Variability in cuticular hydrocarbons and phenotypic discrimination of *Ixodes ricinus* populations (Acarina: Ixodidae) from Europe

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ABSTRACT

The cuticular hydrocarbon composition and a stepwise discriminant analysis are used to elucidate the phenotypic relationships of 66 populations of *Ixodes ricinus* in Europe. The method correctly allocates *Ixodes persulcatus* (outgroup) populations away from the main cluster of *I. ricinus* samples and separates the samples into ten relatively defined clusters of specimens. Populations from Poland are inseparable from samples collected in Germany, Switzerland and the Italian Alps, while individuals from Slovakia and the Czech Republic come into separate groups of phenotypic similarity. Irish and British specimens are separated but highly related and Spanish populations show an unexpectedly high distance from the remaining clusters.

Key words: *Ixodes ricinus*, ticks, phenotypic variability, Europe, cuticular hydrocarbons

INTRODUCTION

Ixodes ricinus is the main vector of some members of the tick-borne encephalitis subgroup: louping-ill virus and European tick-borne encephalitis virus, as well as the spirochete *Borrelia burgdorferi* and a wide variety of other pathogenic agents. A recent overview of *I. ricinus* concluded that much of the ecology and biology of this tick species still remains unclear (Gray, 1991). The general distribution of *I. ricinus* is through most parts of Europe eastward to the Volga River. In Asia, it is present in Turkey, northern Iran, Caucasia and western Kopet-Dag. In Africa, it is recorded in Madeira, Morocco, Algeria and Tunisia (Kolonin, 1981). Healy (1979) showed that an unexpectedly high variation in enzyme polymorphism is present in specimens from Ireland and that this variation may be related to the ability to become active at different periods of the year.

Ixodes ricinus has received much attention in recent years as the main vector in Europe of *B. burgdorferi*, causing Lyme borreliosis. Using various methods,

at least four genospecies of *Borrelia* have been described in Europe. Recent studies have demonstrated that various phenotypes of *B. burgdorferi* may be present in the tick population of one endemic area (Humair *et al.*, 1995; Kahl *et al.*, unpublished) and that their relative prevalences in ticks greatly differ from one area to another, some phenotypes being more frequent in some foci than in others (Hu *et al.*, 1994). Furthermore, in Europe, a great heterogeneity in the expression of the main outer surface proteins of *B. burgdorferi* is present among tick isolates and individual adult ticks may harbour more than one genospecies (Leuba-Garcia *et al.*, 1994). The ecological and evolutionary backgrounds for such a diversity in a focus still remain largely unknown, but are of interest in relation to the epidemiology and distribution of foci of Lyme disease.

Of biological and epidemiological interest are questions of gene flow and niche divergence in zones of overlap or range contiguity between *I. ricinus* populations and the genetic relationships between peripheral and central populations of this tick species. Cuticular hydrocarbon analysis is one of the newest techniques available to taxonomists for arthropod systematics. Several papers (Lockey, 1978a,b, 1988) have demonstrated that the hydrocarbon composition in insects is related to the taxonomic grouping to the extent that hydrocarbon mixtures are species specific and that closely related species tend to have compounds in different proportions. The method has been used for taxonomic studies of several tick species and populations (Estrada-Peña *et al.*, 1992a, 1993, 1994). In a previous paper aimed to ascertain the degree of genetic similarity between sympatric populations of *I. ricinus* from Poland (Estrada-Peña *et al.*, 1994) a very low genetic distance was noted. This paper assesses the coarse geographic structure in the populations of the tick *I. ricinus* by examining cuticular hydrocarbons from samples collected in several countries of Europe.

MATERIALS AND METHODS

Unfed adult *I. ricinus* specimens were examined in this study. They were collected from the field to avoid the higher degree of homogeneity expected in laboratory colonies. Most ticks were collected from vegetation by standard methods; some samples were obtained from their hosts, although partly engorged specimens were avoided. A total of 1558 ticks were studied, from 66 different European localities distributed over 14 European countries.

Some preliminary tests were done to check for the integrity of the cuticular hydrocarbons as typical of each population studied. In such a way, *I. ricinus* nymphs fed on different hosts were collected in a single site of Spain (Villoslada de Cameros) and allowed to moult under different temperature and relative humidity conditions. It was found that although the amount of each cuticular compound in the adult tick was affected by climate characteristics during the moulting period, the pattern of hydrocarbons (presence/absence of compounds) remained unaltered. Furthermore, it was shown that the source of a blood meal

did not affect the hydrocarbon pattern nor its profile. Thus, only the pattern of hydrocarbons (not the chromatographic profile) was used for computations in the present paper. The term 'sample' is used here to describe the specimens collected together in a given locality. The number of ticks used, the collection site and the code name of the final cluster after statistical analysis (see below) are given in Table 1 and Fig. 1. *Ixodes persulcatus* specimens were used as control (outgroup) samples.

For the hydrocarbon extraction procedures and analysis, the reader is referred to the papers of Kruger and Pappas (1993) and Estrada-Peña *et al.* (1993). Each specimen was processed separately, results from ticks of the same population being averaged for the frequency of the presence of each hydrocarbon.

The hydrocarbon data were initially screened for any obvious outlying individuals by applying the principal component analysis. Any unusual patterns were kept for further analysis. In later discriminant analysis, the SAS package was used to calculate, by stepwise selection of hydrocarbon peaks, the linear classification function for characterizing the differences between the populations involved in the analysis. To test the reliability of the separation made by the discriminant function, a jackknifed estimator of the proportion of correct classification was used (Kendal *et al.*, 1983). In this approach, error rates are gauged by 'leaving out' an individual when it is to be classified and using the classification function derived from the remainder of the cases in classifying the missing specimen. This ensures that the success rates are unbiased. Following the same procedure, each population was separately ascribed to a greater genetic assemblage. Each time a sample was included into a given aggregate, calculations were performed again to test for the integrity of the results. The final results of the stepwise discriminant analysis provided us with several assemblages, each of them composed of one or several of the samples listed in Table 1. The term groups of samples is used to describe the cluster of specimens, genetically very related and undistinguishable by the discriminant analysis, that provide the better statistical arrangement.

RESULTS

The cuticular hydrocarbon composition of *I. ricinus* has been described in a previous paper (Estrada-Peña *et al.*, 1994). The results of the stepwise discriminant analysis and the centroids of each group of samples is presented in Fig. 2. This analysis resolved the samples into ten phenotypically related groups. One cluster includes all the specimens from Germany, Switzerland, Poland, the Italian Alps and one sample from France (FR4), other aggregates include samples from Norway and Sweden and the third main group comprises populations from Denmark and The Netherlands. The remaining groups are composed of samples that cannot be ascribed to greater phenotypic assemblages: EI (Ireland), UK (United Kingdom), CH (Czech Republic), SLO

TABLE 1

Origin, code and number of specimens of *Ixodes ricinus* used in this paper

Country and collection site	Code	Number of males/females
Spain		
Villoslada de Cameros (Rioja)	S	35,22
Oyárzun (Guipúzcoa)	S	18,19
Oteo (Alava)	S	21,29
Oiz (Vizcaya)	S	19,32
Carranza (Vizcaya)	S	21,19
The Netherlands		
Ameland	NL	20,20
Switzerland		
Staatswald Forest (Ins)	SW	20,20
United Kingdom		
Winborne St Giles (Scotland)	UK	20,20
Haweswater (Cumbria)	UK	20,20
Sweden		
Dalarö	SE	9
Södertälje	SE	12
Norrtälje	SE	8
Trosa	SE	12,4
Hemse	SE	8,2
Söderköping	SE	6,2
Säle	SE	4
Sundbyberg (Uppsala)	SE	5,5
Tranas	SE	2,4
Italy		
North of the Alps	I	18,32
Ireland		
Killarney (County Kerry)	EI	20,20
Portumna (County Galway)	EI	20,20
Glencree (County Wicklow)	EI	20,20
Norway		
Bjondullu (Kuanne)	N	15,10
Skjaradalen (Kuanne)	N	12,8
Denmark		
Torbenfeldt-Zeeland	DE	20,20
Engharen-Zeeland	DE	20,20
France		
Rambouillet Forest (Paris)	FR	8,4
Azay le Ferran (Indre)	FR	4,10

Continued.

TABLE 1 (Continued)

Country and collection site	Code	Number of males/females
Paingost Forest (Ille-et-Vilaine)	FR	9,12
Alsace	FR	7,9
Slovakia		
Bratislava	SLO	12
Bánovce/Bebrava	SLO	14
Moldava/Bodrou	SLO	17,4
Topoliany	SLO	12
Ruská Poruba (Humenné)	SLO	21,2
Muzla	SLO	13,12
Plastovce	SLO	9
Sverepec (Povazska Bystrica)	SLO	15,3
Germany		
Gatow (Berlin)	G	20,20
Frohnau (Berlin)	G	20,20
Czech Republic		
Vyskov	CH	12,15
Leonice (Moravia)	CH	8,2
Svata, Beroum (Central Bohemia)	CH	18
Piske (Central Bohemia)	CH	14
Ceske Budejovice (South Bohemia)	CH	20,20
Strázkovice (South Bohemia)	CH	20,20
Poland		
Bialowieza Forest (Bialystok)	PO	2,5
Bytom-Stroszek (Katowice)	PO	5,1
Krzyna (Czestokova)	PO	16,12
Dolina Zachwytu (Kraków)	PO	5,6
Góra Rusztowa (Kraków)	PO	8,8
Kaliszak (Czestokova)	PO	11,7
Laznia (Bialystok)	PO	6,6
Murcki (Katowice)	PO	5,3
Okulinka (Chelm)	PO	2,8
Popielno (Suwalki)	PO	20,20
Sawin (Chelm)	PO	10,20
Skowronki (Elblag)	PO	10,10
Laki Sulistrowickie (Wroclaw)	PO	6,6
Szczyglice (Kraków)	PO	9,12
Troszyn (Szczecin)	PO	3,7
Wal Ruda (Tarnow)	PO	15,15
Zalas (Kraków)	PO	20,20
Zuraw (Czestokova)	PO	6,6
Control Populations		
I. persulcatus, former USSR	CLT	12,14
I. persulcatus, Vladivostock (Russia)	CLT	16,14

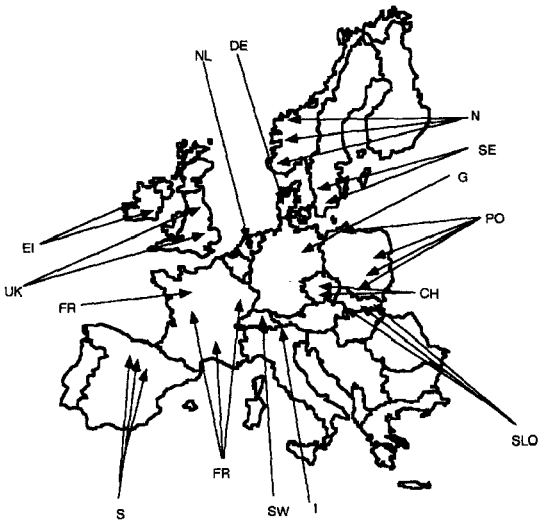

Fig. 1. Map of Europe showing the collection sites of the *I. ricinus* samples used in this study. For the codes, refer to Table 1.

(Slovakia), FR (all but one French sample), S (Spanish representatives) and CTL (*I. persulcatus* specimens). The method correctly allocated the control (outgroup) samples of *I. persulcatus* as separate from the remaining samples of *I. ricinus* in Europe, with a security of 100% according to the jackknifed estimator. The main assemblage of this species is composed of the samples collected in the central portion of Europe, the United Kingdom and Ireland. It must be noted that the technique is able to discriminate specimens from both the Czech Republic and Slovakia each as separate entities and also different from other Central Europe representatives. As already mentioned, Polish samples came into the broad category of CE specimens. The relative distance of both Sweden and Norway (NN group) to this main assemblage of populations is noteworthy. Furthermore, the unexpectedly high distance of Spanish samples from the main group of specimens is a striking feature of this analysis.

Discriminant analysis and population grouping give an output close to a geographic arrangement of the samples. Thus, G/SW/PO/I specimens remain close to the UK, CH and SLO groups, leaving N/SE in an intermediate position. Although statistically separate from this main cluster of populations, S ticks became closest to the FR assemblage.

DISCUSSION

We have presented here an indirect analysis of the genetic variation of 66 populations of *I. ricinus* through most of its European range, using cuticular hydrocarbon gas–liquid chromatography and stepwise discriminant analysis of

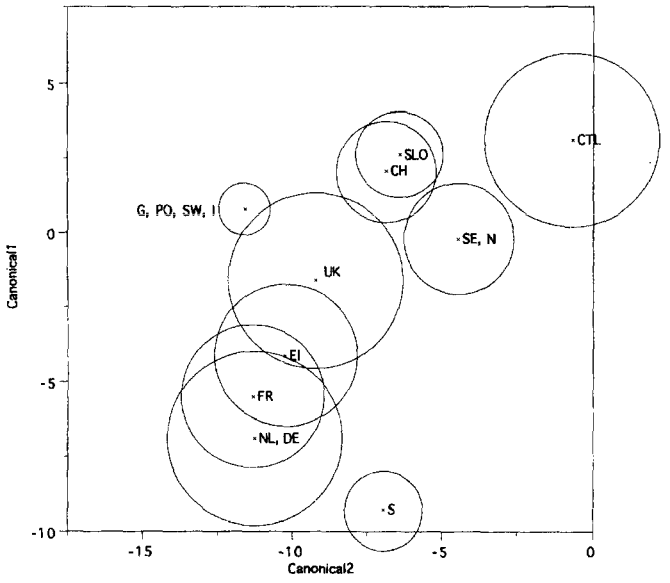

Fig. 2. Multivariate relationships of the *I. ricinus* populations by means of the stepwise discriminant analysis. Each circle is the 95% of the Gaussian bivariate and contains the populations grouped into a given assemblage; the abbreviations refer to the codes in Table 1.

the chemical patterns. Despite the lack of population-defining cuticular hydrocarbons and the small variation between samples (Estrada-Peña *et al.*, 1994), the method is able to separate several assemblages of *I. ricinus* and to distinguish these from *I. persulcatus* samples, which was used as an outgroup. Using discriminant analysis, the samples are separable into ten clusters of specimens. Samples from neighbouring localities are indistinguishable and the distance between groups of specimens is strongly correlated with the geographic distance between captures.

Previous results corroborate the validity of cuticular hydrocarbon analysis as an indirect tool for investigating the genetic relationships between populations of the same tick species, whatever the degree of geographical relationship. The cuticular hydrocarbon pattern has been used for taxonomic purposes (Phillips *et al.*, 1988) in insects, with some reports on ticks (Hunt, 1986; Estrada-Peña *et al.*, 1992a, 1993, 1994). Gas chromatography of surface hydrocarbons has been used for cluster analysis in the *Drosophila virilis* species group (Bartelt *et al.*, 1986), the results being in close agreement with the known phylogeny of the group. The method has also been tested for population relationships in *Aedes* species (Kruger and Pappas, 1993). In addition, the method is suitable for identifying hybrid ticks in nature (Estrada-Peña *et al.*, 1992b) or for recognizing the genetic distance between laboratory bred hybrid ticks (Estrada-Peña and Dusbábek, 1993). The hydrocarbon composition may be considered as an expression of genotype and, as such, it is available as a taxonomic character.

Ixodes ricinus is a member of the *I. ricinus/I. persulcatus* group (reviewed by Filippova (1991) and Keirans *et al.* (1992). These ticks are all members of the subgenus *Ixodes sensu stricto* and are included in an 'artificial' taxonomic construct containing 13 members. The phylogenetic distances of species within this group are not well defined yet, therefore discussion of the putative dispersal routes and intragroup relationships remain speculative. Filippova (1990) suggested that the roots of the *I. persulcatus* group could extend to the Palaeocene and also hypothesized (Filippova, 1991) that the distribution ranges (and subsequent disjunction) of a number of these species are related to the paleological distribution of nemoral mesophilic broad-leaved deciduous forest. *Ixodes persulcatus* is taxonomically closely related to *I. ricinus*, which it replaces in the north east of Europe, from the Baltic Sea shore and extending across northern Asia to Japan (Balashov, 1972).

Our data reveal a genetic subdivision on a continental scale that may imply restricted gene flow between regions. While some representatives remain in a relatively indistinguishable cluster (samples from the United Kingdom, Ireland, France, The Netherlands and Denmark) with considerable overlap of the samples involved, some others rest at a higher distance from the main group, such as ticks from Sweden and Norway and to a higher degree, Spanish representatives. In other words, peripheral populations are separated from the gene flow of the main group, the relative distance between sample phenotypes being proportional to the geographic distance. It must be noted that all the specimens collected in 'Central Europe' (Germany, Switzerland, Poland and the Italian Alps) are in the same cluster; however, specimens from the Czech Republic and Slovakia remain separated between each other and from CE individuals.

An apparent partial isolation may represent a non-equilibrium stage in the introgression of formerly isolated eastern and western types in the middle-eastern region. A similar pattern of variation has been noted for *Ixodes scapularis* populations in the USA (McLain *et al.*, 1995) with restricted gene flow between the different geographic regions of colonization of this species or, alternatively, recent introgression between northern and southern types in the middle-eastern part of the species range.

The geographic variation reflects the past action of microevolutionary forces that may act on various spatial scales (Sokal *et al.*, 1987) that may depend on either of two factors: habit discontinuity (Liebherr, 1988) or species vagility (Zera, 1981). Among ticks, there appears to be little genetic variation across species ranges (e.g. Hilburn and Sattler, 1988) in spite of their limited vagility. Because both the larvae and nymphs of *I. ricinus* readily feed on mammals, birds and reptiles, there may be few barriers to host-mediated dispersal across a variety of habitats.

The 'phylogenetic discontinuities' observed in our results may correspond to the current barriers inferred from regional geology and palaeoclimatic reconstruction. The samples of *I. ricinus* from Spain are unexpectedly separated

from the main stream of the species, even from geographically connected territories such as France. Spanish specimens seem to fall into a relic category, perhaps because of isolation by the last glaciation. The results suggest that gene flow between Spain and other European regions is now or was until recently restricted, permitting a relatively independent evolution of populations.

The present finding that greater genetic differentiation exists between geographically separated samples suggested that stochastic rather than deterministic forces maintain the pattern of variation in the cuticular hydrocarbons of *I. ricinus* The previously mentioned protocols carried out on the nymphal moulting phase, before the extensive analysis of the samples reported here, are suggestive that the climatic conditions are not responsible for the variation of the tick cuticular hydrocarbon pattern.

The method used in the present study may be of value as a genetic marker for studying the distribution and dispersal patterns of *I. ricinus* in Europe. In addition, genetic variation might be employed as a useful adjunct to morphological characters to resolve relationships between species of the *I. ricinus* complex. Additional work on the differential ability of tick strains to transmit certain *B. burgdorferi* genospecies is being carried out.

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