.06	0.06	0.05	0.11
EalMA	0.04	0.04	0.04	0.04	0.03	0.03	0.01	-	0.06	0.05	0.06	0.06	0.06	0.05	0.10
EgeOB	0.41	0.49	0.54	0.64	0.56	0.49	0.31	0.31	-	0.00	0.00	0.00	0.03	0.06	0.11
EgeBO	0.39	0.47	0.52	0.62	0.54	0.47	0.30	0.30	0.00	-	0.00	0.01	0.03	0.06	0.11
EgeVO	0.41	0.49	0.54	0.64	0.56	0.49	0.31	0.31	0.00	0.00	-	0.00	0.03	0.06	0.11
EgeME	0.51	0.62	0.69	0.81	0.70	0.61	0.39	0.39	0.00	0.01	0.00	-	0.04	0.06	0.11
EgeCR	0.38	0.46	0.50	0.36	0.40	0.45	0.19	0.18	0.06	0.07	0.07	0.08	-	0.06	0.12
EgaKO	0.25	0.20	0.22	0.25	0.19	0.20	0.10	0.09	0.43	0.36	0.43	0.54	0.22	-	0.10
EflLA	0.45	0.79	0.86	0.65	0.62	0.65	0.48	0.45	0.65	0.59	0.62	0.52	0.65	0.46	-

clade" (mean = 5.68, s^2 = 1.41, N = 111). This corresponded to a unimodal distribution of this character in the "eastern clade" (common Dp = 6) versus bimodal in the "western clade" (Dp = 5 or 6). ♂♂ of the "eastern clade" also had significantly higher number (t = 1.73, P = 0.045) of pectinal teeth (mean = 7.58, s^2 = 2.1, N = 24) than ♂♂ of the "western clade" (mean = 6.98, s^2 = 1.97, N= 60). This corresponded to a bimodal distribution of this character in the "eastern clade" (Dp = 7 or 8) versus unimodal in the "western clade" (common Dp = 7).

The average number of trichobothria on the ventral aspect of the pedipalp patella ((Pv_{left} + Pv_{right}) / 2) showed more variation. Among populations belonging to the "eastern clade", the number of ventral trichobothria ((Pv_{left} + Pv_{right}) / 2) was more constant (mean = 4.96, s^2 = 0.24, N = 126) and no geographic pattern was detectable. Within the "western clade" the mean Pv was higher and more variable (mean = 5.60, s^2 = 0.34, N = 146) and also exhibited a considerable geographic variation. In the center of its geographical range (Bergamascan Alps) the Pv character was fixed around 6. However, in the marginal populations of the "western clade", this character was fixed at ca. 5. This holds true for both, the westernmost (Fontainemore and Gondo), as well as the easternmost populations (Tavernola, Molina di Ledro, Bezzecca, Marling and Bad Salz).

DISCUSSION

TAXONOMIC SUBDIVISION OF *EUSCORPIUS GERMANUS* (C. L. KOCH)

Before the advent of chaetotaxy (trichobothrial pattern) analysis, taxonomy of *Euscorpius* species was extremely confusing and was based mainly on morphosculpture and coloration characters. Large and conspicuous trichobothria of *Euscorpius* as taxonomic characters were first studied in detail by Hadzi (1929, 1931) and Caporiacco (1950), who used overall trichobothrial counts of pedipalp chela and patella for identification of species and subspecies.

Euscorpius germanus (C. L. Koch, 1837) has been originally described from "southern Tirol [i.e. today's Trentino - Alto Adige in Italy] and northern Italy"; see Fet & Braunwalder (1997) for the detailed taxonomic history and authorship discussion. This species traditionally included several subspecies with rather unclear diagnostics (Caporiacco, 1950).

Although Birula (1900) already clearly demonstrated species-level differences between *E. germanus* and the Caucasian *E. mingrelicus* (Kessler, 1876), several authors later often confused these two species. Until 1980, *E. germanus* was treated as a widely distributing species (from Italy to Caucasus) with a number of subspecies (Hadzi, 1929; Caporiacco 1950; Curcic, 1971; Kinzelbach, 1975). Bonacina (1980) limited *E. germanus* (C. L. Koch) to the Alpine regions of Italy (from Piemonte in the West to Goriziano in the East), Switzerland, and Austria, plus some Balkan populations; and reestablished as a "good" species *E. mingrelicus* (western Balkans and Anatolia to Caucasus, with notable absence in Greece) (Bonacina, 1980; Fet, 1993; Fet & Sissom, 2000). Most recently, we (Scherabon *et al.*, 2000) demonstrated

presence of more than one species within the "*E. mingrelicus* complex", including *E. gamma* Caporiacco, 1950.

Hadzi (1929) was the first to establish subspecies of *E. germanus*; however, his names (*polytrichus*, *mesotrichus* and *oligotrichus*) are invalid since they are homonyms, and therefore replacement names are necessary. Moreover, they were not sufficiently defined to allow identification of these taxa (Fet, 1997). Besides, most populations of "*E. germanus*" from ex-Yugoslavia treated by Hadzi (1929) and by Curcic (1971) do not belong to this species as it is currently defined (Bonacina, 1980; Fet & Sissom, 2000; Scherabon et al., in press).

Capra (1939) separated *E. germanus* from Italy into four forms (A, B and C, as well as a "typical form") based on the number of pectinal teeth (Dp) and the number of trichobothria on the ventral aspect of the pedipalp patella (Pv). Within Italy, the "typical form" of *E. germanus* (Pv = 5) was limited by Capra to the region of Trentino, Alto-Adige and Cadore; the 'A-form' (Pv = 6) to the Lombardian Alps; the 'B-form' (Pv = 5) was assigned to the Piemontese Alps and the 'C-form' (Pv = 5 or 6) to the Goriziano and the Karawanken Alps. Caporiacco (1950) confirmed all of Capra's forms and formally described three new subspecies (*E. g. alpha*, *E. g. beta*, *E. g. gamma*) in addition to the nominotypical *E. g. germanus*. Caporiacco (1950) also described *E. g. croaticus* from Croatia. Finally, Valle *et al.* (1971) described *E. g. marcuzzii* from the Dolomites and Slovenia.

Bonacina (1980) in his revision restricted *E. germanus* to four subspecies: *E. g. germanus* (= *E. g. beta*), *E. g. alpha*, *E. g. marcuzzii* and *E. g. croaticus*. *E. g. gamma* was treated as a subspecies of *E. mingrelicus* (see Scherabon *et al.* (in press) for a detailed taxonomic history). Bonacina (1980) synonymized the disjunct western (Piemonte) *E. g. beta* with the eastern *E. g. germanus* since both taxa shared character of Pv = 5. Furthermore, he suggested hybridization between the taxa *E. g. alpha* and *E. g. germanus*. Bonacina (1980) also carried out a thorough statistical study of trichobothrial numbers (on ventral and external surfaces of the pedipalp patella) for numerous populations in Piemonte and Lombardy, Italy. He postulated that the number of ventral patellar trichobothria is Pv = 5 for *E. g. germanus* and Pv = 6 in *E. g. alpha*. He also suggested a hybridogenic origin for populations in the Bergamascan Alps (Valle Brembana, north of Bergamo), because of intermediate forms (5-5 or 6-6) and a high number of asymmetric (5-6 or 6-5) individuals. However, our allozyme and DNA data do not confirm any assumptions of hybridogenic origin by Bonacina (1980), or by Kinzelbach (1975); see also Gantenbein *et al.* (1999a).

Applying methods based on models of evolutionary change (pairwise distance methods / maximum likelihood) and the maximum parsimony criterion, in our study, two independent systems of molecular markers (allozymes and 16S mtDNA sequences) revealed an almost identical phylogenetic pattern. The phylogeny based on both mtDNA and allozyme data suggests a clear, distinct topology of two major clades (Figs 2-3). These clades are well supported statistically. They include parapatric population groups which are geographically separated by the river Adige (Etsch). The genetic distance which is found between these two groups is comparable to the genetic distance found between each of these clades and the outgroup species

E. gamma. Therefore, we propose to treat these two population groups as two species: *Euscorpius germanus* (C. L. Koch, 1837) sensu stricto ("eastern" clade) and *Euscorpius alpha* Caporiacco, 1950 **stat. nov.** ("western" clade).

The deep divergence and parapatry of the "eastern" and "western" clades inferred by using independent genetic markers is not entirely consistent with geographical ranges of the subspecies *E. germanus germanus* and *E. g. alpha* as shown by Caporiacco (1950) and Bonacina (1980). Nevertheless, type localities of these two taxa (as designated below) would fall well inside the ranges of two papapatric clades, thus making the existing taxonomic names applicable to the monophyletic clades.

THE STATUS OF *EUSCORPIUS GERMANUS BETA* CAPORIACCO, *E. G. CROATICUS* CAPORIACCO, AND *E. G. MARCUZZII* VALLE *ET AL.*

(a) *Euscorpius germanus beta* Caporiacco, 1950. Originally delineated by Capra (1939) as "Form B" from Val d'Aosta, including the marginal, westernmost populations of "*E. germanus*" (sensu lato). It was formally described as a subspecies by Caporiacco (1950) and also limited to Piemonte populations. Bonacina (1980) synonymized *E. g. beta* with eastern *E. g. germanus* since both taxa shared the character Pv = 5. Other diagnostic characters listed by Caporiacco (1950) (i.e. metasomal granulation and carination of chela) were considered too variable to be diagnostic. This synonymy, however, created a disjunct distribution for *E. g. germanus* sensu Bonacina (1980).

Our molecular analysis shows that "*E. g. beta*" populations from Italy (Fontainemore) and bordering Switzerland (Gondo, Zwischbergental) occupy the most derived position in the "western clade", or *E. alpha*. Both populations from this area for which allozyme and DNA data were available (i.e. Fontainemore and Gondo) grouped together and were supported by boostrap values in all analyses (Figs 2-3). Thus, we cannot confirm Bonacinaís synonymy *E. g. beta* = *E. g. germanus*. On the other hand, assigning a separate taxonomic status to these two populations would create a paraphyletic subspecies "*E. alpha beta*". Subsequently we would be required to treat other *E. alpha* subclades as monophyletic assemblages as well and assign at least three other new "subspecies" names. Thus, it seems reasonable not to retain *E. g. beta* as a valid taxon, but to place it into synonymy: *Euscorpius alpha* stat. nov., elevated from *Euscorpius g. alpha* Caporiacco, 1950 = *Euscorpius g. beta* Caporiacco, 1950 **syn. nov.**

(b) *Euscorpius germanus croaticus* Caporiacco, 1950. This taxon remained enigmatic since its description. It was mentioned but not revised by Bonacina (1980). We analysed the morphology of the only existing type specimen of this taxon (MZUF 5580, a male from Mali Halam, Velebit Mountains, Croatia; here designated as lectotype, see below). Its trichobothrial pattern on the pedipalps, i.e. number of ventral trichobothria on patella (Pv = 6) and position of trichobothria on the fixed finger, is identical with that of many populations of *E. alpha* (but not of *E. germanus* s. str.). The external face of the pedipalp patella in *E. g. croaticus* bears 22 trichobothria (Pe = 22) in the following serial arrangement: *et* = 5, *est* = 4, *em* = 3, *esb* = 2,

eb_a = 4, *eb* = 4). However, the number *et* = 5 is not found in other populations of *E. germanus* or *E. alpha*, which have *et* = 4 or even *et* = 3. A number of other morphological characters, first of all the very clear presence of carinae on the metasomal segments, shape of pedipalps and spination of legs, indicate that this form is not close to *E. germanus* and falls into the "species complex" of *E. carpathicus* (L., 1767). Similar forms have been observed by one of us (V.F.) from the Rhodope Mountains in Bulgaria.

Reduction of trichobothrial numbers is not uncommon in *E. carpathicus*. In fact, reduction of the trichobothria in the series em from 4 to 3 in *E. c. banaticus* from Romania has been the reason for confusion (Vachon & Jaques, 1977) since this single character was considered to be diagnostic for *E. germanus* (sensu lato, including *E. alpha*, *E. gamma* and *E. mingrelicus*). A detailed study of *E. carpathicus* and related taxa is now being carried out by us (V. Fet, M. Soleglad, B. Gantenbein, in preparation). Pending the completion of this study, we treat *E. germanus croaticus* Caporiacco, 1950 as a form belonging to "*E. carpathicus* complex", but not to *E. germanus* C. L. Koch. Its exact taxonomic status has to be determined.

(c) *Euscorpius germanus marcuzzii* Valle, Berizzi, Bonino, Gorio, Gimmilaro-Negri & Percassi, 1971. Marcuzzi & Fabris (1957) first recorded a form of *E. germanus* from the Dolomites (Italy) with 20 trichobothria (in contrast to the common 21) on the external face of the pedipalp patella (Pe = 20). Valle *et al.* (1971, p. 95-96) very briefly (one line!) described this subspecies from the "refugial massifs of the Venetian Pre-Alps (Italy) and from northern Slovenia", without designating any type specimens (Valle's syntypes of this subspecies are in the Museo Civico di Scienze Naturali "Enrico Caffi", Bergamo). The sole morphological character distinguishing this taxon from other subspecies is the presence of 3 trichobothria instead of 4, in the accessory basal series (eb_a = 3) on the external face of the pedipalp. This character is unique and indeed accords with the general trend of trichobothrial number reduction in the subgenus *Alpiscorpius* (see Gantenbein *et al.* 1999a). Bonacina (1980) mentions a number of populations from northeastern Italy and Slovenia, some "pure" *E. g. marcuzzii* and others mixed (and "hybrid", i.e. asymmetric eb_a=3-4 or 4-3) with *E. g. germanus*.

Our molecular analysis of *E. alpha* from Italy presently includes assymetrical specimens (eba = 3-4 or 4-3) which were found to be rare at Bezzecca (only one find) but are much more common at Auronzo di Cadore, Belluno, Schluderns, Voltago, Vetriolo, Völs, and Crnice. None of the populations mentioned corresponds with a monophyletic, clearly divergent clade. The only Slovenian locality studied (Crnice) includes both symmetric "*E. g. marcuzzii*" (eb_a = 3-3) and regular *E. germanus* specimens. However, further molecular analysis of all populations with eb_a = 3 and a thorough analysis of the syntype series and of additional material are necessary to clarify the status of *E. g. marcuzzii*, which currently appears to be a taxon of dubious validity.

DESIGNATION OF TYPE SPECIMENS

None of the taxa treated in here have a holotype or lectotype specimen. Types designated by C. L. Koch (1837) are lost. For the taxon described by Caporiacco (1950) only syntypes were designated; they are deposited in the collection of the Museo Zoologico "La Specola" dell'Università de Firenze, Florence, Italy (MZUF) (Bartolozzi *et al.*, 1988). Here we designate the necessary type specimens for the following taxa:

Euscorpius germanus (C. L. Koch, 1837)

Originally described as *Scorpius germanus* C. L. Koch, 1837 (pp. 110-112, plate 108, figs 250-252) from "southern Tyrol (now Trentino-Alto Adige, Italy) and upper (= northern) Italy".

Neotype: ♂, Brixen (Bressanone), Trentino - Alto Adige, Italy, 9. 10. 98, coll. B. Gantenbein & I. Gantenbein, sample No. BG-109-07, deposited in Naturhistorisches Museum Bern, Switzerland, accession number Sc1. Trichobothrial formula: Pv = 5, Pe = 21 (*et* = 4, *est* = 4, *em* = 3, *esb* = 2, eb_a = 4, *eb* = 4).

Euscorpius alpha Caporiacco, 1950 **stat. nov.**

Originally described as *Euscorpius germanus alpha* Caporiacco, 1950: 211.

Lectotype: ♀ (MZUF 5569), Lago di Como near Varenna, Lombardy, Italy (August 1879, coll. Cantoni). Trichobothrial formula: Pv = 6, Pe = 21 (et = 4, est = 4, em = 3, esb = 2, eba = 4, eb = 4).

Paralectotypes: 1 ♂ (MZUF 5571), 5 ♀♀(MZUF 5568, 5570-5574), from the type locality; 6 ♂♂, 5 ♀♀(MZUF 5575-5579), Varese, Lombardy, Italy (1879, coll. Cantoni); 1 ♀ (MZUF 5567), Monte Stelvio, Trentino - Alto Adige, Italy (August 1877, coll. P. Magretti).

Euscorpius germanus beta Caporiacco, 1950: 211

Here considered as **syn. nov.** of *Euscorpius alpha* Caporiacco, 1950.

Lectotype: ♂ (MZUF 5588), Monte Massone (Cesara, Novara), Piemonte, Italy (12 August 1879, coll. C. Parona). Trichobothrial formula: Pv = 5, Pe = 21 (*et* = 4, *est* = 4, *em* = 3, *esb* = 2, eb_a = 4, *eb* = 4).

Paralectotypes: 5 ♂♂ and 5 ♀♀ (MZUF 5589, 5590-5593), type locality; 1 ♂, 1 ♀ (MZUF 5584, 5585), Colle della Piccola Mologna, 2000 m, (Biella, Vercelli), Piemonte, Italy; 2 ♀♀ (MZUF), Lamorano, 1879, Piemonte, Italy.

Euscorpius germanus croaticus Caporiacco, 1950: 215

Lectotype: ♂ (MZUF 5580), Mali Halam, Velebit Mountains, Croatia (other specimens were listed in the original description but are absent in the MZUF collection). Probably belongs to *Euscorpius carpathicus* (L., 1767) "complex" (see above). Trichobothrial formula: Pv = 6, Pe = 22 (*et* = 5, *est* = 4, *em* = 3, *esb* = 2, eb_a = 4, *eb* = 4).

MORPHOLOGICAL VERSUS MOLECULAR DATA

The classical morphological characters in the species of *Euscorpius*, such as the number of pectinal teeth and the number of trichobothria, are quantitative threshold characters similar the number of bristles in *Drosophila* (Futuyma, 1986). Such traits are expressed discontinuously at the phenotypic level, but are affected by a continuous distribution of some underlying trait. Capra (1939), Caporiacco (1950) and Bonacina (1980) have found geographic variation in these characters and also asymmetries which are very common. We confirm their observations that within the *E. alpha* clade the number of the ventral trichobothria of the patella (Pv) is about 6 in the region of the Bergamscan Alps (Valle Brembana) and changes to Pv = 5 in the western populations (Fontainemore and Gondo). The same pattern is found in the eastern populations at Molina di Ledro, Bezzecca (Lago di Garda) and Marling, Bad Salz (Trentino-Alto Adige). Here the subclades detected by using molecular markers (Figs. 2-3) correspond to the observed pattern of the Pv character.

However, no hybridization or gene flow was observed between *E. alpha* and *E. germanus* clades. Bonacina (1980) gave a detailed morphological account of the distribution of Pv = 5-5, 6-6 or 5-6 individuals within the Bergamascan Alps. Analysing the same populations (Fig. 1, box) we found no confirmation of his assumption of hybridisation between two distinct taxa; it appears that the described morphological variation occurs at the phenotypic level within a single genetically coherent species, *E. alpha*. Apparently character states Pv = 5 or 6 alone are not sufficient to define both parapatric species since *E. germanus* usually has Pv = 5 but in *E. alpha* this character varies from 5 to 6 with various degree of fixation. The same is true for the pectinal teeth number, Dp (see Results).

Neither trichobothrial nor pectinal teeth scores give us a clear-cut delineation which would reflect the deep divergence of two clades revealed by molecular data and treated here as species-level taxa. In order to characterize these two clades morphologically, more complex characters should be searched for and analysed. Among these, the morphology of the hemispermatophore could possibly be applied as a species-level character set for *Euscorpius* as suggested by some previous authors (Kinzelbach, 1975; Bonacina, 1980; Scherabon 1987).

PHYLOGENETIC IMPLICATIONS ON BIOGEOGRAPHY

In their analysis of molecular phylogeny and historical biogeography of the genus *Euscorpius*, Gantenbein *et al.* (1999a) noticed the deep split between the endemic Alpine clade (subgenus *Alpiscorpius* Gantenbein, Fet, Largiadèr & Scholl, 1999 which included traditional *E. germanus*) and the major Asia Minor-Trans-mediterranean lineage (subgenera *Euscorpius* Thorell, 1876 and *Polytrichobothrius* Birula, 1917). This led to the assumption that in the ancestors of modern *E. germanus* (sensu lato) ecological differentiation and adaptation to orophylic and mesophylic habitats (in contrast to xerophylic habitats occupied by *E. carpathicus* and especially *E. italicus*) could have been an ancient event. In other words, modern forms inhabiting the Alpine zone of Europe are not necessarily a result of speciations due to recent (Pleistocene) glacial events (Klicka & Zink, 1997) but these taxa may have

evolved in this area since the beginning of the Alpine orogenesis. This long time scale can explain the high level of genetic divergence observed within the subgenus Alpiscorpius, which we separate here into two species, *Euscorpius (Alpiscorpius) germanus* (C. L. Koch, 1837) and *E. (A.) alpha* Caporiacco, 1950.

The period of divergence time between these two parapatric taxa can be estimated by using the genetic distance and by assuming a constant evolutionary rate through time. The calibration of a molecular clock for *Mesobuthus gibbosus* (Brullé, 1832) (Scorpiones, Buthidae) in the mainland Greece and Turkey and on several Aegean islands reveals an average rate of about 3% sequence divergence per Myr for the 16S rRNA gene (our data, unpublished). A comparable rate was reported for another scorpion species, *Buthus occitanus* (Amoreux, 1789) (Buthidae) across the Strait of Gibraltar (Gantenbein *et al.*, 1999b). Applying this "scorpion clock" to the mtDNA sequence divergence between *E. germanus* and *E. alpha* (ca. 7%) reveals a separation time of about 2-3 million years. Such a time scale contradicts the Late Pleistocene Origin model (LPO), which is widely accepted today (see also Klicka & Zink, 1997). The deep split between the evolutionary lineages in *E. germanus* and *E. alpha* remains high even when genetic distances for superimposed substitutions are corrected by using the most appropiate model (TrN93 in this case), as proposed by Arbogast & Slowinski (1998). A similar deep split (> 1.6 Myrs) was uncovered by a recent allozyme survey of Alpine species of *Glomeris* (Diplopoda: Glomeridae) (Hoess & Scholl, 1999) which presumably have similar dispersal rates as *Euscorpius* species.

Taberlet *et al.* (1998) identified several so-called "suture zones" in Europe, including the Alps, where different taxa meet after postglacial isolation. The situation for *Euscorpius* is similar but lacks hybridisation. No gene flow was detected between the two clades, which appear to be true parapatric species separated by the geographic divide of the Adige River valley. Hedin (1997) recently demonstrated for *Nesticus* spiders (Aranae, Nesticidae) in the Appalachian Mountains that mtDNA analysis allows to discover considerable genetic divergence, both between and within recognized morphological species; most of these probably predate the Pleistocene. The divergence between *E. alpha* and *E. germanus* clearly predates the Pleistocene glaciations, as it is the case for a number of other Alpine taxa (Taberlet *et al.*, 1998). This confirms the hypothesis that speciation events mainly occurred during the Pliocene (Zink & Slowinski, 1995).

Possible Pleistocenic refugia for these two species were the Bergamascan Alps for *E. alpha* and the Venetian Prealps for *E. germanus*. Evidence that these regions could have served as refugia for small terrestrial arthropods comes from a palynological analysis (Kral, 1989) which indicates that a relatively mild climate prevailed during the Pleistocene. Other arguments for this interpretation are provided by the genetic data presented in this study. The genetic variability, expressed in the average heterozygosity and the mean number of alleles per locus, in *E. alpha* populations is relatively higher in the region around Bergamo than in regions near the edges of the refuge. Low levels of heterozygosity in *E. germanus* indicate possible genetic bottlenecks in the history of this species. Further investigations are required to test this hypothesis.

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APPENDIX I

Allele frequencies at 18 allozyme loci and sample sizes of populations analysed. Also given are the identified 16S rDNA haplotypes.

Nr.	Sample	Country	N	Sequence	alpdh		ark				ddh		gapdh	aat1				
	Western clade = *E. alpha* stat. nov.				100	95	104	100	98	93	101	100	100	78	88	96	100	104
1	Gondo	CH	(9)	*Eal* GO		1.00		1.00			1.00		1.00			1.00		
2	Fontainemore	CH	(10)	*Eal* FO		1.00		1.00			1.00		1.00			1.00		
3	Rancate	CH	(15)	*Eal* RA		1.00		1.00			1.00		1.00			1.00		
4	Monte	CH	(11)			1.00		1.00			1.00		1.00			1.00		
5	Fornace	CH	(10)			1.00	0.05	0.95			1.00		1.00			1.00		
6	Pontide	I	(5)			1.00		1.00			1.00		1.00	0.10		0.90		
7	Sottoponte	CH	(13)	*Eal* SO		1.00		1.00			1.00		1.00			1.00		
8	San Carlo	CH	(12)			1.00		1.00			1.00		1.00			1.00		
9	Brembilla	I	(5)			1.00		1.00			1.00		1.00			1.00		
10	Gerosa	I	(2)			1.00		1.00			1.00		1.00			1.00		
11	Peghera	I	(2)			1.00		1.00			1.00		1.00			1.00		
12	San Pellegrino	I	(4)	*Eal* SP		1.00		1.00			1.00		1.00		0.25	0.75		
13	San Giovanni Bianco	I	(11)	*Eal* SG		1.00		1.00			1.00		1.00	0.09	0.05	0.82	0.05	
14	Camerata Comello	I	(1)			1.00		1.00			1.00		1.00			1.00		
15	Olmo al Brembo	I	(10)	*Eal* OL		1.00		1.00			1.00		1.00			1.00		
16	Piazzatorre	I	(13)			1.00		1.00			1.00		1.00			1.00		
17	Mezzoldo	I	(7)			1.00		1.00			1.00		1.00			1.00		
18	Isola di Fondra	I	(13)			1.00		1.00			1.00		1.00			1.00		
19	Carona	I	(13)	*Eal* CA		1.00		1.00			1.00		1.00			1.00		
20	Valleve	I	(1)			1.00		1.00			1.00		1.00			1.00		
21	Selvino	I	(5)			1.00		1.00			1.00		1.00			1.00		
22	Membro	I	(3)			1.00		1.00			1.00		1.00	0.17		0.83		
23	Tavernola	I	(4)	*Eal* Ta		1.00		1.00			1.00		1.00			1.00		
24	Vigolo	I	(3)			1.00		1.00			1.00		1.00			1.00		
25	Bezzeca	I	(5)			1.00		1.00			1.00		1.00			1.00		
26	Molina di Ledro	I	(10)	*Eal* ML		1.00		1.00			1.00		1.00			0.75		0.25
27	Marling	I	(10)	*Eal* MA		1.00		1.00			1.00		1.00			0.30		0.70
28	Bad Salz	I	(1)			1.00		1.00			1.00		1.00					1.00
	Eastern clade = *E. germanus*																	
29	Sta Maria	CH	(22)	*Ege* SM	1.00			1.00			1.00		1.00	1.00				
30	Lichtenberg	I	(5)		1.00			1.00			1.00		1.00	1.00				
31	Schluderns	I	(10)	*Ege* SH	1.00			1.00			1.00		1.00	0.55	0.35	0.10		
32	Schlanderns	I	(1)			1.00		1.00			1.00		1.00	1.00				
33	Verdins	I	(10)		0.05	0.95		1.00			1.00		1.00	1.00				
34	Brixen	I	(4)			1.00		1.00			1.00		1.00	1.00				
35	Völs	I	(10)	*Ege* VO	0.40	0.60		1.00			1.00		1.00	0.95		0.05		
36	Bremer	I	(7)		0.21	0.79		1.00			1.00		1.00	1.00				
37	Veltiolo	I	(9)	*Ege* VE		1.00		0.94	0.06		1.00		1.00	1.00				
38	Belluno	I	(4)			1.00		1.00			1.00		1.00			1.00		
39	Voltago	I	(5)			1.00		1.00			1.00		1.00	0.70		0.30		
40	San Tomaso	I	(3)			1.00		1.00			1.00		1.00	0.67		0.33		
41	Mezzocanale	I	(2)	*Ege* ME		1.00		1.00			1.00		1.00	0.50		0.50		
42	Borca di Cadore	I	(10)	*Ege* BO		1.00		1.00			1.00		1.00	0.65		0.35		
43	Auronzo di Cadore	I	(6)			1.00		1.00			1.00		1.00			1.00		
44	Starkenbach	A	(4)	*Ege* ST		1.00		1.00			1.00		1.00			1.00		
45	Tarrenz	A	(2)	*Ege* TZ		1.00		1.00			1.00		1.00	1.00				
46	Kranzach	A	(4)	*Ege* KR		1.00		1.00			1.00		1.00	1.00				
47	Dellach	A	(8)	*Ege* DE		1.00		1.00			1.00		1.00			1.00		
48	Oberdrauburg	A	(5)	*Ege* OB		1.00		1.00			1.00		1.00			1.00		
49	Dobratsch	A	(4)			1.00		1.00			1.00		1.00			1.00		
50	Schütt	A	(6)			1.00		1.00			1.00		1.00			1.00		
51	Federaun	A	(4)			1.00		1.00			1.00		1.00			1.00		
52	Crnice	SLO	(8)			1.00	1.00				1.00		1.00			1.00		
	E. gamma																	
53	Koschuta	A	(6)	*Ega* KO	1.00					1.00	1.00		1.00			1.00		
	E. flavicaudis																	
	Lauris	F	(33)	*Efl* LA	1.00			1.00				1.00	1.00				1.00	

APPENDIX I (2)

Nr. Sample	aat2			gtdh			hk			idh1			idh2			mdh1			
	88	100	107	113	90	95	100	100	107	94	95	100	87	93	100	76	87	89	100
Western clade = *E. alpha* stat. nov.																			
1 Gondo				1.00	1.00			1.00		1.00				1.00				1.00	
2 Fontainemore				1.00	1.00			1.00		1.00				1.00				1.00	
3 Rancate				1.00	1.00			1.00		1.00				1.00				1.00	
4 Monte				1.00	1.00			1.00		1.00				1.00				1.00	
5 Fornace				1.00	1.00			1.00		1.00				1.00				1.00	
6 Pontide				1.00	1.00			1.00		1.00				1.00				0.80	0.20
7 Sottoponte				1.00	1.00			1.00		1.00				1.00				1.00	
8 San Carlo				1.00	1.00			1.00		1.00				1.00				1.00	
9 Brembilla				1.00	1.00			1.00		1.00				1.00				0.90	0.10
10 Gerosa				1.00	1.00			1.00		1.00				1.00				1.00	
11 Peghera				1.00	1.00			1.00		1.00				1.00				1.00	
12 San Pellegrino		0.25		0.75	1.00			1.00		1.00				1.00				1.00	
13 San Giovanni Bianco				1.00	1.00			1.00		1.00				1.00				1.00	
14 Camerata Cornello				1.00	1.00			1.00		1.00				1.00				1.00	
15 Olmo al Brembo				1.00	1.00			1.00		1.00				1.00				1.00	
16 Piazzatorre				1.00	1.00			1.00		1.00				1.00				1.00	
17 Mezzoldo				1.00	1.00			1.00		1.00				1.00				1.00	
18 Isola di Fondra			0.08	0.92	1.00			0.92	0.08	1.00				1.00		0.04		0.92	0.04
19 Carona				1.00	1.00			1.00		1.00				1.00				1.00	
20 Valleve				1.00	1.00			1.00		1.00				1.00				1.00	
21 Selvino				1.00	1.00			1.00		1.00				1.00				0.80	0.20
22 Nembro				1.00	1.00			1.00		1.00				1.00				0.83	0.17
23 Tavernola				1.00	1.00			1.00		1.00				1.00		0.12		0.88	
24 Vigolo		0.33		0.67	1.00			1.00		1.00				1.00				1.00	
25 Bezzecca				1.00	1.00			1.00		1.00				1.00				1.00	
26 Molina di Ledro				1.00	1.00			1.00		1.00				1.00				1.00	
27 Marling				1.00	1.00			0.95	0.05	1.00				1.00				1.00	
28 Bad Salz				1.00	1.00			1.00		1.00				1.00				1.00	
Eastern clade = *E. germanus*																			
29 Sta Maria			1.00		1.00			1.00		1.00			1.00						1.00
30 Lichtenberg			1.00		1.00			1.00		1.00			1.00						1.00
31 Schluderns			1.00		1.00			1.00		1.00			1.00						1.00
32 Schlanderns			1.00		1.00			1.00		1.00			1.00						1.00
33 Verdins			1.00		1.00			1.00		1.00			1.00						1.00
34 Brixen			1.00		1.00			1.00		1.00			1.00						1.00
35 Vôls			1.00		1.00			1.00		1.00			1.00						1.00
36 Bremer			1.00		1.00			1.00		1.00			1.00						1.00
37 Vetriolo			1.00		1.00			1.00		1.00			1.00						1.00
38 Belluno			1.00		1.00			1.00		1.00			1.00						1.00
39 Voltago			1.00		1.00			1.00		1.00			1.00						1.00
40 San Romaso			1.00		1.00			1.00		1.00			1.00						1.00
41 Mezzocanale			1.00		1.00			1.00		1.00			1.00						1.00
42 Borca di Cadore			1.00		1.00			1.00		1.00			1.00				0.20	0.10	0.70
43 Auronzo di Cadore			1.00		1.00			1.00		1.00			1.00				0.33		0.67
44 Starkenbach			1.00		1.00			1.00		1.00			1.00						1.00
45 Tarrenz			1.00		1.00			1.00		1.00			1.00						1.00
46 Kranzach			1.00		1.00			1.00		1.00			1.00						1.00
47 Dellach			1.00		1.00			1.00		1.00			1.00						1.00
48 Oberdrauburg			1.00		1.00			1.00		1.00			1.00						1.00
49 Dobratsch			1.00		1.00			1.00		1.00			1.00						1.00
50 Schütt			1.00		1.00			1.00		1.00			1.00						1.00
51 Federaun			1.00		1.00			1.00		1.00			1.00						1.00
52 Crnice				1.00	1.00			1.00		1.00			1.00					1.00	
Outgroup species																			
E. gamma																			
53 Koschuta				1.00		1.00			1.00	1.00					1.00				1.00
E. flavicaudis																			
Lauris	0.03	0.97					1.00	1.00			0.09	0.91			1.00				1.00

APPENDIX I (3)

		mdh2		mpi									pep						
	Nr. Sample	100	105	100	101	107	110	112	118	125	130	135	78	87	94	98	100	104	107
	Western clade =																		
	***E. alpha* stat. nov.**																		
1	Gondo	1.00		0.17		0.83										1.00			
2	Fontainemore	1.00				1.00										1.00			
3	Rancate	1.00				0.54			0.27		0.12	0.08				1.00			
4	Monte	1.00		0.14		0.77						0.09				1.00			
5	Fornace	1.00				0.30			0.10		0.60					1.00			
6	Pontide	1.00							0.70	0.10		0.20				0.40			0.60
7	Sottoponte	1.00				0.92			0.08							1.00			
8	San Carlo	1.00				0.14			0.86							0.95			0.05
9	Brembilla	1.00			0.25			0.50	0.13	0.13						0.90			0.10
10	Gerosa	1.00							0.75	0.25						1.00			
11	Peghera	1.00			0.25		0.25		0.25	0.25						0.75			0.25
12	San Pellegrino	1.00			0.13	0.13		0.13	0.64					0.50		0.25			0.25
13	San Giovanni Bianco	1.00		0.05				0.18	0.59	0.18						0.59			0.41
14	Camerata Cornello	1.00							1.00					1.00					
15	Olimo al Breembo	1.00					0.05	0.45	0.35	0.15						0.95			0.05
16	Piazzatorre	1.00					0.12	0.39	0.31	0.12	0.08					0.89			0.12
17	Mezzoldo	1.00						0.64	0.14	0.14		0.07				0.83			0.17
18	Isola di Fondra	1.00			0.08			0.58	0.12	0.23						0.92			0.08
19	Carona	1.00						0.65	0.19	0.15						1.00			
20	Valleve	1.00						1.00								1.00			
21	Selvino	1.00						0.10	0.70	0.20						1.00			
22	Membro	1.00							0.50	0.50						1.00			
23	Tavernola	1.00						0.50	0.50										1.00
24	Vigolo	1.00						0.50	0.50										1.00
25	Bezzecca	0.90	0.10		0.10				0.40	0.50						0.20			0.80
26	Molina di Ledro	1.00					0.10	0.35	0.45	0.10						0.10			0.90
27	Marling	1.00		0.10		0.30	0.10		0.50										1.00
28	Bad Salz	1.00										1.00							1.00
	Eastern clade =																		
	E. germanus																		
29	Sta Maria	1.00										1.00						1.00	
30	Lichtenberg	1.00										1.00						1.00	
31	Schluderns	1.00										1.00						1.00	
32	Schlanderns	1.00										1.00						1.00	
33	Verdins	1.00										1.00						1.00	
34	Brixen	1.00										1.00						1.00	
35	Völs	1.00										1.00						1.00	
36	Bremer	1.00										1.00						1.00	
37	Vetriolo	1.00										1.00						1.00	
38	Belluno	1.00										1.00			0.50				0.50
39	Voltago	1.00										1.00			0.60			0.40	
40	San Tomaso	1.00										1.00			0.17			0.83	
41	Mezzocanale	1.00										1.00			0.25			0.75	
42	Borca di Cadore	1.00										1.00			0.05			0.45	0.50
43	Auronzo di Cadore	1.00										1.00						0.83	0.17
44	Starkenbach	1.00										1.00						0.38	0.62
45	Tarrenz	1.00										1.00						1.00	
46	Kranzach	1.00										1.00						1.00	
47	Dellach	1.00										1.00						0.31	0.69
48	Oberdrauburg	1.00										1.00						0.80	0.20
49	Dobratsch	1.00										1.00						0.88	0.12
50	Schütt	1.00										1.00						0.70	0.30
51	Fereraun	1.00										1.00						0.75	0.25
52	Crnice	1.00					0.06		0.94									0.42	0.58
	Outgroup species																		
	E. gamma																		
53	Koschuta	1.00					1.00						0.17			0.83			
	E. flavicaudis																		
	Lauris	1.00		0.10											0.13		0.87		

APPENDIX I (4)

Nr.	Sample	6-pgd 88	93	98	100	104	107	pgi 80	88	93	94	100	pgm 80	91	94	98	100	pk 98	100	101
	Western clade = *E. alpha* stat. nov.																			
1	Gondo			1.00						1.00				0.83	0.06	0.11			1.00	
2	Fontainemore		1.00							1.00				1.00					1.00	
3	Rancate			1.00						1.00				0.57		0.43				1.00
4	Monte			1.00						1.00				0.73		0.27				1.00
5	Fornace			1.00						1.00				0.70		0.30				1.00
6	Pontide			1.00					0.10	0.90				0.40	0.30	0.30				1.00
7	Sottoponte			1.00						0.23	0.77			0.85		0.15				1.00
8	San Carlo			1.00						0.62	0.38			0.75		0.25				1.00
9	Brembilla			1.00					0.10	0.90				0.38	0.13	0.50				1.00
10	Gerosa			1.00					0.25	0.75				0.75		0.25				1.00
11	Peghera			1.00					0.25	0.75				0.50		0.50				1.00
12	San Pellegrino			1.00					0.50	0.50				0.50	0.25	0.25				1.00
13	San Giovanni Bianco			0.91			0.09		0.09	0.91				0.05		0.96				1.00
14	Camerata Cornello			1.00					1.00					1.00						1.00
15	Olmo al Brembo			1.00					0.15	0.85				0.75		0.25				1.00
16	Piazzatorre			1.00					0.08	0.92				0.65		0.35				1.00
17	Mezzoldo			1.00						1.00				0.71		0.29				1.00
18	Isola di Fondra			1.00					0.73	0.27				0.08		0.92				1.00
19	Carona			1.00					1.00					0.13		0.88				1.00
20	Valleve			1.00					1.00											1.00
21	Selvino			1.00					0.10	0.90				0.13	0.13	0.75				1.00
22	Nembro			1.00					0.17	0.83				0.17	0.33	0.50				1.00
23	Tavernola			1.00						1.00				0.50	0.50					1.00
24	Vigolo			1.00						1.00					0.50	0.50				1.00
25	Bezzecca			0.90		0.10				1.00			0.10	0.50	0.20	0.20				1.00
26	Molina di Ledro			1.00						1.00				0.25	0.30	0.45				1.00
27	Marling			1.00					0.05	0.95				0.61	0.22	0.17				1.00
28	Bad Salz			1.00						1.00				1.00						1.00
	Eastern clade = *E. germanus*																			
29	Sta Maria			1.00						1.00				0.80		0.21		1.00		
30	Lichtenberg			1.00						1.00				0.40		0.60		1.00		
31	Schluderns			1.00						1.00				0.93		0.07		1.00		
32	Schlanderns			1.00						1.00				1.00				1.00		
33	verdins			1.00						1.00				1.00				1.00		
34	Brixen			1.00						1.00				0.75	0.13	0.13		1.00		
35	Völs			1.00						1.00				0.70	0.20	0.10		1.00		
36	Bremer			1.00						1.00				0.86	0.14			1.00		
37	Vetriolo			1.00						1.00				0.89		0.11		1.00		
38	Belluno			1.00						1.00						1.00		1.00		
39	Voltago			1.00						1.00				0.40		0.60		1.00		
40	San Tomaso			1.00						1.00						1.00		1.00		
41	Mezzocanale			1.00						1.00				1.00				1.00		
42	Borca di Cadore			1.00						1.00				0.85		0.15		1.00		
43	Auronzo di Cadore			1.00						1.00				0.08		0.92		1.00		
44	Starkenbach			1.00						1.00				0.75		0.25		1.00		
45	Tarrenz			1.00						1.00				1.00				1.00		
46	Kranzach			1.00						1.00				0.50		0.50		1.00		
47	Dellach			1.00						1.00			0.06	0.94				1.00		
48	Oberdrauburg			1.00						1.00			0.10	0.30	0.40	0.20		1.00		
49	Dobratsch			1.00					0.13	0.88			0.25	0.75				1.00		
50	Schütt			1.00						1.00			0.33	0.25		0.42		1.00		
51	Federaun			1.00						1.00				0.75		0.25		1.00		
52	Crnice			1.00						1.00				0.56		0.44		1.00		
	Outgroup species																			
	E. gamma																			
53	Koschuta		1.00					1.00								1.00				1.00
	E. flavicaudis																			
	Lauris	0.35			0.65							1.00					1.00		1.00	

APPENDIX II

Polymorphic sites (indels, parsimony informative sites, transitions (ti), and transversions (tv)) in the 16S rRNA gene sequences analysed. Abbreviations for the haplotypes are explained in appendix I.

```
position          111 1111111111 1111111112 2222222222 2222222222 2222223333333333
position     33378012 2223344455 5555666890 1111122222 2333346677 7789990222335555
position    312352760 2381812923 4569056598 0123723467 9023471901 2322461457020 29
Indel       ===D===== ========== ========== =====D==== ======D=== ================
Informative====FF=F= ==FFFF===F F=FFFFFFFF FF==F==F== ==FFFF==F= FFFF=F=F=======
Ti          N===NNNNN =NNNNN=NNN NNNNN=NNNN NN=NN==N=N NNNNNN==N= NNNNNNNNN=NN=NN
Tv          =VV==V=== V==V=VV==V V====VV=VV V=V===VVV= V=VV===V=V ========V=V==V=V

EalFO       TTATATTGT TTACGTTGAT CCTCATATAG GGTTA*TGTA ATAGAAGAAT AACAAGAGGTTTCGA
EalGO       --------- ---------- ---------- ---------- ---------- ---------------
EalSP       --------- ---------- T--T------ ---------- --G-----G- -------A------G
EalSG       --------- ---------- T--T------ ---------- --G-----G- -------A-----AT
EalRA       ---*----- ---------- T--T----G- -A-------- --G-----G- ---G-----------
EalCA       --------- ---------- T--T----G- -A-------- --------G- ---G-----------
EalOL       --------- ---------- T--T----T- -A-------- --G-----G- ---G--------A--
EalSO       --------- ---------- T--T----G- -A-------- --G-----G- ---G-----------
EalTA       -------A- ---------- T--T------ AA-------- G-G-----G- G--------------
EalML       -------A- ---------- T--------A -A-------- --G-----G- G--------------
EalMA       -------A- ---------- T--------A -A-------- --G-----G- G--------------
EgeOB       -----G-A- ----AG---C T-C-G-G-GT A---G--A-- --GAG----- GGT--A-T-------
EgeDE       -----G-A- ----AG---C T-C-G-G-GT A---G--A-- --GAG----- GGT--A-T-------
EgeST       -----G-A- ----AG---C T-C-G-G-GT A---G--A-- --GAG----- GGT--A-T-------
EgeBO       -------A- ----AG---C T-C-G-G-GT A---G--A-- --GAG----- GGT--A-T-------
EgeSM       -----G-A- ---TAG---- T-C-G-G-GT A---G--A-- --GAG----- GGT--A-T-------
EgeVE       -----G-A- ---TAG---- T-C-G-G-GT A---G--A-- --GAG----- GGT--A-T-------
EgeSH       -----G-A- ---TAG---- T-C-G-G-GT A---G--A-- --GAG----- GGT--A-T-------
EgeVO       -----G-A- ---TAG---- T-C-G-G-GT A---G--A-- --GAG----- GGT--A-T-------
EgeME       -----G-A- ---TAG---- T-C-G-G-GT A---G--A-- --GAGG---- GGT--A-T-------
EgeKR       -----G-A- ---TAG---- T-C-G-G-GT A---G--A-- --GAG-*--- GGT--A-T-------
EgeTZ       -----G-A- ---TAG---- T-C-G-G-GT A---G--A-- --GAG----- GGT--A-T-------
EgeCR       ----GG-A- ----AGG--A T-C-G--C-T AA-----A-- -C-AG---G- GGT--A-T-------
EgaKO       -----A-A- G-GT---A-A AT---A-C-T AA-----A-- --G-----G- G-T-GA-T-------
EflLA       CGC-GAC-C -CGAA---G- A----AT--T TAACGTGTAG T-TT-G-T-A G-T--A---GCC---
```