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**Evolutionary biology as a tool towards a more customized biological control strategy
of weeds: *Lepidium draba* as a case study**

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Summary: *Lepidium draba* (Brassicaceae), native to Eurasia, is one of the most invasive noxious weeds in North American rangelands and croplands. As part of the management plan for its control, one of its natural enemies, the collar gall weevil *Ceutorhynchus assimilis* (Coleoptera: Curculionidae) has been targeted and its genetic structure in the mitochondrial COI gene explored. Results clearly gave evidence of different genetic entities within this recognized species, one being restricted to the host *L. draba* and distributed in Southern France, Northern Spain and Italy. Furthermore, these results were supported by host plant spectrum studies in laboratory that showed specific and generalist populations. Underlying partial reproductive isolation which were also tested, probably explains the genetic differences that we observed between these morphocryptic entities. Likewise, a study was jointly undertaken to trace the origin of the *L. draba* American populations and to evaluate the phylogeography of the species in its native range. Results tend to show some similarities in the evolutionary pattern of both the weed and the weevil. These multiple approaches will provide a valuable interest in biological control strategy and in the risk assessment.

La biologie évolutive en appui d'une stratégie globale de lutte biologique contre les mauvaises herbes: le cas d'étude de *Lepidium draba*. Résumé: *Lepidium draba* (Brassicaceae), originaire d'Eurasie est une des plus nocives mauvaises herbes envahissant les prairies et cultures Nord Américaines. Dans le cadre de la lutte contre cette mauvaise herbe, un ennemi naturel a été sélectionné, le charançon *Ceutorhynchus assimilis* (Coleoptera: Curculionidae) et sa variabilité génétique de l'ADN mitochondrial COI a été analysée. Nos résultats suggèrent clairement au niveau de cette espèce, l'existence de deux principales entités génétiques dont une spécifique à *L. draba* et distribuée au Sud de la France, Nord de l'Espagne et Italie. De plus, ces résultats ont été confirmés par des études de spectre de plantes hôtes qui ont révélées des populations spécifiques et généralistes. Une isolation reproductive qui a été testée pourrait expliquer en partie les différences génétiques observées entre les entités morphologiquement non différenciables. De même, une étude a été réalisée parallèlement pour retracer l'origine des populations Américaines de *L. draba* et pour évaluer sa phylogéographie dans son aire d'origine en comparaison avec celle du charançon. Les résultats tendent à montrer un schéma de variation génétique similaire à celui observé pour le Charançon en Europe. Ces approches multiples sont d'un

intérêt indéniable pour une stratégie de lutte biologique et de l'évaluation des risques encourus.

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 *Lepidium draba* (L.) Desv. (= *Lepidium draba*) (Brassicaceae) and other Brassicaceae, including crops. Within the framework of a biological control program of *Lepidium draba*, which is considered as an invasive weed in North America (Mulligan and Findley, 1974), *Ceutorhynchus assimilis* 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 *L. draba* in Southern France (Fumanal *et al.*, 2000; Fumanal *et al.*, 2004). 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 natural geographic distribution as well as its host-range. Cross-breeding experiments between the different genetic entities observed were conducted to assess whether reproductive isolation occurs.

Likewise, a study was jointly undertaken to trace the origins of American *L. 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, will provide valuable insights in the insect-plant evolutionary relationships. Furthermore it will allow for a better estimation of risk assessment in biological control strategies.

MATERIALS AND METHODS

Genetic analysis of *L. draba*: A total of 32 populations including 190 individuals of *L. draba* were sampled across its native range in Eurasia (26 populations) and its introduced range in North America (6 populations) (data not shown). The Eurasian populations were chosen to match the presence of *Ceutorhynchus assimilis* at the same location. The genetic structure of the weed populations was studied using Amplified Fragment Length Polymorphism (AFLP) method Total DNA using a CTAB protocol was extracted from dried leaves following by enzyme digestion (ECOR1). AFLP amplifications were separated by electrophoretic migration on polyacrylamide gel and stained with ethidium bromide.

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

Genetic analysis of *C. assimilis*: We analyzed 63 populations including 572 individuals of *Ceutorhynchus assimilis* from natural populations sampled over its European distribution range and a broad host-plant spectrum (data not shown). Insects were identified by taxonomists. Total DNA of weevil larvae stored in 96° ethanol was extracted following a CTAB protocol and was amplified by PCR. A 560bp fragment of mitochondrial *COI* gene was sequenced (two individuals per populations). Double Strand Conformational Polymorphism (DSCP) analysis was performed on shorter fragment of *COI* (317bp) selected to maximize observable polymorphism.

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

Cross-breeding experiments: Four populations (A,C, D, E) of *Ceutorhynchus assimilis* were selected for cross-breeding experiments based on multiple criteria: first, genetic entity that belongs to one of the three distinct main clades (II, III, IV); second, host-plant used in nature including the target weed, *Lepidium 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). Weevils tested were reared from their 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 using a non-invasive method based on direct PCR amplification of insect fecal secretions associated with DSCP typing (Fumanal *et al.*, submitted).

RESULTS

Genetic data on *L. 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. One first cluster (in grey) displays a restricted area centered on Southern France (see figure 1.b) whereas the second main cluster (in white) 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 (Martin, in preparation).

Genetic data on *Ceutorhynchus assimilis*: 31 haplotypes were identified based on DNA sequencing and represented on the Neighbor Joining tree (Fig.1.a). Among the four clades highlighted, clade II is strictly represented by *C. assimilis* populations collected on *C. draba* inside a restricted area (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 ranged from 2.3 to 3.9%, which is unexpected at the intraspecific level.

Biological data – Cross-breeding experiments: We considered only cross-breeding results from populations A, C and E for which controls (i.e. intra-population breedings) 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. Crossbreeding 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 only one small gall and aborted larvae (for the fraction of galls already dissected) have been observed. Crossings of populations A (clade II, on *C. draba*) and C (clade IV, on *B. napus oleifera*) led to abnormal tiny galls on their natural host-plants. Nevertheless, crossings involving female A x male C produced both eggs and aborted neonate larvae on *C. draba* without presence of normal galls. Cross-breeding experiments are still ongoing. The complete development of the hybrid larvae into adults is required to determine their fertility before stating on the level of reproductive isolation.

DISCUSSION AND CONCLUSION

Based on preliminary biological tests during 1999-2002, the specificity of *Ceutorhynchus assimilis* populations from Southern France to *L. 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 with both a large host plant spectrum and geographical distribution. Such genetic diversity together with a strong structure is surprising, given the absence of morphological differentiation of the species in the whole distribution. The level of divergence of the clade II (Fig.1.a) is in favor of an ongoing differentiation based on host-plant specialization which was confirmed through host specificity testing. 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 *L. draba* host-plants (Fig.1.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).

Underlying partial reproductive isolation which were also tested, probably explains the genetic differences that we observed between these morphocryptic entities. The precise extent and influence of barriers to gene flow will 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 species concepts 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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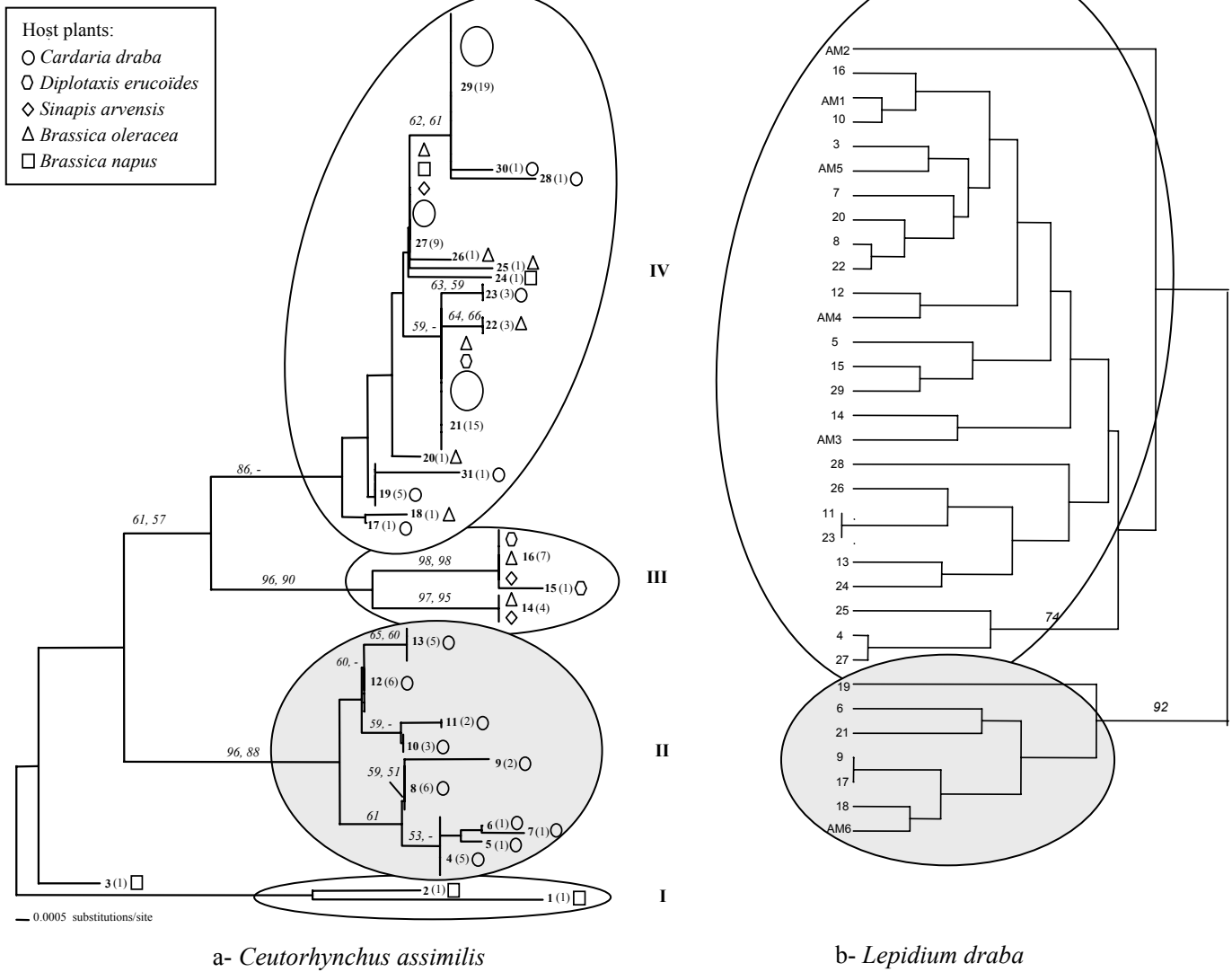


Figure 1: a- The Neighbor Joining tree based on 560 nucleotides of COI (mtDNA) revealed 31 haplotypes of *C. assimilis* representing the geographic distribution and host-ranges. Four clades are identified by roman numbers and delimited by color shapes (White and grey). Haplotype numbers are provided on the tree and the number of individuals with the same sequence are noted in parenthesis and represented by the size of the corresponding host-plant symbol. Bootstrap proportions are provided for each supported branch (>50). b- UPGMA tree of *L. draba*, based on Nei's unbiased genetic distance applied on 15 polymorphic AFLP loci. Bootstrap proportions are provided for each supported branch. The two topologies are confronted on same figure for comparison.