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High genetic variability despite haplodiploidy in primitive sawflies of the genus *Cephalcia* (Hymenoptera, Pamphiliidae)

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Abstract. Hymenoptera are haplodiploid and usually display very low genetic variation. Most data concern social or parasitic *Apocrita*, while the little information available for the primitive phytophagous species of the suborder Symphyta is contradictory. The present study is related to seven species of the genus *Cephalcia*, living in coniferous forests of Northern Eurasia and sharing spruce (*Picea* sp. pl.) as host plant. Individuals from 22 populations belonging to *Cephalcia abietis*, *C. alashanica*, *C. arvensis*, *C. erythrogaster*, *C. fallenii*, *C. fulva*, *C. klugii* from Europe and China were surveyed for genetic variation at 28 loci using enzyme electrophoresis. Pairs of sibling species were recognized within *C. arvensis* and *C. fallenii*, corresponding to different phenological and morphological forms. In the latter case, reproductive isolation in sympatry occurs despite low genetic distance ($D = 0.059$). Large genetic distances and fixed alternate alleles were observed between Chinese and European populations of *C. abietis* and *C. arvensis*. Expected heterozygosity of *Cephalcia* populations (0.197, SD 0.064) is significantly higher than that of other Symphyta (Tenthredinoidea) (average H_{exp} 0.059, SD 0.032) (two-tailed Mann-Whitney test, $Z = 4.39$, $p < 0.01$). These data suggest that haplodiploidy per se does not reduce the genetic variation in most *Cephalcia* populations. Most of the factors that can lower the potential for genetic diversity in a haplodiploid genetic system are not so effective in *Cephalcia* populations, which seem to be comparable to diplodiploid insect populations in diversity. In a few isolated populations the large number of fixed loci and the large genetic distances may support the predicted faster rate of fixation, as a consequence of haplodiploidy.
Key words. Hymenoptera; Symphyta; haplodiploidy; heterozygosity; allozymes.

Hymenoptera are haplodiploid (the male is haploid and the female diploid). They have often been found to exhibit very low genetic variation at the enzyme level in comparison to other insect populations[1,2]. The causes of this lack of genetic variability have been discussed extensively over the last 20 years. The major explanations proposed[3] include: (i) the intrinsic properties of a haplodiploid genetic system, i.e. increased selection due to hemizygous expression of alleles in males[4], increased effects of genetic drift and reduced recombination rates[5,6]; (ii) the environmental and population consequences of social life, i.e. buffered life conditions of colonies[7], inbreeding[8] and reduced effective population size[9]; (iii) the effects of periodic genetic bottlenecks[10]. However, the premise that Hymenoptera as a group are scarce in genetic polymorphism is derived from a phylogenetically biased species sample[3,11], as the available data, even if based on a very large number of electrophoretic markers[12,13], come mainly from eusocial bees and ants or parasitic wasps, both of which may be predisposed to reduced genetic variability for reasons other than haplodiploidy[2,5,7]. Nonsocial outbreeding species of the suborder Symphyta are more suitable for ascertaining the levels of genetic variability in Hymenoptera, because they lack many of the confounding factors present in eusocial or parasitic *Apocrita*.

The results of genetic screening of Symphyta are by now rather ambiguous[3], since in some cases data are consistent with average levels found for diploid insects[11,14], while in others they are similar to the low estimates for other Hymenoptera[2,10,15,16]. In this paper, we report the results of an electrophoretic survey of seven species of web-spinning sawflies in the genus *Cephalcia* Panzer, sampled in Europe and Northern China. *Cephalcia* species may display different levels of larval gregariousness as the only trait indicative of social behavior. The purpose of this study was to clarify the genetic relationships within taxonomically troublesome species complexes and to yield information for further biosystematic work. The genetic data presented here are the first available from sawflies in the superfamily Megalodontoidea, which is considered to be within the most primitive Hymenoptera[17], and thus may help to answer the question of whether haplodiploidy per se sufficiently explains the general dearth of genetic variation commonly observed in this order.

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Materials and methods

Insects

Natural history. The species studied live in Europe and Northern Asia on spruce (*Picea abies* and *P. koraiensis*, respectively) and may cause severe damage to the forests. The sawfly adults emerge from the ground in early spring to summer, with a sex ratio of approximately 1. The females lay eggs on spruce needles, singly or in small groups in the solitary and semi-gregarious species, respectively. Females can fly to other, distant sites after some of the eggs are laid. Solitary larvae live in silk tubes, while semigregarious and gregarious larvae spin a more or less compact nest filled with frass. The time of development from hatching to mature larva is temperature-dependent and lasts up to two months. Mature larvae then drop to the ground and immediately enter the soil under the tree crown, where they transform into a prepupa and overwinter. Only some of these individuals transform into pupa and adult the following spring; the others remain in prolonged diapause for one or more years.

Taxonomy. The group has recently been revised on the basis of European[18,19] and Chinese material[20]. *C. erythrogaster* (Hartig) is a rather rare species with solitary habits and early emergence, clearly recognizable by peculiar morphological characteristics of the adults. *C. fallenii* (Dalman) is also solitary and characterized by a very early emergence and a distinct pigmentation pattern in adults; at least two forms, light and dark, with different emergence times, may be recognized in Europe (unpublished data), where both are rather common. *C. arvensis* Panzer is a complex of several solitary forms sharing a peculiar adult morphology. Within these, a rare species, *C. fulva* Battisti and Zanocco, has recently been described by both morphological and biological features[21]. Several population outbreaks of *C. arvensis* occurred recently in Europe, characterized by the different emergence periods of adults, which led to the recognition of a spring form characterized by earlier emergence, peculiar prepupal morphology and a tendency to develop gregarious larval behaviour[22]. Another form emerging in summer is known[23], but its morphological identification is difficult. Chinese populations also show slight differences in behaviour and morphology[20]. *C. abietis* (Linnaeus), primitively gregarious, is the most widespread and economically important species. *C. alashanica* (Gussakovskji) is also primitively gregarious and known to produce population outbreaks only in China. *C. klugii* (Hartig), a rare species recently redescribed[24], is close to *C. alashanica*, differing mainly in its markedly gregarious larval behaviour and earlier emergence.

Sampling and rearing. Prepupae of *Cephalcia* sp. pl. were sampled from the soil in 12 spruce or mixed forests from Europe and Northeastern China (table 1). Fresh

Table 1. Genetic variability at 28 loci in 22 populations belonging to seven species of *Cephalcia*.

Species	Population[a]	N	A	P	H_{obs}	H_{exp}
C. abietis	Bodenmais (5)	16.2 (2.1)	1.7 (0.1)	42.9	0.089 (0.024)	0.138 (0.035)
	Nachod (6)	4.1 (0.3)	1.4 (0.1)	35.7	0.062 (0.020)	0.140 (0.043)
	Jesenik (7)	17.2 (1.8)	1.6 (0.1)	46.4	0.086 (0.022)	0.230 (0.057)
	Xinlin (9)	4.6 (0.4)	1.3 (0.1)	21.4	0.095 (0.051)	0.094 (0.038)
	Liangshui (10)	20.1 (2.4)	1.7 (0.2)	32.1	0.068 (0.025)	0.140 (0.042)
C. alashanica	Liangshui (10)	4.8 (0.4)	1.6 (0.1)	46.4	0.136 (0.046)	0.226 (0.050)
C. arvensis	Asiago (1)	29.7 (2.7)	1.8 (0.2)	46.4	0.123 (0.033)	0.197 (0.046)
	Cansiglio (2)	44.3 (3.9)	2.1 (0.2)	46.4	0.097 (0.027)	0.188 (0.044)
	Nachod (6)	7.0 (0.4)	1.9 (0.2)	57.1	0.059 (0.029)	0.247 (0.050)
	Jesenik (7)	10.2 (0.7)	2.2 (0.2)	50.0	0.164 (0.035)	0.286 (0.055)
	Skripov (8)	52.0 (2.5)	2.6 (0.3)	53.6	0.174 (0.034)	0.276 (0.053)
	Laoyelin (11)	9.0 (1.0)	1.5 (0.1)	42.9	0.077 (0.029)	0.162 (0.042)
	Changbaishan (12)	14.6 (1.2)	1.8 (0.2)	53.6	0.159 (0.050)	0.235 (0.047)
spring form	Nachod (6)	19.7 (0.9)	2.2 (0.2)	60.7	0.171 (0.039)	0.264 (0.053)
C. erythrogaster	Nachod (6)	5.2 (0.2)	1.2 (0.1)	21.4	0.057 (0.031)	0.063 (0.027)
C. fallenii	Changbaishan (12)	10.1 (0.7)	2.1 (0.3)	53.6	0.202 (0.051)	0.286 (0.056)
dark form	Nachod (6)	43.4 (2.5)	2.3 (0.3)	46.4	0.141 (0.036)	0.221 (0.048)
light form	Nachod (6)	20.8 (1.5)	1.9 (0.2)	46.4	0.105 (0.031)	0.215 (0.048)
C. fulva	Skripov (8)	5.5 (0.3)	1.6 (0.1)	53.6	0.125 (0.055)	0.232 (0.047)
C. klugii	Montasio (3)	5.8 (0.5)	1.1 (0.1)	10.7	0.030 (0.021)	0.041 (0.023)
	Vrsic (4)	7.9 (0.7)	1.5 (0.1)	32.1	0.094 (0.041)	0.160 (0.044)
	Liangshui (10)	11.0 (1.0)	1.7 (0.2)	50.0	0.105 (0.029)	0.202 (0.046)

[a]Populations are identified by the name of the locality. Figures within brackets refer to the following countries and regions: 1, 2: Italy, Veneto = 3: Italy, Friuli; 4: Slovenia; 5: Germany, Bavaria; 6: Czech Republic, Bohemia; 7, 8: Czech Republic, Moravia; 9: China, Inner Mongolia; 10, 11: China, Heilongjiang; 12: China, Jilin.
N = mean sample size per locus, S.E. within brackets.
A = mean and S.E. of number of alleles per locus, S.E. within brackets.
P = proportion of polymorphic loci, 95% criterion.
H_{obs} = mean proportion of heterozygous females per locus, S.E. within brackets.
H_{exp} = unbiased expected number of heterozygous genotypes per locus, S.E. within brackets.

weight, head diameter, pigmentation of frons and subanal plate, and colour of the body were recorded for each living prepupa. The data were used for sex determination and for the preliminary identification of taxa[20,23]; specimens identified as different forms were considered as separate samples. Specimens of each taxon are in the collection of the Institute of Agricultural Entomology of the University of Padova. Feeding larvae and adults collected in the field were reared on spruce seedlings in outdoor conditions in Padova. Rearings were used to assess emergence period, larval behaviour, and correspondence between prepupae and adults of each taxon. Dried adult specimens of each taxon are also preserved in the Institute collection.

Electrophoretic analysis

Living prepupae were preserved in single plastic tubes at 8–10 °C until genetic analysis was performed. Whole individuals were macerated manually in 0.05 ml of a 0.01 M Tris-HCl buffer, pH 8.0, solution with a glass pestle in Eppendorf tubes kept on ice. The uncentrifuged homogenate was absorbed onto Whatman no. 3 paper wicks and immediately used in electrophoresis for about half of the markers. The remaining homogenate was frozen at −30 °C and used to complete electrophoretic analysis within 2 or 3 days of homogenization. Horizontal starch gel electrophoresis was performed on 10% starch gels (Connaught hydrolysed potato starch) using established techniques[25]. Twenty one enzymatic proteins (28 presumptive gene loci) were assayed using material from the same individuals: α-glycerophosphate dehydrogenase (*G3pdh*), lactate dehydrogenase (*Ldh*), malate dehydrogenase (*Mdh-1*, *Mdh-2*), malic enzyme (*Me-1*, *Me-2*), isocitrate dehydrogenase (*Idh*), 6-phosphogluconate dehydrogenase (*6Pgdh*), octanol dehydrogenase (*Odh*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), hydroxybutyrate dehydrogenase (*Hbdh*), xanthine dehydrogenase (*Xdh*), superoxide dismutase (*Sod-1*, *Sod-2*), aspartate aminotransferase (*Aat-1*, *Aat-2*), pyruvate kinase (*Pk*), arginine phosphokinase (*Apk*), phosphoglucomutase (*Pgm*), esterase (*Est-1*, *Est-2*, *Est-3*), peptidase 1 (*Pep-1*) (substrate: leucine-leucine-leucine), peptidase 2 (*Pep-2*) (substrate: phenylalanine-proline), peptidase 3 (*Pep-3*) (substrate: glycine-leucine), leucine aminopeptidase (*Lap*), aldolase (*Ald*), glucose phosphate isomerase (*Gpi*). Buffer systems, running conditions and staining techniques are described elsewhere[26,27]. Isozymes and allozymes were designated respectively in numerical and alphabetical order. On occasion, a locus was not resolved in a given population. In this case, the most common allele was assigned to allow computerized matrix data operations[10]. Thus any distortions generated tend to underestimate genetic distances and genetic variability. Allozymic data were analyzed with the BIOSYS-1 program[28]. Genotype frequency data obtained from females alone were used to calculate observed heterozygosity (H_{obs}). Allele frequencies from both sexes were used to compute the unbiased[29] expected number of heterozygous genotypes at all polymorphic loci; this value was compared with direct counts of heterozygous female genotypes using χ^2 test. Allozyme frequency data from all samples were also used for calculating other genetic indices (A, P (95%)), unbiased genetic distances[29] and gene flow estimates[30].

Results

Patterns. Allele frequencies at 28 loci from 22 populations are available upon request. Only one locus (*Me-2*) was invariant across all populations and taxa studied. *Sod-2* was invariant across all populations, except in *C. fallenii* from Changbaishan, where an alternative allele is fixed. Fixation frequently occurs also at the enzyme loci *Ldh*, *Idh*, *Aat-2*, *Mdh-1*, *Pep-3*, *Sod-1*.

Within the four better represented species or species complexes a few loci are fixed for two alternative alleles in Chinese and European populations (e.g. *Idh* and *Mdh-1* in *C. abietis*, *Ldh* in *C. arvensis*, *Sod-2* in *C. fallenii*, *Pep-2* in *C. klugii*). Within Europe, *Mdh-1* is near fixation in the Italian and Czech populations of *C. arvensis*, and *Gpi* is near fixation between the light and dark forms of *C. fallenii*. Other cases of within species fixation of alternative alleles were observed in three populations only: *C. abietis* (Xinlin), *C. arvensis* (Laoyelin), *C. klugii* (Vrsic).

C. erythrogaster possesses the highest number of unique alleles (*Idh*, *Aat-2*, *Ald*, *G3pdh*); unique alleles are also present in populations of *C. abietis* (Liangshui: *Est-3*; Xinlin: *Pep-3*; Bodenmais: *Ldh*, *Aat-1*), *C. fallenii* (dark form, Nachod: *Ald*; light form, Nachod: *G3pdh*; Changbaishan: *Sod-2*, *Pk*), *C. arvensis* (Laoyelin: *Ldh*), *C. klugii* (Liangshui: *Apk*), *C. fulva* (Skripov: *Gpi*).

Genetic variability. Table 1 shows mean sample size per locus, mean number of alleles per locus, percentage of polymorphic loci and mean expected and observed heterozygosity values for each population. The lowest genetic variability was detected in *C. erythrogaster* (H_{exp} 0.063): the values of all genetic indices for this species, however, should be viewed as provisional and are probably underestimated (only one population examined, with small sample size). Small samples may be responsible for the low genetic variation detected in two other populations (*C. abietis*, Xinlin; *C. klugii*, Montasio). In all other populations values are high to very high (H_{exp} ranges from 0.138 to 0.286), even when few individuals are sampled, such as in *C. alashanica* and in *C. fulva*. Populations of *C. fallenii* and *C. arvensis* display the highest variation. In the five European populations of *C. arvensis*, and mainly in those from the Czech Republic, variation can be enhanced by the mixing of typical

and summer forms in unknown proportions. Chinese populations display more variation than the conspecific samples from Europe in *C. fallenii* and *C. klugii*.

Correlations between genetic variability indices and biological or ecological parameters of *Cephalcia* populations are not evident. There is only a slight tendency to display higher genetic variation in early emerging species with solitary larval behaviour. Within single populations, individual observed heterozygosity (in females) varies with no apparent relation to the size of the prepupae (fresh weight and head diameter). Introgressive hybridization as a possible source of genetic variability was also considered, but no evidence was found.

Hardy-Weinberg analysis. Twelve significant departures (four with $p < 0.01$, eight with $p < 0.05$) from Hardy-Weinberg expectations, always involving a deficiency of heterozygotes, were observed, out of 251 analysed cases (4.8%) using a Bonferroni procedure[31]. This percentage of departures is almost what would be expected by chance alone. Such deviations were detected in nine populations (41%). Two populations display deviations from Hardy-Weinberg proportions for more than one locus: *C. abietis* (Jesenik: *Est2* ($p < 0.01$), *Odh* ($p < 0.01$)) and *C. fallenii* dark form (Nachod: *Pep-2* ($p < 0.01$), *6Pgd*, *Pk* ($p < 0.05$)). In the other populations, only one deviation was detected: *C. abietis* (Bodenmais, Liangshui: *Est-2*), *C. arvensis* (typical form, Nachod: *G3pdh*; Cansiglio: *Est-2*; Skripov: *G3pdh*; spring form, Nachod: *Est-1*; Laoyelin: *Aat-1*). In former cases, sample sizes were large compared to the other populations in this study, suggesting that departures from Hardy-Weinberg expectations are unlikely to be due to sampling errors. Mixing of species in these cases also seems unlikely.

Gene flow. Gene flow estimates based on the average frequency of private alleles (i.e. alleles that are present in one population only[30]) were calculated (table 2). The estimated number of migrants was calculated for conspecific populations within geographic regions (Europe and China) and over the whole range. Estimated values predict a rather high number of migrants for *C. arvensis*, both within Europe and between Europe and China. Values are lower for *C. abietis* and far lower for *C. klugii*.

Different phenological sympatric 'forms' within species complexes are often reproductively isolated as well. This was observed within *C. fallenii*, where the dark and light forms are separated by different alleles at the diagnostic locus *Gpi* (only one heterozygous female was observed, out of a total of 52). The dark and light forms sympatrically living in Nachod, moreover, display a high number of private alleles: 14 in the dark form, with average frequency $p(1) = 0.122$ (SD = 0.265) and 5 in the light form, with $p(1) = 0.175$ (SD = 0.157). The estimated number of migrants per generation is low ($Nm_{est} = 0.53$).

Table 2. Gene flow estimates on the basis of the frequencies of rare alleles[30].

Species	Region	n of populations	n private alleles	$p(1)$	Nm_{est}
C. abietis	Europe	3	10	0.060	1.047
	China	2	21	0.266	0.054
	All	5	17	0.226	0.075
C. arvensis	Europe	5	19	0.057	2.653
	All	6*	17	0.135	0.443
C. klugii	Europe	2	14	0.311	0.021
	All	3	18	0.189	0.071

$p(1)$ = average frequency of private alleles.
Nm_{est} = estimated number of migrants per generation.
*The population from Laoyelin was omitted from the calculation of the gene flow because it is likely to be a different species.

The same is true for the typical and spring forms of *C. arvensis*, which at Nachod are characterized by 8 and 18 private alleles with an average frequency $p(1) = 0.360$ (SD = 0.244) and 0.205 (SD = 0.171), respectively. This corresponds to a very low value of Nm_{est} (0.06).

Genetic distances. Coefficients of unbiased genetic identity and distance[29] were calculated for all pairwise combinations of populations. The complete matrix is available from the authors upon request. The UPGMA cluster shown in figure 1 has a percentage SD of 22.532[32]. With the exception of *C. klugii*, genetic distances between European and Chinese populations belonging to the same morphological species are close to or higher than values observed between species. However, reproductive isolation (and therefore speciation) may also occur without sharp genetic divergence, as shown by the pairs dark form-light form of *C. fallenii* (I = 0.942, D = 0.059), and spring form-typical form of *C. arvensis* (I = 0.845, D = 0.168), and *C. arvensis*–*C. fulva* (I = 0.818, D = 0.201). A few populations display higher values of genetic distance within species and within the same geographic region. This is the case for *C. abietis* populations from Xinlin and Nachod, *C. arvensis* from Laoyelin and Nachod, *C. klugii* from Vrsic.

Discussion

The mean genetic variation of *Cephalcia* species (average H_{exp} 0.197, SD 0.064) is significantly higher than that of other Symphyta (suborder Tenthredinoidea) (average H_{exp} 0.059, SD 0.032) (two-tailed Mann-Whitney test, $Z = 4.39$, $p < 0.01$). The latter data were obtained from[2,10,11,16]. Unpublished data from the authors concerning the genus *Pontania* A. Costa ($H_{exp} = 0.057$, 0.035, 0.056 for *P. montivaga* Kopelke, *P. reticulatae* Malaise, *P. retusae* Benson, respectively) were also considered. In some populations and species of *Cephalcia* H_{exp} reaches the highest levels observed for diplodiploid insects[13,33]. These data clearly suggest that, at least

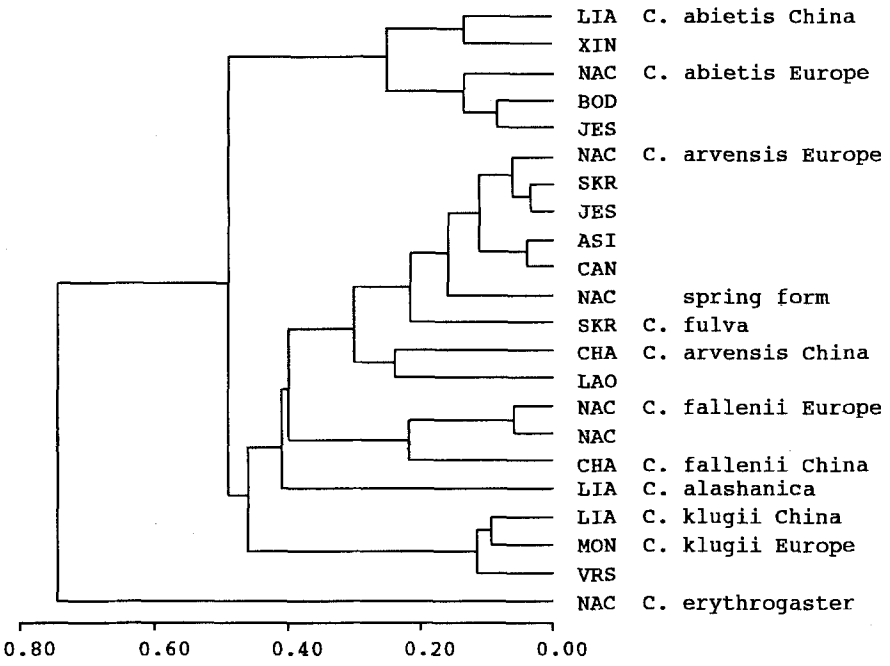

Figure 1. UPGMA cluster of the unbiased Nei's[29] genetic distances. Populations are identified by the first three letters of the locality names listed in table 1.

under particularly favourable conditions, haplodiploidy per se does not reduce genetic variability in a strong way.

Why is there such high genetic variation in populations in this genus? We suspect that different deterministic and stochastic factors may contribute to this situation. The populations of *Cephalcia* are characterized by a wide variation in the physical environment, enhanced by the plantation of the host plant outside its natural range, and are fully exposed to selection at all developmental stages. Moreover, they display a rather high colonization capability. This is supported by the high levels of gene flow detected within European populations of *C. abietis* and *C. arvensis*, with Nm_{est} values comparable to those calculated in *Drosophila*[30]. It is well known that different types of spatially and temporally varying selection regimes, coupled with migration, may influence the nature and the level of genetic polymorphism[34].

Overlapping generations may also be important in maintaining genetic variability through diversifying selection. It has been demonstrated that the progeny of a single female can emerge over 1–4 years according to the temperature of the soil when mature larvae enter it[35,36]. Thus, the individuals arising from a single mating event and experiencing the same developmental conditions as eggs and larvae, can test their fitness as prepupae and adults in different years. Consequently, individuals of different age (up to 3 and 5 years in solitary and gregarious species, respectively) participate each year in the mating process.

Thus, if genotypes are selected, many other types of natural selection can maintain polymorphism, even if classical models of balanced polymorphism or heterosis are more difficult to establish under haplodiploidy[37]. The loss of variability due to increased selection against alleles exposed in haploid males[4] may be counterbalanced by gene flow between sub-populations of different age or arising from exchange of migrants between adjacent populations.

Because of their mating system and dispersal capability, *Cephalcia* populations have a potentially large effective population size. Haplodiploidy only may result in a decreased effective population size, as the sex ratio is balanced, and levels of neutral genetic variation reduced by about 25% are expected[33]. This argument may be relevant in small ecological isolates, where the observed reduction in heterozygosity is conspicuous. In fact, the populations of *C. arvensis* (Laoyelin), *C. abietis* (Xinlin) and *C. klugii* (Vrsic, Montasio) display a higher number of fixed alleles and/or a lower genetic variation than other populations belonging to the same species. The small sample size or the way the samples have been collected may be in part responsible for this situation. However, genetic data for these populations are more in line with those already summarized for other sawflies[16]. In all these isolates, the high number of loci fixed and the high genetic distances within conspecific populations may support the predicted consequences of haplodiploidy (shorter time to fixation and faster rate of evolution)[5,38].

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