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Original article

Enzymatic polymorphism in natural populations of the sawfly *Diprion pini* L (Hymenoptera: Diprionidae)

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Summary – *Diprion pini* L is a sawfly whose larvae cause sudden, brief and spectacular defoliation on *Pinus sylvestris*. In France, bivoltine populations in lowland areas and univoltine populations in mountain areas cohabit, all living in forests located at varying distances from each other. The characteristics of the diapause of mountain populations are different from those of lowland populations. Six natural populations were studied using enzymatic electrophoresis to identify markers reflecting genetic heterogeneity in the French *D pini* populations: three lowland (Rambouillet, Romorantin, Lorris) and three mountain populations (Saint-Just-Saint-Rambert, Ceillac, Fontchristianne). The study of enzymatic polymorphism concentrated on six loci: three polymorphic esterase loci, a monomorphic malate dehydrogenase locus, a monomorphic and a polymorphic amino-peptidase loci. The determination of genetic distance between populations did not allow us to discriminate between bivoltine lowland populations and univoltine mountain populations. The populations fell into two subgroups: those from the Alps and Rambouillet, and those from central France (Lorris, Romorantin and Saint-Just-Saint-Rambert).

***Diprion pini* / Hymenoptera / natural populations / enzymatic polymorphism**

Résumé – Polymorphisme enzymatique des populations naturelles de la tenthrède *Diprion pini* (Hymenoptera, Diprionidae). *Diprion pini* L est une tenthrède dont les larves causent des défeuillaisons brutales, brèves et spectaculaires sur *Pinus sylvestris* L. En France, coexistent des populations bivoltines en plaine et univoltines en montagne, toutes inféodées à des massifs forestiers plus ou moins distants les uns des autres. Les populations de montagne présentent des caractères de diapause différents de celles de plaine. Pour tenter d'identifier des marqueurs reflétant l'hétérogénéité génétique des populations françaises de *D pini*, six populations naturelles ont été étudiées par électrophorèse enzymatique : trois populations de plaine (Rambouillet, Romorantin, Lorris) et trois populations de montagne (Saint-Just-Saint-Rambert, Ceillac, Fontchristianne). L'étude du polymorphisme enzymatique porte sur six loci : trois loci estérasiques polymorphes, un locus malate

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deshydrogénase monomorphe et deux loci amino-peptidase dont l'un est monomorphe et l'autre polymorphe. Les distances génétiques entre populations n'ont pas permis de différencier les populations bivoltines de plaine et univoltines de montagne. Deux sous-groupes de populations peuvent être distinguées : celles des Alpes et de Rambouillet et celles du centre de la France (Lorris, Romorantin et Saint-Just-Saint-Rambert).

***Diprion pini* / Hymenoptera / populations naturelles / polymorphisme enzymatique**

INTRODUCTION

Diprion pini L (Hymenoptera: Diprionidae) is widespread in the whole paleartic area of *Pinus sylvestris* L, its main host plant. Several hundred thousands hectares of Scots pine are defoliated each year and the annual cost due to the reduced tree growth alone would represent about 300 millions FF in the European Community.

In France *D pini* is bivoltine in lowland plains, whereas it is univoltine in mountain sites. For example, in the Paris Basin, the first generation of this sawfly develops from April to July and the second from August to the following April; above 1 000 m altitude, in the Alps, only one generation occurs, mainly from June to the following June. It is commonly assumed that *D pini* outbreaks in Atlantic and Central Europe are related to the bivoltine cycle. This theory is supported by the fact that in France outbreaks start in the plains and that most damage occurs in autumn (Géri, 1988).

On the other hand, *D pini* life cycle is controlled by a complex phenomenon of diapause. Indeed, a proportion of the individuals of each generation undergo a diapause ranging from a few months up to several years (up to 6 years in high altitude populations).

Considering the intensity and the duration of this diapause, Eichhorn (1976–1977, 1979) described several ecotypes in European populations. However, this classification may be defective owing to the permanent dependence of the sawfly reaction to the photoperiod and temperature conditions previously experienced by the insect during its whole life cycle, as shown by Géri and Goussard (1988, 1991).

The objective of the present study was to use isozyme patterns to obtain a more objective characterization of six *D pini* populations living in various geographical areas and to study its relationship with the voltinism attribute.

Up till now, isozymes have only been studied in some Diprionidae, such as *Neodiprion* sp or *Diprion similis* (Pamilo et al, 1978; Kuenzi and Coppel, 1986; Woods and Guttman, 1987). *D pini* has only been the subject of brief study of individual allozymic variability (Steinhauer, 1979).

MATERIALS AND METHOD

As Diprionidae have haplodiploid sex determination (Maxwell, 1956) and as the offspring of *D pini* live grouped in colonies during larval development, the data analysed are the parental genotypes obtained from their offsprings.

A sample of 129 *D pini* colonies were collected from *Pinus sylvestris* between June and September 1988 from three plain sites, Romorantin, Loiret (115 m asl, $n = 18$), Lorris, Loiret (130 m, $n = 25$) and Rambouillet, Yvelines (150 m, $n = 22$) and in three mountain sites, Ceillac, Hautes-Alpes (1643 m, $n = 21$), Fontchristiane, Hautes-Alpes (1400 m, $n = 22$) and Saint-Just-Saint-Rambert, Haute-Loire (600 m, $n = 21$) (fig 1).

The colonies were collected on rather weakly infested trees to avoid the possibility that they were issued from several females laying eggs together and only colonies with a number of larvae corresponding to one laying were taken.

Larvae from each colony were bred on Scots pine needles until adult emergence in an external

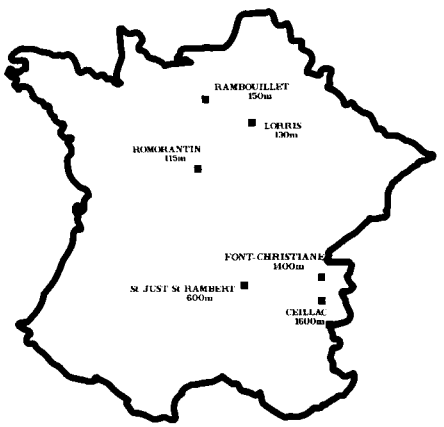

Fig 1. Geographical localization of sampling sites.

shelter at the INRA Station in Olivet (France). Newly emerged males and females were frozen alive at –20 °C and stored at this temperature until further analysis. As stated previously, all the individuals belonging to one colony were confirmed by zymograms to be brother or sister issued from the same parental couple.

Non-specific esterases, malate dehydrogenase and leucine amino-peptidase were investigated.

The analysis of 583 males and 1 174 females grouped according to their origin was performed, colony by colony, using polyacrylamide gel electrophoresis. Entire individual sawflies were ground at +4 °C with an Eppendorf grinder in 400 µL of 0.2 M phosphate buffer (pH 7.4) containing saccharose (10% v/v), mercapto-ethanol (1.10^{-5} % v/v) and a drop of polyethyleneglycol. Each sample was centrifuged at 12 400 *g* for 20 min at +4 °C. Supernatants were then stored at –80 °C until analysis.

Electrophoresis were performed in 8.5% acrylamide gel vertical slabs (180 × 140 × 1.5 mm) in a Pharmacia apparatus (GE 2/4 LS) at + 4 °C under 450 V. For each analysis, 30 µL of extract were applied to the gel strips and electrophoresis was performed using Tris Borate EDTA buffer (pH 8.3) for the electrode and the gel (Beaudoin, 1990).

Non-specific esterases (EC 3111) were visualised at room temperature by staining for 3 min with a solution of α-naphtylacetate (0.2%) and ß-naphthylacetate (0.15%) in 0.1 M Tris HCl buffer (pH 7.4) containing acetone (40%) and for 15 min with a solution of Fast Blue RR salt (0.2%) as dye-coupler in 0.1 M Tris-HCl buffer (pH 7.4).

For the visualisation of malate dehydrogenase (EC 11137), gels were incubated at 37 °C in darkness in an appropriate staining solution containing malic acid (0.067%), NAD (0.025%), NBT (0.015%), PMS (0.001%) in 0.5 M Tris-HCl buffer (pH 7.1).

For the visualisation of leucine amino-peptidase (EC 34111), the gels were immersed in a solution of 0.5 M boric acid. The acid solution was removed after 15 min and replaced by a staining solution containing 0.2 M anhydrid maleic, $MgCl_2$ (0.1%), L-leucine ß-naphthylamide-HCl (0.05%) and Fast Black K salt (0.07%) in 0.2 M Tris-HCl buffer (pH 5.3) (Cheliak and Pitel, 1985). After enzyme revelation, staining gels were fixed using 10% acetic solution and they were then stored in darkness at + 4 °C.

Electrophoresis data were analysed using classical parameters, ie, heterozygoty (H) and enzymatic polymorphism (P). Nei distances (Nei, 1972), within and between populations, were calculated. The results were expressed in matrical form and a dendrogram was elaborated using the method of Sneath and Sokal (1973).

The observed and expected genotypic frequencies calculated under the hypothesis of panmixia were compared using the chi square test. If the difference between expected and observed values was not significant, this result was accepted. If the test showed a significant heterogeneity, the frequences of the less frequent alleles were pooled and the test was repeated. In every case, Yates' correction was used (Yates, 1934).

RESULTS

Four esterase isozyme patterns were identified in males (E_1, E′, E_2, E_3) and five (E_1, E′, E_2, E_3, E_4) in females. Esterase E_1 was diallelic, and E_2 and E_3 were triallelic. E′ seemed to possess a null allele. The specific female esterase E_4 was monomorphic and proceeded from the female cementary gland (Beaudoin and Allais, 1991).

The patterns obtained from leucine amino-peptidase showed two loci: Lap-1, which was representated by four alleles, and Lap-2, which was monomorphic. All the alleles were found in each of the six populations. The malate dehydrogenase system was monomorphic for all the analysed individuals.

Offspring genotypes were identified for each colony. This gave us the opportunity to determine parental genotypes. Indeed, in a colony issued from a E_1–E_2 female and a E_1 male 50% of the female offspring will possess a E_1–E_1 genotype and 50% will have an E_1–E_2 genotype. Among the males, the abundance of E_1 and E_2 genotype will be identical. The same pattern applies for the offspring of E_1–E_2 females and E_2 males.

The allele frequencies of the allozymes in the six french *D pini* populations are given in table I. The allelic frequencies fitted well the panmictic expectations (chi square test).

Table II gives the observed and expected (under panmictic hypothesis) female heterozygoties for the four polymorphic loci. In four populations (Lorris, Rambouillet, Romorantin, Saint-Just-Saint-Rambert), we observed that the observed heterozygoty was higher than expected. We observed an apparent deficiency of heterozygotes in the two alpine populations. However, the deviations between the expected and observed heterozygoties were not significant (Wilcoxon test, Scherrer, 1984). We can therefore reasonably conclude that there was no differences between the observed values and the expected ones.

Table I. Allele frequency data for six natural *Diprion pini* populations.

Loci	*Alleles*	*Populations*					
		Romorantin	*Rambouillet*	*Lorris*	*Saint-Just-Saint-Rambert*	*Ceillac*	*Fontchristiane*
		n = *54*	n = *60*	n = *69*	n = *60*	n = *51*	n = *24*
Est E_1	E_{1-1}	0.71	0.69	0.80	0.77	0.74	0.79
	E_{1-2}	0.29	0.31	0.20	0.23	0.26	0.21
Est E_2	E_{2-1}	0.71	0.29	0.66	0.50	0.27	0.17
	E_{2-2}	0.27	0.65	0.32	0.50	0.61	0.62
	E_{2-3}	0.01	0.06	0.02	0.00	0.12	0.21
Est E_3	E_{3-1}	0.52	0.70	0.67	0.40	0.70	0.75
	E_{3-2}	0.26	0.10	0.12	0.27	0.22	0.25
	E_{3-3}	0.22	0.20	0.21	0.33	0.08	0.00
Lap-1	Lap-$_{1-1}$	0.37	0.34	0.35	0.49	0.28	—
	Lap-$_{1-2}$	0.30	0.26	0.21	0.35	0.25	—
	Lap-$_{1-3}$	0.23	0.31	0.34	0.16	0.47	—
	Lap-$_{1-4}$	0.10	0.09	0.10	0.00	0.00	—
Lap-2		100	100	100	100	100	100
Mdh		100	100	100	100	100	100

Table II. Observed and expected heterozygoty of the polymorphic loci (under panmictic hypothesis) for the females of the six populations.

Loci		*Population*					
		Romorantin	*Rambouillet*	*Lorris*	*Saint-Just-Saint-Rambert*	*Ceillac*	*Fontchristiane*
Est E_1	OH	0.4667	0.3529	0.5	0.3	0.3889	0.3750
	CH	0.4109	0.4306	0.3163	0.3578	0.3841	0.3299
	d	0.1358	–0.1804	0.5808	–0.1615	0.0125	0.1367
Est E_2	OH	0.4667	0.75	0.6818	0.65	05882	0.3750
	CH	0.4227	0.4913	0.4541	0.5	0.5413	0.5382
	d	0.1041	0.5266	0.5014	0.3	0.0866	–0.3032
Est E_3	OH	0.6429	0.5294	0.6364	0.7	0.4118	0.125
	CH	0.6111	0.4537	0.4959	0.6578	0.4491	0.375
	d	0.052	0.1669	0.2833	0.0642	–0.0831	–0.6667
Lap-1	OH	0.7895	0.9615	0.8889	0.7826	0.55	—
	CH	0.7122	0.7117	0.7072	0.6108	0.6394	—
	d	0.1085	0.3510	0.2569	0.2813	–0.1398	—

OH: observed heterozygoty; CH: calculated heterozygoty (according to Hardy-Weinberg equilibrum); *d* = (OH–CH)/CH.

The six populations presented allelic and genotypic distributions that conform to the Hardy Weinberg distribution and the heterozygote rate was always the same for all the populations (between 0.41 and 0.54). There was no significant difference between plain and mountain populations.

The Nei genetic distance matrix is given in table III and figure 2 presents the UPGMA dendrogram.

DISCUSSION

The matrix and the dendrogram show that there was no evident difference between plain and mountain populations. This fact agrees with our knowledge of the ecophysiological control of *D pini* diapause. It shows that the same population is able to be bivoltine or univoltine, under plain and mountain conditions, respectively (Géri, 1988; Géri and Goussard, 1988, 1991; Beaudoin et al, 1992). However, this situation is not exclusive of a strengthening of the plain and mountain population characteristics by genetical factors.

On the whole, the six populations have the same genotype. However, the results show that, in 1988, there were two groups of populations. The first one was present in central France (Massif Central, Romorantin and Lorris), whereas the second one was representated by both the Alps and Rambouillet populations. The low relatedness between the first three populations could be explained by the 1982–1984 outbreaks, which occurred from the south center of France to the north as described previously (Géri and Goussard, 1984). During the same

Table III. Nei genetic distance between the six populations.

	Rambouillet	*Lorris*	*Romorantin*	*Saint-Just-Saint-Rambert*	*Ceillac*	*Fontchristiane*
Rambouillet	0					
Lorris	0.0361	0				
Romorantin	0.0516	0.0105	0			
Saint-Just-Saint-Rambert	0.0368	0.0327	0.0221	0		
Ceillac	0.0097	0.0400	0.0589	0.0503	0	
Fontchristiane	0.0231	0.0855	0.1127	0.0791	0.0061	0

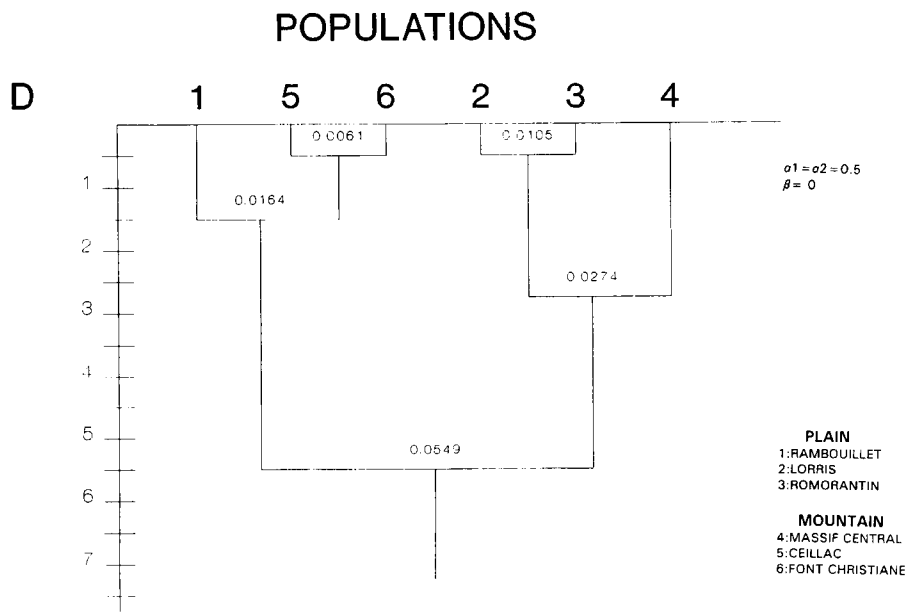

Fig 2. UPGMA dendrogramm computed for the six loci of Est E_1, Est E_2, Est E_3, Lap-1, Lap-2 and Mdh-1.

period, the Rambouillet and the two alpine populations were not affected or only slightly and their genetic polymorphism would represent some previous unknow relation between these populations or a more general status, which would have existed in France before the outbreak.

Our results do not exclude the hypothesis of the existence of adult migrations speading the outbreak and of population exchanges between mountains and lowlands. For the three populations of central France, we could accept the hypothesis that there was a migration of some individuals from mountains to

plain and that the newly formed populations developed an outbreak.

However, from a methodologic point of view, the study shows a reduced enzymatic polymorphism of *D pini* and illustrates the difficulty in using enzymatic electrophoresis to investigate *D pini* population diversity, so that it may be necessary to explore it further in order to envisage more sophisticated methods, such as mitochondrial DNA. This finding is in accordance with the low level of genetic diversity observed within the sawflies and other Hymenoptera as compared to other insects (Pamilo and Crozier, 1981; Woods and Guttman, 1987). Furthermore, it is reasonable to suppose that this species, which is rather homogeneous from a morphological and a biological point of view in the whole of Europe, has a higher genetic uniformity than the American genus *Neodiprion* sp previously studied by enzymatic electrophoresis, whose species or species complex present many variable populations (Knerer and Atwood, 1973).

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