Aquat. Sci. 64 (2002) 66–75
1015-1621/02/010066-10
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Aquatic Sciences

Research Article

Isolation by distance, gene flow and phylogeography in the *Proasellus coxalis*-group (Crustacea, Isopoda) in Central Italy: allozyme data

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Received: 5 February 2001; revised manuscript accepted: 11 October 2001

Abstract. Fifteen populations belonging to the *Proasellus coxalis*-group were surveyed for genetic variation at 19 enzymatic loci in order to clarify the degree of genetic structuring of this species at the scale of Central Italy. With Neighbour-Joining analysis, it was possible to identify two main clusters, the first grouping populations sampled in coastal areas of Central Italy, the second comprising several populations from inland areas of the Apennines. In the first group, genetic relationships follow a geographic scheme, with populations arranged according to the proximity of the collecting sites. The inland populations have less clear geographic relationships and a higher degree of genetic differentiation. In view of the paleogeography of the study areas, I hypothesise a more recent penetration of this taxon into the Central Italian alluvial coastal plains (subjected to cyclical marine ingressions during the Quaternary) from sources of colonisation in the inland areas. The higher genetic differentiation of the inland populations presumably reflects a longer evolutionary history and could be explained by the periodic fragmentation of river catchments during Pleistocene climatic fluctuations. Such an evolutionary scenario is supported by *F-statistics* analyses. Effective dispersal of individuals between populations can be hypothesised for the coastal populations but not for the inland ones. On the whole, there is a pattern of isolation by distance, but a large amount of gene flow seems to reflect historical rather than ongoing gene exchange.

Key words. *Proasellus coxalis*; allozymes; gene flow; isolation by distance; phylogeography.

Introduction

Gene flow plays a central role in the evolution of species (Slatkin, 1987). A detailed understanding of its levels and patterns is a basic tool for the assessment of the genetic structuring, distribution and dispersal of a species at a given geographic scale. Data from allozyme electrophoresis have been widely used over the past two decades to elucidate the population genetic structure of many species.

In this paper, I focus on the freshwater isopod *Proasellus coxalis* (Dollfus, 1892), a polytypic species widely distributed in the Mediterranean region. Taxonomic studies, based mainly on the analysis of morphological characters, have led to the description of no less than 24 subspecies (Herhaus, 1977; Stoch, 1989). Other studies, using more accurate morphological traits and genetic characters (Volpi et al., 1989; Stoch et al., 1996), have revealed the inconsistency of this taxonomic arrangement, leading to the proposal of several well-differentiated species hidden under the name *coxalis*. More recently, the analysis of allozyme polymorphism was applied to several populations from Sardinia, Central Italy and Jordan (Ketmaier et al., 2001 a); it revealed a high degree of genetic differentiation among geographic groups, suggesting a complex evolutionary scenario. That paper presented data on six Central Italian populations:

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Published on Web: April 22, 2002

five of them were genetically homogeneous, whereas an Umbrian population was highly differentiated genetically. In the present paper, I report on data for 15 populations, sampled in different rivers of Central Italy, east and south of Umbria, on both sides of the Apennine Chain. The aim is to provide a more accurate description of the degree of genetic structuring of this taxon. The preliminary genetic data on the Central Italian populations reported in Ketmaier at al. (2001a) should assure that we are dealing, in this part of the genus range, with only one species, although its formal name is far from being established. For simplicity, I will indicate the study populations as belonging to the *P. coxalis*-group.

Freshwater species are of central interest in the study of the possible role of intrinsic (i.e., ability to disperse, reproductive behaviour, habitat specificity) and extrinsic factors (i.e., tectonic and/or glacial phenomena) in shaping the variation of allele frequencies. For instance, unidirectional gene flow due to water flow has been proposed as the main cause of genetic heterogeneity among populations of the mosquito fish *Gambusia holbrooki* (Hernandez-Martich and Smith, 1997). Isopods lack a larval stage that usually accounts for high rate of dispersal (Avise, 1994). *P. coxalis* is typical of mesosaprobic water and its presence indicates low hydrodynamism. In these conditions, dispersal is mainly due to water-column movements of juveniles and is limited to relatively short distances (Palmer et al., 1996). Such an ecological scenario could lead to an effective decrease of gene flow among geographically distinct populations, promoting the onset of local genetic differentiation. Besides these ecological and life history factors, one has to consider the changing environmental and ecological conditions (such as marine cyclical ingressions, fragmentation of river catchments due to glacial phenomena) that affected Central Italy during the Quaternary and that might have acted as biogeographic barriers to dispersal.

The aim of this paper is to determine the degree of genetic structuring and the levels of gene flow among 15 populations of the *P. coxalis*-group from neighbouring catchments. I intended to test if the geographic distance between populations is the main factor affecting their genetic structure, i.e., the presence of isolation by distance (Slatkin, 1993), or if exchanges throughout the study area are sufficient to counteract genetic differentiation.

Materials and methods

Fifteen populations, each identified by a three-letter code, were studied for genetic divergence and polymorphism. Details of the collecting sites and sample sizes are reported in Table 1. Populations from springs were all epigean and collected in surface water. Specimens were collected by hand and transported live to the laboratory and then stored at $-80\,°C$. The geographic locations of the study populations are illustrated in Figure 1. 11%–12% horizontal starch gel electrophoresis was performed on the crude homogenate of the whole body in TRIS HCl, 0.05 M, pH 7.5. Samples were screened for 13 enzymes, namely: Acid phosphatase (EC 3.1.3.2; *Acph-1, Acph-2*); α-Amylase (EC 3.2.1.1; *α-Amy*); Alkaline phosphatase (EC 3.1.3.1; *Aph-1, Aph-2*); Esterase (EC 3.1.1.1; *Est*); β-Glucosidase (EC 3.2.1.21; *β-Gluco*); Exokinase (EC 2.7.1.1; *Hk*); Isocitrate dehydrogenase (EC 1.1.1.42; *Idh-1, Idh-2*); Leucine amino peptidase (EC 3.4.11.1; *Lap*); Lactate dehydrogenase (EC 1.1.1.27; *Ldh*); Non-specific dehydrogenase (EC 1.6.99.1; *No-Dh-1, No-Dh-2*); Peptidase (EC 3.4.11; *Pep-1, Pep-2, Pep-3*); Phosphoglucomutase (EC 2.7.5.1; *Pgm*); Phosphohexose isomerase (EC 5.3.1.9; *Phi*).

Details of the buffer systems and staining techniques are the same as in Ketmaier et al. (2001a). The alleles at each locus were ranked according to electrophoretic mobility. The neutrality of the set of polymorphic loci was verified with the Ewens-Watterson test for neutrality

Table 1. Details on sampling localities, sample size and population codes.

Locality	Province – Region	Sample	Code	Altitude
Cantiano – Spring	Pesaro – Marche	30	CAN	360 m
San Polo – Stream Laia	Rieti – Latium	30	POL	514 m
Fiamignano – Spring	Rieti – Latium	30	FIA	988 m
Orvinio – Spring	Rieti – Latium	35	ORV	840 m
Veiano – Spring	Viterbo – Latium	20	VE1	400 m
Veiano – River Mignone	Viterbo – Latium	20	VE2	400 m
Bussi sul Tirino – River Tirino	Pescara – Abruzzo	26	BUS	344 m
Popoli – River Giardino	Pescara – Abruzzo	26	GIA	254 m
Tivoli – River Aniene	Roma – Latium	30	TIV	232 m
Sambuci – Stream Fiumicino	Roma – Latium	25	SAM	434 m
Roma – River Tiber Ponte del Grillo	Roma – Latium	36	TE1	sea level
Ostia – River Tiber Idroscalo	Roma – Latium	30	TE2	sea level
Circeo National Park – Pond	Latina – Latium	25	CIR	sea level
Sonnino – Spring	Latina – Latium	25	SON	sea level
Posta Fibreno – Lake Posta Fibreno	Frosinone – Latium	32	FIB	430 m

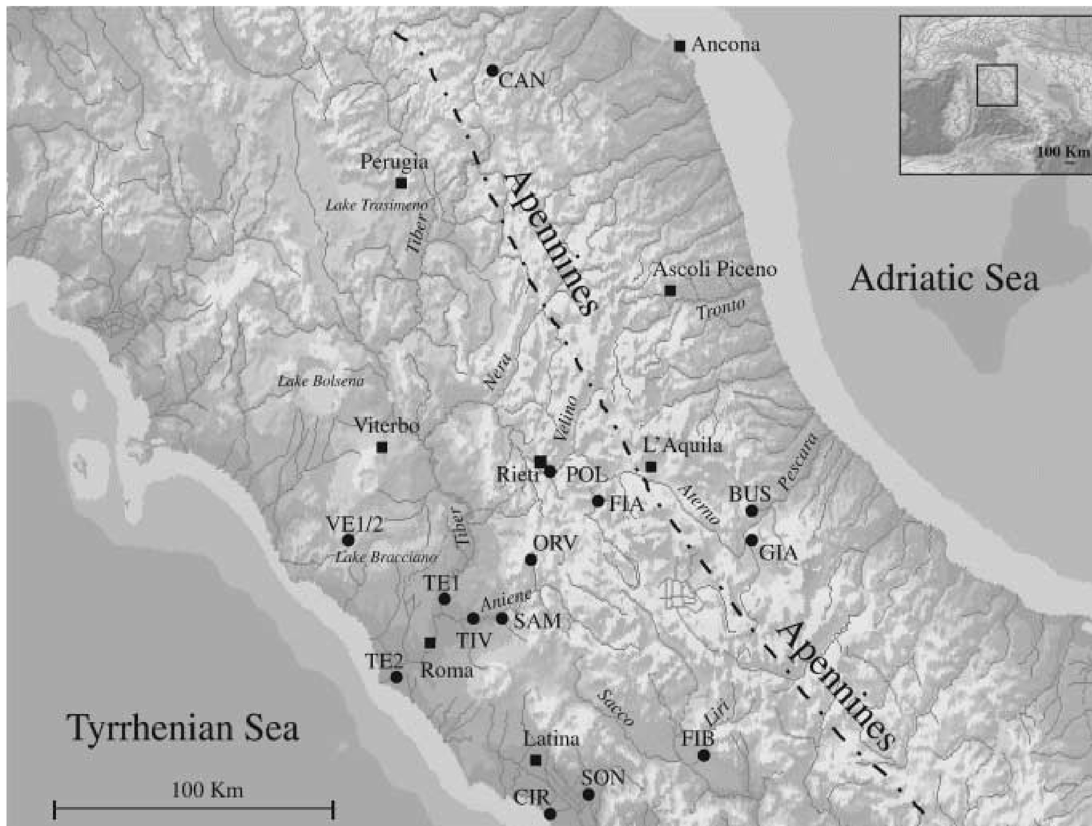

Figure 1. Sampling localities of study populations. For population codes see Table 1.

using the algorithm given in Manly (1985), as implemented in Popgene v 1.21 (Yeh et al., 1997). The upper and lower 95% confidence limits for the test were estimated by 1000 simulations. The genetic variability of samples was estimated by H_e (expected mean heterozygosity under Hardy-Weinberg equilibrium); H_o (observed mean heterozygosity); P (proportion of polymorphic loci according to the 0.99 criterion) and A (mean number of alleles per locus). Genetic distances between populations were estimated with the index D (Nei, 1978). Allele frequencies, variability estimates and D values were calculated with Biosys-1 (Swofford and Selander, 1981). D values were used to reconstruct evolutionary relationships among study populations by Neighbour-Joining (NJ, Saitou and Nei, 1987). The robustness of the NJ tree was tested by 1000 bootstrap replications (Felsenstein, 1985) by using Phylip 3.5 (Felsenstein, 1995). The degree of genetic structuring of study populations was investigated by Wright's *F-statistics* (Wright, 1978) calculated with the estimators of Weir and Cockerham (1984). These statistics partition total heterozygote deficiency into components due to deficiencies within populations (F_{is}) and subdivision among populations (F_{st}). F_{is} and F_{st} correspond to f and θ in Weir and Cockerham's notation. Jack-knifing per locus and over loci and relative standard errors were calculated. A 95% confidence interval of the f and θ values over all loci was also built, according to Weir (1990), by 1000 bootstrap replications. Estimates of gene flow were computed by θ values, according to the equation $Nm = 0.25\,(1-\theta)/\theta$ (Wright, 1931). θ and Nm values were also computed per pair of populations. *F-statistics* were calculated with the options available in Fstat (Goudet, 1995) and Genepop v.2 (Raymond and Rousset, 1995). To test for the existence of isolation by distance, I plotted the log-transformed pairwise gene flow values against the geographic distance values (*logNm* and *logKm* respectively) according to Slatkin (1993). The significance of the resulting regression (r) was tested with a Mantel test between the two matrices (Mantel, 1967). One thousand randomisations of the *logNm* and *logKm* matrices were performed to yield a distribution of regression coefficients. These distributions were compared with the observed ones. A one-tailed test was used because a hypothesis of isolation by distance predicts a negative correlation between gene flow and geographic distance.

Geographic distances (*Km*) between populations were determined from a 1:500,000-scale map using a planimeter. Distances between populations were calculated along watercourses and, whenever necessary, crossing major watersheds. Isolation by distance analyses were performed with Genepop v.2 (Raymond and Rousset, 1995) and Statistica for Windows.

Results

The 13 enzymes gave 19 activity zones interpreted as the product of as many gene loci. Allele frequencies and variability estimates are presented in Table 2. Three loci were monomorphic in all populations (*Acph-2; No-dh-1; Pep-1*). *No-dh-1* and *Pep-1* were fixed for the same allele in all populations; at *Acph-2*, POL was fixed for an alternative allele with respect to the other study populations. Sixteen loci (*Acph-1; α-Amy; Aph-1; Aph-2; Est; β-Gluco; Hk; Idh-1; Idh-2; Lap; Ldh; No-dh-2; Pep-2; Pep-3; Pgm; Phi*) were polymorphic in one population at least. At *β-Gluco*, CAN was fixed for an alternative allele. In several cases, there were strong differences in allele frequencies among populations. According to the results of the Ewens-Watterson test for neutrality: no loci deviated significantly from neutrality and they were all retained for further analyses. Table 3 reports the matrix of genetic distances *D* (Nei, 1978): *D* ranged from 0.447 (CAN vs POL) to 0.002 (VE1 vs VE2). CAN and POL

Table 2. Allele frequencies and genetic variability estimates (Standard Error in parentheses) for study populations. Letters are alleles at single loci; for each locus is given the sample size; for variability estimate codes see Materials and methods.

Pops. Locus	CAN	POL	FIA	ORV	VE1	VE2	BUS	GIA	TIV	SAM	TE1	TE2	CIR	SON	FIB
Acph-1	30	30	25	35	20	20	26	26	24	25	36	30	25	25	32
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.875	1.000	1.000	1.000	1.000	1.000	1.000
Acph-2	30	30	30	35	20	20	26	26	30	25	36	30	25	25	32
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
α-Amy	30	30	30	25	20	20	26	26	30	20	20	25	25	25	32
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.900	0.975	0.980	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.025	0.020	0.000	0.000	0.000
Aph-1	30	30	30	35	20	20	26	26	30	22	36	30	25	25	25
A	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.038	0.283	0.273	0.000	0.000	0.000	0.000	0.000
B	1.000	0.000	1.000	1.000	1.000	1.000	1.000	0.962	0.717	0.727	1.000	1.000	1.000	1.000	1.000
Aph-2	30	30	30	35	20	20	26	26	30	25	36	30	25	25	32
A	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.260	0.180	0.000
B	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.740	0.820	1.000
Est	30	30	30	29	20	20	22	21	30	25	36	25	25	20	25
A	0.000	0.000	0.000	0.103	0.900	1.000	0.136	0.262	0.000	0.000	0.000	0.040	0.000	0.000	0.000
B	1.000	1.000	1.000	0.897	0.100	0.000	0.864	0.738	1.000	0.480	1.000	0.960	1.000	0.875	0.260
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.520	0.000	0.000	0.000	0.125	0.740
β-Gluco	30	30	30	30	20	20	26	21	30	25	36	25	25	25	32
A	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.000	1.000	1.000	0.983	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.920	1.000	1.000	1.000
C	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.080	0.000	0.000	0.000
Hk	20	30	30	30	20	20	25	21	30	25	36	20	24	20	23
A	0.184	0.000	0.567	0.000	0.767	0.778	0.880	0.786	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.816	1.000	0.433	1.000	0.233	0.222	0.120	0.214	0.983	1.000	1.000	0.667	0.896	0.781	0.935
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.333	0.104	0.219	0.065
Idh-1	30	27	30	35	20	20	25	24	24	25	26	29	25	25	20
A	1.000	0.000	0.891	0.838	0.650	0.577	0.000	0.846	0.896	1.000	0.212	0.345	0.360	0.000	0.294
B	0.000	0.741	0.109	0.162	0.350	0.423	1.000	0.154	0.104	0.000	0.788	0.655	0.640	1.000	0.706
C	0.000	0.259	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Idh-2	30	27	30	35	20	20	25	24	24	25	26	29	25	25	20
A	0.950	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Lap	30	30	30	35	20	20	26	26	30	25	36	30	25	25	32
A	1.000	1.000	1.000	0.931	0.925	0.933	0.975	0.942	0.933	1.000	0.865	0.865	0.781	1.000	1.000
B	0.000	0.000	0.000	0.069	0.075	0.067	0.025	0.058	0.067	0.000	0.135	0.135	0.219	0.000	0.000
Ldh	20	30	30	30	20	20	25	21	30	25	36	20	24	20	23
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.025
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.975	1.000	0.940	1.000	0.975
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.060	0.000	0.000
No-dh-1	30	30	30	35	20	20	26	26	30	25	36	30	25	25	32
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table 2 (continued)

Pops.-Locus	CAN	POL	FIA	ORV	VE1	VE2	BUS	GIA	TIV	SAM	TE1	TE2	CIR	SON	FIB
No-dh-2	30	30	30	35	20	20	26	26	30	25	36	30	25	25	32
A	0.974	1.000	1.000	1.000	0.300	0.556	0.750	1.000	1.000	1.000	1.000	1.000	0.960	1.000	0.800
B	0.026	0.000	0.000	0.000	0.700	0.444	0.250	0.000	0.000	0.000	0.000	0.000	0.040	0.000	0.200
Pep-1	28	30	30	33	20	20	25	21	30	22	35	30	20	20	20
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pep-2	28	30	30	33	20	20	25	21	30	22	35	30	20	20	20
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000
B	0.914	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	0.086	1.000	1.000	0.983	1.000	1.000	1.000	1.000	0.733	0.980	1.000	1.000	0.975	1.000	1.000
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.267	0.020	0.000	0.000	0.000	0.000	0.000
Pep-3	28	30	30	33	20	20	25	21	30	22	35	30	20	20	20
A	1.000	1.000	1.000	0.983	0.850	0.643	1.000	1.000	1.000	0.980	1.000	1.000	0.800	1.000	0.500
B	0.000	0.000	0.000	0.017	0.150	0.357	0.000	0.000	0.000	0.020	0.000	0.000	0.200	0.000	0.500
Pgm	30	30	30	35	20	20	26	26	30	25	36	30	25	25	32
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000
B	0.000	1.000	1.000	1.000	1.000	1.000	0.925	1.000	0.857	0.717	0.968	0.778	0.800	1.000	0.844
C	1.000	0.000	0.000	0.000	0.000	0.000	0.075	0.000	0.143	0.283	0.032	0.222	0.180	0.000	0.156
Phi	30	30	30	35	20	20	26	26	30	25	36	30	25	25	32
A	1.000	1.000	1.000	0.833	0.969	0.950	0.920	0.942	1.000	1.000	1.000	0.820	1.000	1.000	1.000
B	0.000	0.000	0.000	0.167	0.031	0.050	0.080	0.058	0.000	0.000	0.000	0.180	0.000	0.000	0.000
Variability estimates (SD)															
A	1.2	1.1	1.1	1.4	1.4	1.3	1.3	1.3	1.4	1.3	1.3	1.4	1.5	1.2	1.4
	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)
*P**	21.1	5.3	10.5	36.8	36.8	31.6	31.6	31.6	36.8	31.6	26.3	42.1	47.4	15.8	36.8
H_o	0.020	0.008	0.013	0.039	0.062	0.090	0.047	0.035	0.035	0.048	0.025	0.047	0.089	0.025	0.056
	(0.011)	(0.008)	(0.011)	(0.018)	(0.023)	(0.036)	(0.019)	(0.015)	(0.013)	(0.021)	(0.014)	(0.015)	(0.030)	(0.016)	(0.022)
H_e**	0.033	0.021	0.062	0.255	0.098	0.052	0.101	0.112	0.069	0.037	0.086	0.084	0.039	0.110	0.122
	(0.018)	(0.021)	(0.026)	(0.053)	(0.047)	(0.022)	(0.037)	(0.045)	(0.029)	(0.028)	(0.032)	(0.038)	(0.021)	(0.038)	(0.038)

* a locus is considered polymorphic if the frequency of the most common allele does not exceed 0.99.
** unbiased estimate (see Nei, 1978).

Table 3. Genetic distance values *D* (Nei, 1978) between study populations (below the diagonal).

Pops	CAN	POL	FIA	ORV	VE1	VE2	BUS	GIA	TIV	SAM	TE1	TE2	CIR	SON	FIB
CAN	****														
POL	0.447	****													
FIA	0.177	0.240	****												
ORV	0.172	0.204	0.020	****											
VE1	0.297	0.211	0.082	0.105	****										
VE2	0.303	0.193	0.083	0.104	0.002	****									
BUS	0.266	0.223	0.055	0.089	0.072	0.071	****								
GIA	0.200	0.223	0.006	0.036	0.054	0.052	0.045	****							
TIV	0.151	0.198	0.027	0.011	0.130	0.133	0.107	0.048	****						
SAM	0.163	0.192	0.042	0.024	0.120	0.120	0.131	0.056	0.021	****					
TE1	0.208	0.182	0.044	0.024	0.124	0.121	0.051	0.062	0.036	0.059	****				
TE2	0.179	0.198	0.035	0.022	0.113	0.110	0.047	0.047	0.034	0.053	0.009	****			
CIR	0.189	0.204	0.041	0.023	0.121	0.115	0.060	0.059	0.033	0.054	0.007	0.010	****		
SON	0.241	0.180	0.060	0.046	0.128	0.121	0.041	0.074	0.061	0.080	0.008	0.014	0.014	****	
FIB	0.247	0.196	0.087	0.064	0.106	0.092	0.090	0.096	0.081	0.053	0.049	0.055	0.045	0.046	****

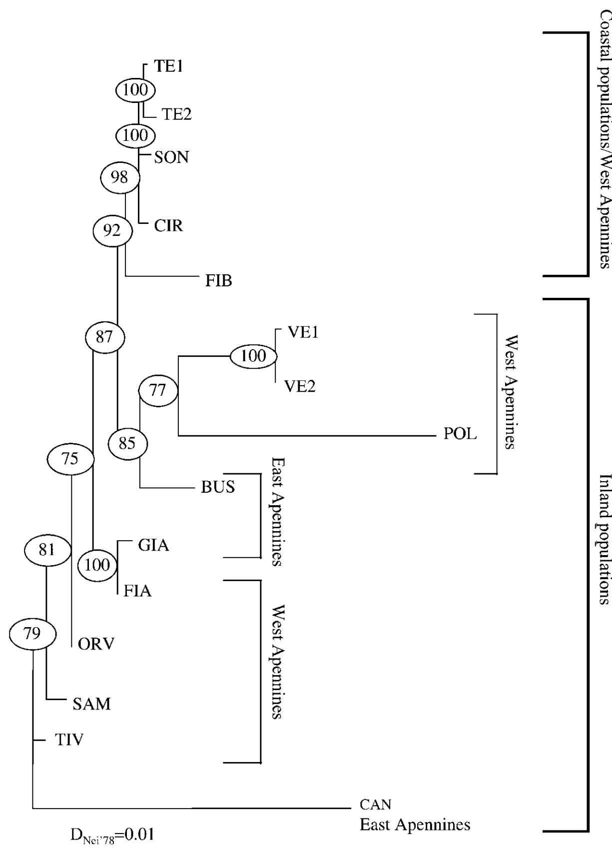

Figure 2. Majority rule consensus tree obtained by NJ bootstrap analysis. Circled nodes include bootstrap percentages of 1000 replications, only nodes with a bootstrap support above 50% are shown. The tree is unrooted.

were, on average, the most differentiated populations, with mean *D* values of 0.231 and 0.220, respectively, versus the other populations. Figure 2 illustrates the 50% majority rule consensus tree obtained by NJ bootstrap analyses. Due to the lack of a clear outgroup, this tree is unrooted. Nodes of this tree were well supported by the associated bootstrap values. CAN is basal in the tree, followed by TIV and SAM. The two populations from the eastern slope of the Apennine chain (BUS and GIA) do not cluster together. The other populations are generally grouped according to the proximity of their sampling catchments; there are two main clusters, the first including the more coastal populations (TE1, TE2, SON, CIR and FIB) and the second with some of the inland populations (VE1, VE2 and POL). Populations from the same river (i.e., TE1, TE2 and VE1, VE2) cluster together.

The estimates of f and θ, and the associated jack-knifing values at single loci and over all loci, are summarised in Table 4. The f values were significant at $p \leq 0.05$ for all loci except *α-Amy; β-Gluco; Idh-2; Lap; Ldh; Pep-2; Pep-3*. Generally large positive f values were obtained, indicating heterozygote deficiency. The f value over all loci (0.434, $p \leq 0.05$) supports this trend and falls within the bootstrapped 95% Confidence Interval (0.275–0.550).

High and significant θ values were obtained at single loci (Table 4), ranging from 0.089 ($p \leq 0.05$, locus *Phi*) to 0.926 ($p \leq 0.05$, locus *β-Gluco*); the θ value over all loci was 0.585 ($p \leq 0.05$), confirming a pattern of strong genetic differentiation. The θ value over all loci was

Table 4. Analysis of θ and f values at single locus and over all loci for the *P. coxalis*-group populations. Jack-knifing and bootstrapping analyses are also reported.

Locus	θ	θ-jack-knifing (SD)	f	f-jack-knifing (SD)
Acph-1	0.099***	0.190 (0.092)	0.631*	1.219 (0.589)
α-Amy	0.055***	0.083 (0.053)	– 0.060	– 0.091 (0.057)
Aph-1	0.723***	0.998 (0.501)	0.703***	0.748 (0.101)
Aph-2	0.197***	0.202 (0.065)	0.427**	0.335 (0.541)
Est	0.712***	0.726 (0.112)	0.296***	0.317 (0.112)
β-Gluco	0.926***	1.678 (0.824)	– 0.053	– 0.091 (0.054)
Hk	0.563***	0.566 (0.089)	0.545***	0.554 (0.093)
Idh-1	0.523***	0.527 (0.097)	0.671***	0.673 (0.065)
Idh-2	0.033*	0.063 (0.030)	– 0.033	– 0.064 (0.031)
Lap	0.058***	0.056 (0.025)	0.064	0.065 (0.075)
Ldh	0.026***	0.035 (0.022)	– 0.028	– 0.039 (0.023)
No-dh-2	0.432***	0.499 (0.219)	0.555***	0.575 (0.137)
Pep-2	0.719***	1.046 (0.512)	0.290	0.456 (0.341)
Pep-3	0.329***	0.388 (0.154)	0.060	0.108 (0.195)
Pgm	0.575***	0.784 (0.457)	0.471***	0.478 (0.112)
Phi	0.089***	0.091 (0.025)	0.373***	0.394 (0.276)
Over all loci	0.585***	–	0.434***	–
Jack-knifing over all loci	–	0.587 (0.049)	–	0.445 (0.080)
Bootstrapping over all loci (95% CI)	0.478–0.676	–	0.275–0.550	–

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Table 5. θ (above the diagonal) and *Nm* (below the diagonal) values between all study populations.

Pops.	CAN	POL	FIA	ORV	VE1	VE2	BUS	GIA	TIV	SAM	TE1	TE2	CIR	SON	FIB
CAN	****	0.973	0.819	0.794	0.798	0.813	0.832	0.782	0.710	0.732	0.856	0.697	0.696	0.847	0.751
POL	0.017	****	0.875	0.834	0.776	0.799	0.840	0.823	0.768	0.777	0.846	0.745	0.730	0.847	0.727
FIA	0.055	0.036	****	0.289	0.531	0.571	0.526	0.088	0.293	0.408	0.532	0.307	0.337	0.580	0.517
ORV	0.065	0.050	0.614	****	0.569	0.596	0.599	0.363	0.136	0.257	0.341	0.214	0.214	0.476	0.429
VE1	0.065	0.072	0.221	0.189	****	0.011	0.441	0.364	0.549	0.530	0.647	0.471	0.472	0.609	0.452
VE2	0.057	0.063	0.187	0.169	21.626	*****	0.458	0.376	0.558	0.550	0.676	0.470	0.457	0.634	0.409
BUS	0.050	0.047	0.225	0.167	0.316	0.295	****	0.391	0.567	0.613	0.518	0.330	0.374	0.424	0.481
GIA	0.070	0.054	2.563	0.438	0.436	0.413	0.388	****	0.364	0.401	0.541	0.322	0.361	0.529	0.481
TIV	0.102	0.075	0.602	1.579	0.205	0.198	0.191	0.437	****	0.172	0.363	0.239	0.214	0.470	0.423
SAM	0.091	0.072	0.362	0.722	0.222	0.204	0.157	0.372	1.197	****	0.494	0.330	0.319	0.529	0.329
TE1	0.042	0.045	0.220	0.481	0.136	0.119	0.232	0.212	0.438	0.255	****	0.121	0.092	0.173	0.397
TE2	0.109	0.085	0.563	0.915	0.281	0.281	0.506	0.525	0.794	0.506	1.813	****	0.066	0.136	0.307
CIR	0.109	0.092	0.491	0.917	0.279	0.296	0.417	0.441	0.918	0.532	2.446	3.501	****	0.143	0.252
SON	0.045	0.045	0.181	0.274	0.160	0.144	0.339	0.222	0.281	0.222	1.195	1.588	1.496	****	0.351
FIB	0.083	0.094	0.233	0.332	0.303	0.360	0.269	0.269	0.341	0.508	0.379	0.562	0.741	0.462	****

confirmed by the bootstrap over all loci analysis (95% Confidence Interval 0.478–0.676). θ and *Nm* values were also calculated for pairwise comparisons to obtain a more detailed description of the pattern of genetic structuring: the results are given in Table 5. θ ranged from 0.011 (VE1 vs VE2) to 0.937 (CAN vs POL); the same comparisons gave the highest and lowest *Nm* values: 21.626 and 0.017, respectively. Only 10 of the 105 pairwise *Nm* values were greater than 1.

The plot of pairwise *logNms* and *logKms* between sites is shown in Figure 3. The regression coefficient (r) was -0.449 ($p = 0.0004$) (linear regression analysis is $logNm = 0.658 - 0.667\ logKm$), indicating isolation by distance between pairs of populations. None of the 1000 regression coefficients resulting from the Mantel test randomisation was less than or equal to the observed value; this indicates a significant negative correlation between *logKms* and *logNms*.

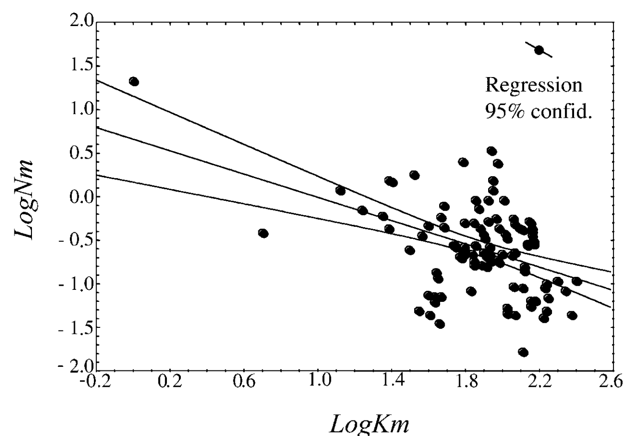

Figure 3. Regression analysis based on *logNms* and *logKms* values for the study populations. Linear regression analysis is $logNm = 0.658 - 0.667\ logKm$, the regression coefficient r is -0.449 ($p = 0.0004$).

Discussion

The analysis of genetic distance values (Table 3) shows that CAN and POL are the two most genetically differentiated populations, with an average $D_{Nei'78}$ value of around 0.200 from the remaining populations. This value is close to the average $D_{Nei'72}$ between subspecies and/or incipient species in invertebrates (Ayala, 1983) and to the values among conspecific populations of several crustacean species (reported in the review of Hedgecock et al., 1982). CAN vs POL also had the highest $D_{Nei'78}$ value (0.447) among all the comparisons. The degree of genetic divergence between these two putative conspecific populations is no less than that observed among morphological species of isopods (Cobolli Sbordoni et al., 1997; Gentile and Allegrucci, 1999; Ketmaier et al., 2001 a). This underlines how populations of the *P. coxalis*-group tend to accumulate electrophoretically detectable differences even at a relatively small geographic scale. Ketmaier et al. (2001 a) hypothesised that the Central Italian *Proasellus* populations belong to at least two different waves of colonisation, the most ancient one being in the Umbria region. Although CAN is quite differentiated from the other populations at the allozyme level, it does not represent the ancient Umbrian lineage. The mean value of $D_{Nei'78}$ between the Umbrian lineage and the other Central Italian populations reported in Ketmaier et al. (2001 a) is 0.912, much higher than the value reported in the present paper between CAN and the remaining populations. It can be hypothesised that CAN is at the extreme of the geographic variation of the *P. coxalis*-group (possibly a new specific entity), as indicated by its basal position in the NJ tree (Fig. 2). However, at present the number of sampled populations is not sufficient to draw definite conclusions. Interestingly, in the NJ tree, POL does not cluster separately, as expected on the basis of the genetic distance values alone, but joins

VE1, VE2 and BUS in a cluster that is well supported in terms of bootstrap values.

On the whole, there is not always a clear geographic pattern in the relationships derived from the NJ analysis. In the cluster grouping TE1, TE2, SON, CIR and FIB, the relationships among populations seem to be determined mainly by the proximity of the collecting sites, as expected under a model of isolation by distance. In contrast, the position of BUS and GIA (the other two populations, with CAN, collected on the eastern slope of the Apennine Chain) in two different groups does not agree with their geographic distribution. The two main clusters identified in Figure 2 (inland/coastal populations) do not show the same degree of differentiation among populations: the first (coastal populations) is less structured than the second. Differences in the paleohistory of the sampling localities could have played an important role in shaping the pattern of relationships revealed by allozymes. TE1, TE2, SON and CIR were sampled at sites in the alluvial plains of Latium close to the coast (and therefore "true" coastal populations), FIB is from a lake at the foot of the Apennines, along the Sacco and Liri alluvial plains, whereas VE1, VE2, POL and BUS are from inland areas of the Apennines. According to Ambrosetti et al. (1979), the Latium alluvial plains were recurrently covered by the sea during the Plio-Pleistocene (from 3.3 to 0.3 million years ago), which could have caused the extinction of almost all the freshwater fauna in the area. It is not unlikely that the present *Proasellus* populations colonised this area at the end of the marine cyclical ingressions, presumably during the Pleistocene. Inland areas, not affected by sea ingressions, could have acted as a source of colonisation when favourable environments became available (La Greca, 1995, 1997). At the same time, inland areas were affected by glacial phenomena that could have led to periodic fragmentation of river catchments. This may have caused the more pronounced genetic structuring of these populations. This hypothesis is supported by the divergence times calculated on the basis of the genetic distance values, applying the allozyme rate proposed for Aselloidea in Ketmaier et al. (1999) ($T_{myr} = 0.234 + 19.262\ D_{Nei'78}$). Though these speculations are usually associated with large errors and need to be considered with caution, the main splits inferred from the NJ topology occurred 2.5 to 1.5 million years ago, which roughly corresponds to the Biber and Donau glacial periods (West, 1977; Ambrosetti et al., 1979). Interestingly, several study cases reported a similar contrasting pattern of strong inland/weak coastal genetic structuring in species whose distribution could have been subdivided by Quaternary glaciations (Alexandrino et al., 2000; Gentile and Allegrucci, 1999; Hurwood and Hughes, 2001). At the same time, more recent events, such as environmental alterations due to human activities, could have played a certain role in shaping the observed pattern of genetic variation (De Matthaeis et al., 2000).

The *F-statistics* analysis revealed a strong pattern of genetic structuring among the study populations, the θ value over all loci (0.585; $p \leq 0.005$) indicating that a large component of the genetic variation is due to differences among populations. Thus the *P. coxalis*-group in Central Italy can be seen as an assemblage of semi-isolated populations, connected by relatively low levels of gene flow. The existence of a clear pattern of isolation by distance among these populations indicates that a stepping-stone model (Kimura and Weiss, 1964) could be adopted to describe the spatial distribution of genetic heterogeneity at the geographic scale considered here. In the stepping stone model, there is a correlation between gene flow and geographic distance, because the probability of gene flow is higher between nearby populations.

Slatkin (1993) reconsidered the theory of isolation by distance to test whether populations with restricted dispersal were in equilibrium between gene flow and genetic drift. In that paper, Slatkin adopted a simulation approach and showed that the results were reliable with an adequate sample size and a low mutation rate. Neither of these assumptions is violated in my study; the sample size (Table1) is always higher than 20 individuals per population and the mutation rate can reasonably be assumed to be low since we are dealing with a relatively short-term evolutionary study case. According to the generalisations proposed by Slatkin (1993), the pattern of genetic structuring revealed in the present study suggests that the populations are in equilibrium between gene flow and genetic drift under relatively low levels of dispersal.

One of the main problems in studies aimed at estimating the degree of genetic connection between natural populations is to understand when a certain estimate of *Nm* indicates high or low levels of ongoing gene flow (Avise, 1994). According to Wright (1931), genetic drift will not produce significant genetic differentiation between populations if $Nm \geq 1$ (i.e., the average exchange of one individual per generation). Although this is a theoretical estimate, it has been successfully applied to describe the genetic structuring of several species (i.e., Cobolli Sbordoni et al., 1997; De Matthaeis et al., 1998, 2000; Hernandez-Martich and Smith, 1997; Ketmaier et al., 2001b). The sampling strategy of this study was designed to obtain individuals from two collecting sites (VE1 and VE2) that are only a few hundred meters apart along the River Mignone (Fig. 1). In this case, it can be assumed that ongoing gene exchange is occurring between the two populations. Similarly, it is reasonable to hypothesise that a certain degree of ongoing gene flow occurs between TE1 and TE2, collected along the River Tiber. The *Nm* value for these populations is lower than the one between VE1 and VE2, because of the greater geographic distance separating TE1 and TE2. Nevertheless their genetic similarity is clear from the NJ branching pattern (Fig. 2). At the same time, the high degree

of genetic differentiation shown by CAN, as well as its position in the NJ tree (Fig. 2), reflect the complete absence of gene flow between it and the remaining populations. In this way, it is possible to quantify, albeit tentatively, a low (0.017–0.109) and a high (1.813–21.626) range of threshold values in which gene flow can be excluded or assumed respectively. This is of particular importance in discriminating between the historical and ongoing gene flow (Larson et al., 1984). If these criteria are adopted, only the group of populations from the alluvial plain of Latium are presently connected by effective gene flow, whereas most of the *Nm* pairwise comparisons fall within or near the low threshold range. Relatively low levels of gene flow are not unusual among freshwater macro-invertebrates, such as crayfish and insects with aquatic larval stages (Scillitani et al., 1996; Lörtscher et al., 1998; Ketmaier et al., 2001 b). All these taxa lack stages able to achieve a high rate of dispersal and their patterns of genetic structuring are mainly shaped by obvious geographic barriers to migration, such as the lack of connections between river systems. Data for the *P. coxalis*-group indicate that when routes for dispersal are available, as in the case of the populations from the Latium alluvial plain, this species is able to achieve rates of gene flow high enough to counteract local genetic differentiation. In contrast, in inland areas, the lack of connections between river basins can be assumed to be the main extrinsic factor responsible for the observed level of genetic fragmentation.

Both the inland and coastal populations are characterised by significant departures from Hardy-Weinberg expectations on account of heterozygote deficiency. Selection was hypothesised for the locus *α-Amy* in the freshwater isopod *Asellus aquaticus* (Christensen et al., 1974; Christensen, 1977), but in the present study this is one of the few loci that does not deviate from Hardy-Weinberg proportions. Some characteristics related to the population ecology of this species could account for these results. According to Solla and Basset (1996), at low density, *P. coxalis* individuals mate earlier and produce more offspring, whereas this pattern is reversed at high density. This suggests a selective action of density on reproductive phenomena. At the sampling sites, the species were always very abundant. In this condition, offspring mortality could be severely affected by the ability to respond to limiting factors (i.e., food availability). It is generally held that the superior fitness of heterozygotes is due to their higher metabolism. However, this might not be a favourable trait in conditions of food scarcity, since a higher metabolic rate corresponds to a higher food demand (Singh and Green, 1984). In contrast, homozygotes could be favoured by selection, because of their lower food demands, leading to an excess of homozygotes at several loci in electrophoretic surveys. A similar explanation was proposed by Falniowski et al. (1999) to account for the homozygote excess in the spring snail *Bythinella*. Further analyses are obviously needed to solve this crucial point in the *P. coxalis*-group.

The results of the present study add to those reported in Ketmaier et al. (2001 a) and lead to some conclusions about the phylogeography and genetic structuring of this taxon at the scale of Central Italy. Populations from inland and coastal areas are characterised by different levels of gene flow. In general, the pattern matches an isolation by distance model, although a large amount of the detected gene flow seems to reflect historical rather than ongoing gene exchange.

Acknowledgments

I thank Roberto Argano (University of Rome “La Sapienza”) for his advice and collaboration and three anonymous reviewers for helpful comments on a first draft of this paper. My deepest debt is to Elvira De Matthaeis and Marina Cobolli (University of Rome “La Sapienza”) for providing continuous advice and support as well as laboratory facilities. Financial support came from the Ministero della Ricerca Scientifica e Tecnologica, MURST ex-40%, 1999 (Variazione geografica e biodiversità: cause evolutive e conseguenze ecologiche) and the National Research Council (C.N.R.) to Elvira De Matthaeis. The data presented are part of VK’s Ph.D. thesis project.

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