Evolutionary biology as a tool towards a more customized biological control strategy of weeds: Hoary cress as a case study

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Introduction

The collar gall weevil *Ceutorhynchus assimilis* (Paykull, 1792) [= *C. pleurostigma* (Marsham,1802) syn,n,] (Colonelli, 1993) is widely distributed in Eurasia where it colonizes an indigenous weed *Cardaria draba* (L.) Desv. (= *Lepidium draba*) (Brassicaceae) and other Brassicaceae, including crops. Within the framework of a biological control program of *C. draba* (Fig.1), which is considered as an invasive weed in North America (Mulligan and Findley, 1974), *Ceutorhynchus assimilis* (Fig.3.a.b) has been targeted as a potential biological control agent. Host-range studies in laboratory (choice and no choice conditions) and open field (choice conditions) displayed specific larval development of this weevil reared from galls of *C. draba* in Southern France (Fig.2) (Fumanal *et al.*, 2000; Fumanal *et al.*, 2002). Such observation was in discrepancy with its pest status given in the literature (Hoffman, 1954). This called for the use of genetic markers to get insights into the population genetic structure of this recognized species.

Genetic variation of *C. assimilis* was examined using mitochondrial *Cytochrome Oxidase I* gene (COI) sequencing and extended here by double strand conformation polymorphism analysis (DSCP) (Barros *et al.*, 1994; Saad *et al.*, 1994) in natural populations representing its geographic distribution as well as its host-range, uncovering several distinct lineages. Interbreeding experiments between the different genetic entities (using a novel noninvasive fecal DNA method) were conducted to assess whether reproductive isolation occurs.

Likewise, a study was jointly undertaken to trace the origins of American *C. draba* populations and to evaluate the genetic structure of the plant based on amplified fragment length polymorphism (AFLP) analysis in its native range in parallel to the study on the insect.

This approach, using information from both the target weed and its potential biological control agent, provides valuable insights in the insect-plant evolutionary relationships. Furthermore it allows for a better estimation of risk assessment in biological control strategies.

Fig.1: a- *Cardaria draba* patch in flowering stage on fallow land.
b- *Cardaria draba* both at the rosette and flowering stages.

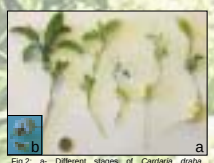

Fig.2: a- Different stages of *Cardaria draba* infestation by the collar gall weevil from uninfested to severely infested plant (from left to right). b- Cross section of galls exhibiting developed mature larvae.

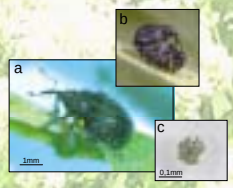

Fig.3: a- *Ceutorhynchus assimilis* adult on plant, characterized by two thoraco-hielytral spots (in yellow). b- Two weevils mating. c- weevil fecal secretion.

Materials and methods

Plant material : A total of 32 populations including 190 individuals of *Cardaria draba* were sampled across its native range in Eurasia (26 populations) and its introduced range in North America (6 populations). The Eurasian samples were chosen to match the presence of *Ceutorhynchus assimilis* at the same location (Fig.4.a.b). The genetic structure of plants was studied using a derived Amplified Fragment Length Polymorphism (AFLP) method.

Data analysis included phylogenetic reconstruction using phenetic UPGMA based on Nei's unbiased distance presented here.

Insect material : We analyzed 63 populations including 572 individuals of *Ceutorhynchus assimilis* from natural populations representing different collection sites throughout its distribution area in Europe and collected on the whole spectrum of host-plants (Fig.4.a). Insects were identified by taxonomists and carried the same morphology. A 560bp fragment of COI mitochondrial gene was sequenced (two individuals per populations) and the genetic characterization was extended by DSCP analysis of a shorter fragment of COI (317bp) selected to maximize observable polymorphism.

Data analysis included phylogenetic reconstruction using Neighbor Joining distance method (NJ) based on the pairwise percentage of divergence presented here.

Interbreeding experiments : Four populations of *Ceutorhynchus assimilis* representing clades II, III and IV were selected as they are living on three host-plants from different sites and corresponding to populations previously studied in host-range experiments. Selection of populations was based on multiple criteria: first, genetic entity that involves the three distinct main clades; second, host-plant used in nature including the target weed, *Cardaria draba* (populations A- Southern France, clade II and E- Toscana, Italy, clade IV) as well as wild and cultivated Brassicaceae, *Sinapis arvensis* (population D, Southern France, clade III) and *Brassica napus oleifera* (population C, Western France, clade IV); third, distance between the populations from close (less than 5 km) to distant (France and Italy). Populations A and E are especially important in interbreeding tests as they represent distinct clades although they use *C. draba* as host-plant in geographically distant area.

Weevil populations were separately reared from the corresponding host-plant galls. The larvae were kept isolated until the emergence of the adults which were sex-determined under binocular. Then, they were genetically identified by a novel noninvasive method (see technical box) based on direct amplification (by PCR) of insect fecal secretions (Fig.3.C) associated with DSCP.

A novel noninvasive method for the genetic characterization of insect feces based on direct-PCR amplification associated with DSCP

Principle: Adult insects from different populations were reared and fed separately using one leaf of their natural host-plant. One fecal secretion per weevil was collected under binocular after two days exposure and was put in PCR mix (10 µM of each primer, 2.5 mM dNTP, 1X Qiagen Buffer, 1 unit of Qiagen Taq Polymerase and ultrapure water qsp 23µL). Thermal cycle parameters for PCR were as follows: 92°C for 1 min followed by 35 cycles of 92°C for 30 s, 48°C for 1 min, 62°C for 30 s. The last elongation step was extended to 7 min. PCR products were checked on 1% agarose gel electrophoresis before determination by DSCP with electrophoretic migration in acrylamide gel (PCR products were electrophorized in TBE 1X buffer, 10% polyacrylamide 49:1 gel (30% acrylamide, 5% Glycerol) for 15 hours at 100V, 30mA in 20x20 cm plates).

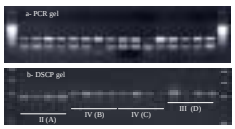

Fig.5: Example of PCR (a) and DSCP (b) gels visualized under 302nm UV light after EtBr impregnation (0,07 %) : a- Direct PCR products (PCR mix and feces) of 317bp checked on 1% agarose gel electrophoresis. b- DSCP polyacrylamide gel (10%, 49:1) with direct-PCR products displaying band migration polymorphism in relation to different clades (corresponding to Fig.6.a) and weevil populations.

Advantages: (i) method based on feces provides a noninvasive tool for the insect or others organism; (ii) direct-PCR is a useful tool to gain both time (no extraction required) and money; (iii) associated DSCP technique is very easy to use, less expensive than sequencing and enough informative in the case study.

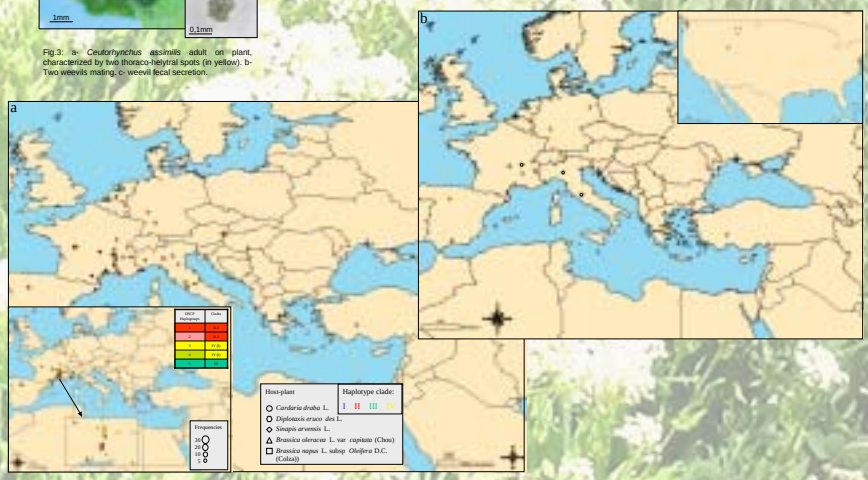

Fig.4: a- Distribution of *Ceutorhynchus assimilis* samples analyzed by sequencing and DSCP technique, showing both the different host-plants including *C. draba* and others Brassicaceae (symbolized by geometric forms) on which the insect (larvae) were collected and the four genetic clades (presence and/or frequencies). b- Distribution of *Cardaria draba* samples analyzed by AFLP in the USA and Europe where they co-occur in most locations (26) with the weevil (analysis of both the insect and its host-plant when it was possible). The different clades are represented by distinct colours as on Fig.6.

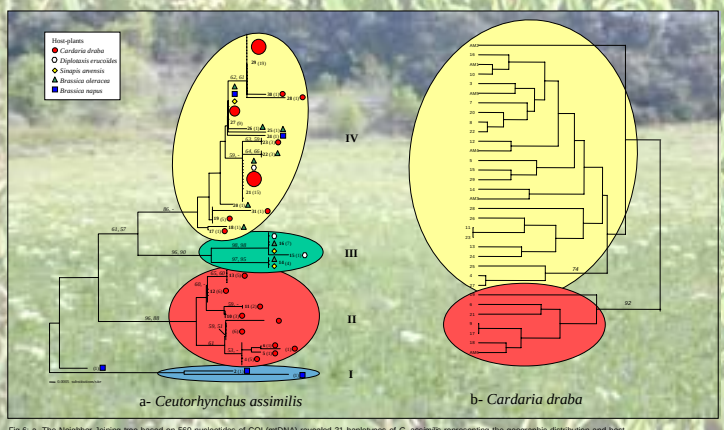

Fig.6: a- The Neighbor Joining tree based on 560 nucleotides of COI (mtDNA) revealed 31 haplotypes of *C. assimilis* representing the geographic distribution and host-ranges. Four clades are identified by roman numbers and delimited by colour shapes. Haplotype numbers are provided on the tree and the number of individuals is represented by the size of the corresponding host-plant symbol. Bootstrap proportions are provided for each supported branch (>50). b- UPGMA tree based on Nei's unbiased genetic distance applied on 15 polymorphic AFLP loci. Bootstrap proportions are provided for each supported branch.

Results

Genetic data

Cardaria draba: Characterization of the genomic polymorphism as highlighted by AFLP reveals two main genetic clusters shaping the relationships between populations widespread in the whole European distribution (Fig.4.b). One first cluster (in red) displays a restricted area centered on Southern France (see figure 6.b) whereas the second main cluster (in yellow) is widely distributed in the whole European distribution. However, the American population samples do not cluster together and are distributed throughout the phenetic tree. Such pattern is also found in other weeds using the same approach based on AFLP markers as for the wild radish that invades Australia (in preparation).

Ceutorhynchus assimilis: 31 haplotypes were identified based on DNA sequencing and represented on the Neighbor Joining tree (Fig.6.a). Among the four clades highlighted, clade II is strictly represented by *C. assimilis* populations collected on *C. draba* inside a restricted area as shown in figure 4.a above (Northern Spain, Southern France and the Northwestern part of Italy). The sequencing analysis was combined with the DSCP technique giving evidence of 5 haplogroups. Two of them represented clade II, two others corresponded to clades I & IV and the latest to clade III. Also, each haplogroup found on DSCP displayed differential migration patterns on polyacrylamide gel in relation to different curvature profiles. The divergence observed between the clade II and others (2.3 to 3.9%) is unexpected at the intraspecific level.

Biological data

Interbreeding experiments: We considered only interbreeding results from populations A, C and E for which controls (i.e. intra-population matings) showed positive gall and larval development on their natural host-plants. On the other hand, population D was not considered because no development has been observed yet. Interbreeding between populations A (clade II) and E (clade IV) both on *C. draba* host-plant showed gall and larval development in the case of males A x females E but so far no development for females A x males E for which one observed small galls and aborted larvae (for the fraction of galls already dissected). Second, crossings of populations A (clade II, on *C. draba*) and C (clade IV, on *B. napus oleifera*) led to abnormal small galls on their natural host-plants. Nevertheless, crossings involving female A x male C produced eggs but also aborted neonate larvae on *C. draba* without presence of normal galls. Interbreeding experiments are still ongoing and we will have to wait for the complete development of the hybrid larvae into adults to determine their fertility.

Conclusion

Based on preliminary biological tests during 1999-2002, the specificity of *Ceutorhynchus assimilis* populations from Southern France to *Cardaria draba* was first hypothesized and later confirmed. Whereas there was no distinct difference in morphology between populations in the distribution area, we found large genetic variability among the analyzed weevil populations using mtCOI marker. This variability was structured in four distinct clades within the NJ tree. In one hand a clade (II) including weevils collected strictly from *C. draba* and found so far in Northern Spain, Southern France and Northern Italy, and on the other hand, populations from several host-plants with a large geographical distribution. Such genetic diversity together with a strong structure is surprising, given the absence of morphological differentiation in the whole distribution. The genetic divergence of the clade II (Fig.6.a) is in favor of an ongoing differentiation based on host-plant specialization. Such a pattern has already been reported in other weevils (data not shown) and it would be interesting to analyze whether this is a common feature in the evolution of this family. Moreover, the weevil genetic divergence seems to be supported by a comparable differentiation of the European *Cardaria draba* host-plants (Fig.6.b). This calls for more detailed testing additional data of the genetic pattern for both the weevil and the plant using microsatellites (currently in progress in the lab for the later).

Hybridization is demonstrated for crossings between populations on *C. draba* from clades II and IV but it is still unknown whether the progeny is fertile. Conversely, populations both from different host-plant and clades (*C. draba*, clade II and *B. napus oleifera*, clade IV), showed only one case of egg laying from females from clade II on *C. draba* but without further gall and larvae development. These results suggest some potential reproductive isolation between the recognized genetic entities from different host-plant. The precise extent and influence of barriers to gene flow could be examined using microsatellite markers.

The couple insect/plant studied here is a good case-study of the role of specialization in the genetic differentiation of phytophagous insects and demonstrates that there may be decoupling in morphology and molecular evolution. At present, no conclusion on the level of differentiation can be drawn from the data presented here when considering the diverse definitions of species found in literature. Thus, this study provides conceptual bases for the development of biological control programs. The combination of both the genetic characterization of the insect and the target and the host specificity testing in the biological control evaluation should be of general interest in limiting the risk of introduction side-effects and reducing both time and cost.

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