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A comparison between allozyme data and phenotypic distances from defensive secretion in *Oreina* leaf-beetles (Chrysomelinae)

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Abstract

The genetic relationships between five *Oreina* species (Chrysomelidae, Coleoptera) were studied. Of these species, four (*O. bifrons*, *O. gloriosa*, *O. speciosa*, *O. variabilis*) feed on Apiaceae and secrete mixtures of autogenous cardenolides from defensive glands, whilst the other (*O. speciosissima*) feeds on Asteraceae and is able both to produce cardenolides and to sequester pyrrolizidines N-oxides (PAs). A dendrogram based on the different mixtures of cardenolides produced by the different species agreed with these genetic relationships. In other words, cardenolide mixtures are good taxonomic markers, since the clustering method based on chemical defense produces a branching pattern similar to that based on genetic relationships.

Introduction

Chrysomeline leaf beetles are aposematic and chemically defended by a secretion oozing from pronotal and elytral glands (Deroe and Pasteels, 1982; Pasteels et al., 1989). In the Chrysolinina, most species produce autogenous cardenolides (Van Oycke et al., 1987; Pasteels, 1993). The genus *Oreina* belongs to this subtribe, and several of its species secrete cardenolides. The wide distribution of cardenolides in the defensive secretions of two different genera of Chrysolinina and in the members of the related subtribe Doryphorina (Timmermans et al., 1992), irrespective of the food plants on which the beetles are specialized (they represent at least 8 different

plant families), suggests that the secretion of autogenous cardenolides might be the primitive condition in these taxa. A few *Oreina* species, however, secrete both cardenolides and pyrrolizidine alkaloid N-oxides (PAs) which are sequestered from the food plants (Pasteels et al., 1988; Rowell-Rahier et al., 1991; Pasteels, 1993) and one species (*O. cacaliae*) secretes only PAs. Those species secreting PAs feed on Asteraceae containing PAs, whereas those species producing cardenolides feed on plants (mostly Apiaceae) containing neither PAs, nor cardenolides.

The main aim of this paper is to compare genetic data with phenotypic distances based on defensive cardenolides, since by comparing phylogenies obtained using different data sets one can gain insight into the evolution of different characters. We evaluate the cardenolide mixtures as taxonomic markers by comparing the topology of dendrogram based on a) chemical defense and b) on allelic data.

We have determined allozyme frequencies and the exact composition of the defensive mixtures in 5 *Oreina* species: four species feeding on Apiaceae and producing cardenolides (*O. bifrons*, *O. gloriosa*, *O. speciosa*, *O. variabilis*), and one species able both to produce cardenolides and to sequester PAs from Asteraceae (*O. speciosissima*).

Material and methods

Collecting sites. The sites where the seven different geographically separated populations belonging to a total of five *Oreina* species were collected are summarized in Tab. 1. Sympatric *Oreina* species are also given in Tab. 1. For the *Oreina* nomenclature we follow Bourdonné and Doguet (1991). The sites are described in Rowell-Rahier (1992) and Eggenberger and Rowell-Rahier (1991). Each beetle population was sampled by collecting insects along a transect. The host plants were common and regularly spaced along this transect.

Table 1. Collecting sites of the seven populations studied in this paper. The abbreviations given here for each population are used in all the other Tables and Figures. * indicates that the species is present but was not included in this study.

Collecting sites:	AP	BV	VA	VD	VO
***Oreina* spp.**					
O. bifrons	*		bi1		bi2
O. gloriosa		*	g1		
O. speciosa	*	sp1	sp2		
O. speciosissima	*			si	
O. variabilis	va				

AP, Swiss Appenzell (Brulisau); BV, southern French Alps near Glacier Blanc (Les Bans, Vallouise); VA, Swiss Valais (Anniviers for bil and sp2 and Saas Fee for g1); VD, Swiss Vauds (La Lécherette); VO, French Vosges (Le Hohwald).

Allozyme electrophoresis. The method was similar to that of Rowell-Rahier (1992). The thoracic muscles of each beetle were dissected and homogenized in a buffer solution (Tris at pH 7 with mercaptoethanol) then absorbed onto filter paper wicks. Starch gel electrophoresis was performed on 6 enzyme loci which were polymorphic in all *Oreina* species studied here: *aconitase* (*acon-1*; E.C. 4.2.1.3), *aspartate aminotransferase* (*aat-1*; E.C. 2.6.1.1, *fumarate hydratase* (*fumh-1*; E.C. 4.2.1.2), *glycerol-3-phosphate dehydrogenase* (*g3pdh-1*; E.C. 1.1.1.8), *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh-1*; E.C. 1.2.1.2), and *malate dehydrogenase* (*mdhp-1*; E.C. 1.1.1.40). The enzyme nomenclature follows the new usage of Murphy et al. (1990). Only polymorphic loci were used because the main purpose of the study was to compare genetic and phenotypic variation rather than to establish a rigorous phylogeny. All gels were run at 50 mA in refrigerated chambers. Three buffer systems were used: tris citrate (pH 6.7, *acon-1*, run time 5 hrs), a discontinuous tris citrate buffer (TCB, pH 8.0, *fumh-1*, *mdhp-1*, run time 4 hrs), and a discontinuous high pH buffer (Tris Borate EDTA I, pH 8.6, *aat-1*, *g3pdh-1*, and *gapdh-1* run time 3 hrs). For more details on the buffer and staining protocols used, see Eggenberger and Rowell-Rahier (1991) and Murphy et al. (1990).

Statistical analysis. Departure from Hardy-Weinberg expectations (HWE) were tested at each locus using BIOSYS software (Swofford and Selander, 1981). Before testing, the genotypes were sorted into homozygotes for the most common allele, heterozygotes of the most common allele and one of the other alleles, and heterozygotes and homozygotes of the rarer alleles. This procedure is a conservative test, because it does not detect departures among the rarer alleles (Weir, 1990a). Exact tests were used in significance testing to avoid the problems posed by low expected frequencies in some genotypic classes. The modified Rogers' distance (Wright, 1978) was calculated. Hierarchical cluster analysis using the unweighted pair-group method with arithmetic averging (UPGMA) was performed to produce a dendrogram of genetic distances between the studied populations. This distance was selected because it is appropriate for the Mantel statistic described below.

Defensive secretions. Seven populations producing only cardenolides and in which the secretion was collected in sufficient amount for quantitative chemical analysis were utilized (bi1, bi2, gl1, si3, sp1, sp2 and va1).

Defensive secretions of freshly field-collected beetles were collected on pieces of filter paper, and at least 15 individual secretions pooled per population to minimize the effects of individual variation (see below) and stored in methanol at −20 C. Before chemical analysis, the methanol was evaporated and the secretion dissolved in 10% acetonitril in water. The samples were analysed by High Pressure Liquid Chromatography (HPLC) as described in Eggenberger and Rowell-Rahier (1992) [two-pump system (Waters 510); detector: photodiode array (Waters 994), 220 nm; column: Macherey-Nagel cartridge, C-18, 3 μm, 4 × 130 mm; eluent: chromatography grade acetonitrile (Baker) and water (Merck), 15–42% acetonitrile linear in 36 min, 0.45 ml/min; data analysis: Maxima 820 data station]. The area of each separate peak was quantified relative to the total area of the peaks produced by the

sample. Only the 38 peaks which had a UV spectrum typical of cardenolides were used. Thus, for each population, the proportion of each of the different cardenolide peaks in the defensive mixture was estimated.

The data were standardized to mean zero and variance 1 and a hierarchical cluster analysis (UPGMA) was performed to obtain an evaluation of phenotypic distances (using Euclidean distances) between the 7 populations.

Comparative analysis of genetic and chemical data. The Mantel non-parametric statistic (Mantel, 1967; Dietz, 1983) was used to compare the two data sets: i) a genetic distance matrix constructed using the modified Rogers' distances (Nei's distance is inappropriate for this test since it is not metric) and ii) a phenotypic distance matrix, based on the proportion of the different cardenolides constructed using Euclidean distances. Because the sample size ($n = 7$) is smaller than 20, we used a special random permutation procedure (Hemelrijk, 1990), rather than a normal distribution, to generate the distribution of the Mantel Z. The program for this procedure was written by C. Hemelrijk (1990).

Principal component analysis was used to describe both the pattern of observed allele frequencies and the pattern of phenotypic variation between populations, estimated by the proportion of cardenolides in the defensive mixture. To assure independence of the genetic data the rarest allele of each loci was excluded from the analysis and only the frequencies of the remaining 18 alleles used. Further, to assure normality both data sets were transformed by arcsin of the square root. In both data sets, the correlation matrix between each of the variables was calculated to check that the variables were indeed independent. To compare the two data sets, the first principal component from the proportions of 38 cardenolides in the 7 populations was regressed with the first principal component of the frequencies of 18 alleles in the same 7 populations.

Natural history

The distribution of each of the 5 species studied here overlaps extensively in the Alps and the Pyrenees, some are also known from the Vosges (2spp.) and the Black forest (3spp.).

O. bifrons feeds on *Chaerophyllum hirsutum* and at least one population (VA) also accepts *Peucedanum ostruthium*. Both plant species are Apiaceae. In the Vosges the insect is found at a relatively low altitude (600 m). In the Valais, this species is uniformly bronze colored, whereas in the Vosges (as well as in the other sites) it is green with blue stripes on the elytra. The defensive secretion of this species is composed of cardenolides.

O. gloriosa is a strict specialist on the Apiaceae species *P. ostruthium*. The complex mixture of cardenolides (circa 20 components) in defensive secretion varies both between populations (Eggenberger and Rowell-Rahier, 1991) and between individuals (Eggenberger and Rowell-Rahier, 1992; Eggenberger et al., 1992; Eggenberger and Rowell-Rahier, 1993). The variation between individuals of one

population is smaller than that between populations. The genetic distance (allozymes) between 5 populations from neighbouring (10 to 30 km) valleys in the Swiss Valais (the Saas population was studied here) are small and correlated with geographical distances (Eggenberger and Rowell-Rahier, 1991). Some but not all populations are polymorphic for color, ranging from totally blue to green.

Table 2. Allele frequencies in seven populations of *Oreina* belonging to five different species from central Europe (see Tab. 1 for population abbreviation, N = number of individuals tested).

Population:	bi1	bi2	gl	sp1	sp2	si	va
Locus							
AAT							
(N)	5	30	174	13	17	40	9
A	.400	.200	.977	.000	.000	.162	.056
B	.600	.800	.023	1.000	1.000	.837	.944
ACO1							
(N)	5	30	181	13	17	41	9
A	.900	.950	.000	.000	.000	.000	.000
B	.000	.000	.953	.154	.000	.000	.000
C	.100	.050	.047	.000	.000	.000	.833
D	.000	.000	.000	.846	1.000	.000	.000
E	.000	.000	.000	.000	.000	.000	.000
F	.000	.000	.000	.000	.000	.902	.167
G	.000	.000	.000	.000	.000	.098	.000
FUM1							
(N)	5	30	180	14	17	41	9
A	.000	.017	.000	.000	.000	.024	.000
B	1.000	.983	.011	.000	.000	.976	.056
C	.000	.000	.000	.143	.000	.000	.000
D	.000	.000	.989	.857	1.000	.000	.944
GAP1							
(N)	5	29	50	13	16	41	8
A	.600	.431	.150	.615	.000	.890	.625
B	.000	.000	.850	.154	.563	.110	.375
C	.000	.000	.000	.231	.438	.000	.000
D	.400	.569	.000	.000	.000	.000	.000
GPD1							
(N)	5	29	181	14	17	41	9
A	.000	.000	.000	.679	.000	.000	.000
B	.000	.000	.000	.321	1.000	.000	.000
C	.000	.000	.006	.000	.000	.000	.667
D	.000	.000	.994	.000	.000	.902	.333
E	1.000	1.000	.000	.000	.000	.098	.000
MDH1							
(N)	5	30	28	14	17	41	9
A	.300	.000	.982	.607	.441	.915	.000
B	.700	.883	.000	.393	.559	.085	.000
C	.000	.117	.018	.000	.000	.000	1.000

O. speciosa is oligophagous on several Apiaceae including *C. hirsutum*, *P. ostruthium*, *Angelica sylvestris*, *Heracleum* spp). The mixture of cardenolides in the defensive secretion is characterized by its extreme complexity (at least 38 components). Elytral red and gold stripes are characteristic of the species, but some individuals can also be completely black.

O. speciosissima feeds on *Petasites albus* and *P. paradoxus* in the field. In the laboratory, it accepts other seneciomal. Typically, the defensive secretion contains small amounts of a relatively simple mixture of cardenolides (circa 13 components). In the laboratory, when this species feeds on the leaves of *Adenostyles alliariae*

Table 3. Observed heterozygotes (Ho), expected heterozygotes (He), and fixation indices (Fi) for polymorphic loci in seven *Oreina* populations belonging to five species. Significance of the deviations from HWE was determined by exact tests. Locality and species abbreviation as in Table 1.

Population:	bi1	bi2	gl	sp1	sp2	si	va
aat-1							
Ho	2	8	4	–	–	.07	1
He	2.40	9.60	7.81	–	–	.27	0.94
Fi	.167	.167	.488	–	–	.73	−.059
P	ns	ns	**			***	ns
acon-1							
Ho	1	3	17	2	–	.15	3
He	.9	2.85	16.20	3.38	–	.18	2.5
Fi	−.111	−.053	−.049	.409	–	.18	−.200
P	ns	ns	ns	ns		ns	ns
fumh-1							
Ho	–	1	4	2	–	.05	1
He	–	.98	3.95	3.43	–	.05	0.94
Fi	–	−.017	−.011	.417	–	0.01	−.059
P	–	ns	ns	ns		ns	ns
gapdh-1							
Ho	4	15	11	6	8	.07	6
He	2.4	14.22	12.75	7.08	7.87	.20	3.75
Fi	−.667	−.055	.137	−.130	−.016	.63	−.600
P	ns	ns	ns	ns	ns	***	ns
g3pdh-1							
Ho	–	–	2	7	–	.10	4
He	–	–	1.99	6.11	–	.18	4.00
Fi	–	–	−.006	−.146	–	.46	.000
P	–	–	ns	ns		*	ns
mdhp-1							
Ho	1	1	1	7	11	.07	–
He	2.1	6.18	.98	6.68	8.38	.16	–
Fi	.524	.838	−.018	−.146	−.312	.54	–
P	ns	***	ns	ns	ns	*	

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.00$

rich in pyrrolizidines N-oxides, these plant allelochemicals are sequestered (Rowell-Rahier et al., 1991; Emhke et al., 1991). A high level of genetic differentiation was observed between geographically isolated populations (Rowell-Rahier, 1992). In most of the known populations, the beetles are green with blue stripes, but a few blue individuals have been observed.

O. variabilis is oligophagous on several Apiaceae but favors *C. hirsutum*. The defensive secretion consists of cardenolides (circa 32 components in the mixture).

Results

Allozyme electrophoresis. Allele frequencies in the 7 populations studied are given in Tab. 2.

The observed and expected heterozygote frequencies as well as the fixation indices for the 5 species (7 populations) are given in Table 3 for each locus.

In 4 of the studied species (*O. bifrons*, *O. gloriosa*, *O. speciosa* and *O. variabilis*; together 6 populations), only 2 of 27 conducted tests indicated a significantly lower heterozygosity than expected [*aat-1* in *O. gloriosa* (VA) and *mdhp-1* in *O. bifrons* (VO)], all the other loci were in Hardy-Weinberg equilibrium (Tab. 3).

In *O. speciosissima* there was a pervasive deficiency (4 of 6 loci) of heterozygotes compared to the expected value under Hardy-Weinberg equilibrium (Tab. 3). This is discussed in detail in Rowell-Rahier (1992) and interpreted as the consequence of the distribution of host-plants and the low mobility of these specialized leaf-beetles.

Figure 1 (right side) shows the dendrogram obtained with Rogers' modified genetic distances (see Tab. 4) for the 7 populations ($F = 5.804$; r coph = 0.94; %sd = 7.30).

Defensive secretions. In each of the 7 samples of pooled secretions analysed by HPLC a total of 38 cardenolides peaks (all present in *O. speciosa*) were measured. The proportion of these peaks in the defensive mixture of each population is given in Tab. 5 and the HPLC traces of one sample (*O. speciosa*, BV) is shown as an example in Fig. 2. The phenotypic distances, reflecting the degree of similarity in the

Table 4. Matrix of genetic distances (Modified Rogers' distance, Wright, 1978) between the seven populations of *Oreina*.

Population	bi1	bi2	gl	sp1	sp2	si	va
bi1	–						
bi2	.153	–					
gl	.835	.904	–				
sp1	.685	.711	.702	–			
sp2	.775	.779	.748	.371	–		
si	.605	.670	.713	.648	.779	–	
va	.755	.761	.737	.595	.672	.693	–

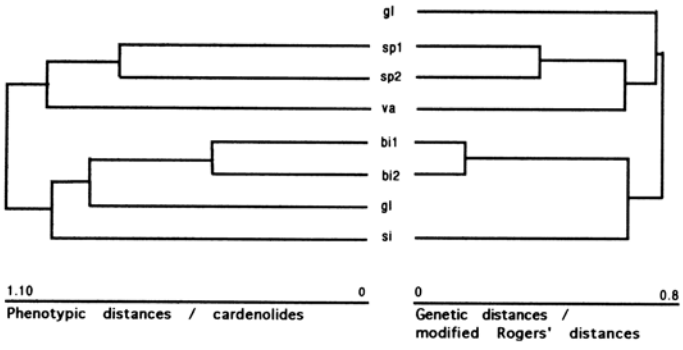

Fig. 1. Right: Genetic distances: UPGMA based on the modified Rogers' distance (Wright, 1978). Goodness of fit statistics (Prager and Wilson (1976) "F" = 5.804, cophenetic correlation = .939). Left: Phenotypic distances: UPGMA based on phenotypic (cardenolides proportions in defensive secretions) distances between seven populations of *Oreina* species producing cardenolides. Locality and species abbreviations as in Tab. 1.

proportion of the different cardenolides, are shown in the dendrogram (Fig. 1, left side). The geographically isolated populations of the same species (here *O. speciosa* or *O. bifrons*) had secretions that were more similar to each other than to those of other species. At the species level, the secretion of *O. bifrons*, *O. gloriosa* and *O. speciosissima* were grouped together as were those of *O. speciosa* and *O. variabilis*.

Comparison of genetic and chemical data. The clustering of the species based on the similarity of the cardenolide mixtures was remarkably similar to the clustering of the species based on genetic distance (Fig. 1). The two topologies were congruent, with the exception of the position of *O. gloriosa* which differs in the 2 clusters; this discrepancy is presumably due to the small sample of enzyme loci studied here. This suggests that the cardenolide mixtures are species-specific and can offer useful taxonomic information.

The genetic (allele frequencies) and phenotypic (proportion of cardenolides in the defensive secretion) distance matrices were indeed congruent, as measured by the Mantel-statistic. In the Mantel Z test, the probability of the random Z being larger than the observed Z was $p = 0.014$ out of 10 000 random permutations. Thus the two clustering procedures produced significantly similar branching patterns.

The first principal component based on the proportion of 38 cardenolides in the defensive mixture (PC1 C) explained 46% of the variation among populations; the first and second components explained 66% of the variation. The first principal component, based on the frequencies of 18 alleles (PC1 G), explained 40% of the variation between populations; the first two components (PC2 G) explained a total of 61%. PC1 C and PC1 G were significantly correlated (Fig. 3, Pearson coefficient = 0.80, $p = 0.03$). There was no other significant correlation between any combination of the first two principal components of the two data sets.

Table 5. Proportion of each cardenolide (% of total cardenolides) in the defensive secretion of seven populations. Each peak is indicated by its retention time "rt".

Population:	bi1	bi2	gl	si	sp1	sp2	va
Peak							
rt4	0.00	0.00	0.00	1.29	2.31	3.07	0.74
rt6	0.00	0.00	0.00	0.00	1.45	0.00	0.65
rt7	39.42	32.08	23.23	40.28	8.10	12.25	36.07
rt8	0.00	0.00	0.00	2.77	1.04	1.03	1.17
rt9	0.00	0.00	0.00	0.00	1.90	1.28	0.00
rt10	0.00	0.00	0.00	0.00	1.24	0.66	0.00
rt11	1.78	1.22	0.00	3.03	3.48	3.12	1.70
rt12	0.44	0.24	4.19	0.00	7.09	4.02	2.78
rt14	0.00	0.00	2.94	0.00	3.42	1.94	0.90
rt15	4.30	2.53	14.50	4.72	12.38	10.48	9.63
rt16	1.54	0.75	0.00	0.00	0.50	1.35	1.16
rt17	0.00	0.00	0.00	0.00	0.30	0.70	0.60
rt18	0.00	0.00	0.00	0.00	1.10	3.59	0.21
rt19	0.00	0.00	0.00	0.00	0.34	0.53	0.26
rt20	1.90	2.45	1.60	24.17	1.71	2.13	1.87
rt22	0.71	0.50	4.80	1.88	2.62	3.31	3.18
rt23	0.83	0.00	0.00	0.00	0.36	0.80	0.00
rt24	0.34	0.00	0.00	13.53	5.52	5.07	0.73
rt25	0.94	0.93	2.28	3.72	1.95	1.70	1.34
rt26	5.04	8.75	4.68	0.99	0.56	1.41	1.27
rt28	0.00	0.00	0.00	0.00	2.36	1.98	0.00
rt29	1.53	2.97	4.77	1.16	2.09	0.78	1.70
rt30	0.00	0.00	0.00	0.00	0.31	0.35	0.24
rt31	0.00	0.00	0.70	0.00	0.49	0.58	1.13
rt32	0.00	0.00	0.00	0.00	0.48	0.00	0.00
rt33	0.00	0.00	0.69	0.00	0.53	0.99	0.00
rt34	11.44	8.28	3.42	0.00	3.41	1.42	5.93
rt35	3.16	4.91	10.44	1.19	2.82	0.60	3.57
rt36	0.00	0.00	0.78	0.00	0.26	0.25	0.86
rt37	0.78	1.85	0.00	0.00	0.44	0.11	0.48
rt38	3.62	8.99	1.81	0.00	1.88	3.81	2.81
rt39	0.00	0.00	1.22	0.00	1.34	1.72	0.15
rt40	0.43	0.70	2.91	0.00	14.31	18.92	6.72
rt41	21.78	22.85	13.27	1.28	5.16	2.92	11.29
rt42	0.00	0.00	0.65	0.00	3.26	4.76	0.23
rt43	0.00	0.00	1.11	0.00	2.26	2.04	0.06
rt44	0.00	0.00	0.00	0.00	0.92	0.33	0.36
rt45	0.00	0.00	0.00	0.00	0.29	0.00	0.22

Discussion

Three different procedures indicate that the genetic and chemical defense data sets are congruent: 1) the topographic similarity between two dendrograms, 2) the result of the Mantel test on the phenotypic and genetic distances matrices and 3) the significant correlation between the first axis of the PCA based on the one hand on

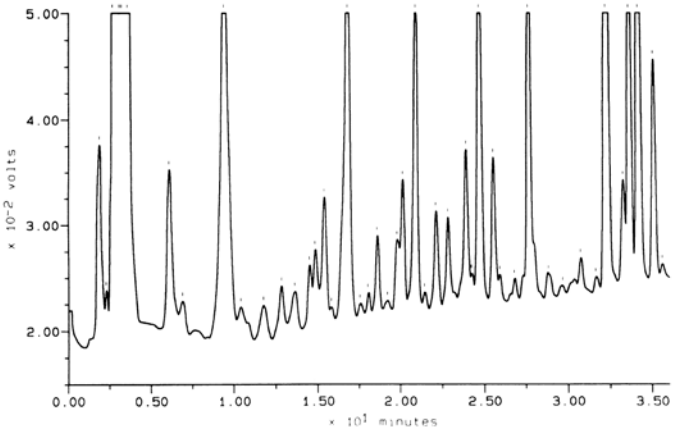

Fig. 2. HPLC traces of the cardenolides mixture in the defensive secretion of *O. speciosa* from BV.

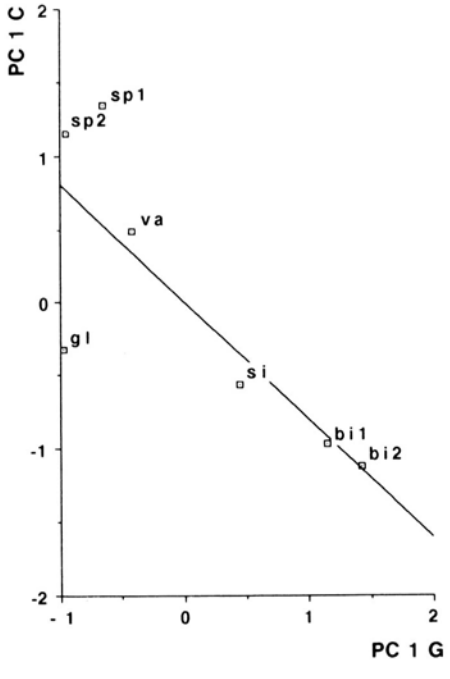

Fig. 3. Linear regression between the first principal component based on the proportion of cardenolides (PC1 C) and the first principal component based on the allele frequencies (PC1 G).

cardenolides mixture and on the other hand on allele frequencies. All of these results support the hypothesis that phenotypic similarities of cardenolide mixtures can be good estimates of overall genetic similarities. This confirms the previous report of Eggenberger and Rowell-Rahier (1991) of an intraspecific correlation between cardenolide mixtures and allozyme frequencies between *O. gloriosa* populations. Such a relation seems to indicate a similar evolutionary pattern in the two characters. It is very likely that geographic isolation and consequent differentiation and speciation processes have influenced both sets of characters in a parallel way. This may be because: 1) both the allozymes investigated and the defensive secretions are subject to similar selective pressures (e.g. some studies indicate that natural selection acts on commonly used allozyme loci in herbivorous insects, Slatkin, 1987 and Rank, 1992); 2) the genetic variation in both allozymes and cardenolides is the result of genetic drift, or a combination of points 1 and 2. Cardenolide biosynthetic steps can presumably be turned on and off or easily modified. We believe alternative 2 to be the more probable; this suggests that the exact composition of the "average" (pooling individuals) defensive mixture in a population is not under strong selection. The main predators of the adult beetles are probably birds (beetles with damaged elytra suggesting pecking by a bird are found in the field) and insectivorous rodents. To date, no parasitoids of adult *Oreina* are known. If a particuar defensive mixture was more effective against natural enemies, we would expect the defensive secretion of sympatric *Oreina* from different species to be more similar to each other (e.g. *O. bifrons*, *O. gloriosa* and *O. speciosa* in the Valais) than to their conspecific from other sites. This is not the case in our samples. At least in *O. bifrons* and *O. speciosa*, the composition of the secretion remains species specific and it is not excluded that the secretion plays a role in inter- or intraspecific communication. It has recently been shown that the proportions and concentrations of cardenolides in the secretions of *O. gloriosa* have a high heritability (Eggenberger and Rowell-Rahier, 1992) and that individual variation is partly explained by sex, age and mating status (Eggenberger and Rowell-Rahier, 1993).

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