Master of Science Thesis

The importance of genetic variation to the risk of Hymenoptera parasitism in bag worm moths (Lepidoptera: Psychidae)

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ABSTRACT

According to the Red Queen hypothesis, coevolution between hosts and parasites maintains genetic variation in the host population. Genetic variation can be an advantage when avoiding parasites, because parasites are expected to infect the most common genotype of the local host population, while rarer genotypes could avoid parasite infection. In this study, I examined the importance of genetic variation to Hymenoptera parasitism in coexisting bag worm moths of *Dahlica* and *Siederia* (Lepidoptera: Psychidae) genera. The objectives were to find out, if there are any differences in the parasitoid infection rate between and among parthenogenetic and sexual bag worm moth species and to examine, if the genetic variation of parasitized individuals differs from non-parasitized individuals. Bag worm moth larvae were collected from three populations from the region of Jyväskylä in the spring 2005. AFLP markers were used to assess the genetic variation and also as tool for identification. According to this study, most of the individuals (~94 %) were parthenogenetic *Dahlica fennicella* species, while only few were sexually reproducing *Siederia rupicolella* species. Only 5.0 % of Sippulanniemi, 5.0 % of Pihta and 15.0 % of Laajavuori population's bag worm moths were parasitized. According to my results, parasitoids were not species-specific and there were not significant differences in the infection rate between sexually reproducing *S. rupicolella* and parthenogenetic *D. fennicella* species. The average heterozygosity varied between 0.095-0.119 and about 30 % of the loci were polymorphic; both parameters were used as measures of genetic variation in parthenogenetic *D. fennicella*. Based on AFLP analysis, some genetic differentiation was observed between parthenogenetic populations, which confirm the poor dispersal ability of these moths. According to AMOVA, there was not a clear population substructure within the two populations under study. All the observed variation was between individuals within trees (~89 %) and among populations (~9 %), which is a respectable amount. No significant difference in genetic variability between non-parasitized and parasitized *D. fennicella* individuals was found (~93 % genetic similarity). There was neither any indication that particular clones would be more vulnerable to parasitoids. Interestingly, parasitized individuals had significantly more alleles compared to non-parasitized ones. If the observed higher amount of alleles in parasitized individuals is due to differential selection, genetic drift or contamination of parasitoids' DNA remains to be investigated in future studies.

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TIIVISTELMÄ

Red Queen-hypoteesin mukaan koevoluutio loisen ja isännän välillä ylläpitää geneettistä vaihtelua isäntäpopulaatiossa. Geneettisestä vaihtelusta voi olla etua loisten välttelyssä, sillä loisten odotetaan infektoivan eniten paikallisen isäntäpopulaation yleisintä genotyyppiä, jolloin harvinaisemmat genotyypit voivat välttyä loisinnalta. Tässä tutkimuksessa tutkittiin geneettisen vaihtelun merkitystä Hymenoptera loisriskiin samalla alueella esiintyvillä *Dahlica* ja *Siederia* (Lepidoptera: Psychidae) suvun pussikehrääjäperhosilla. Tavoitteena oli selvittää onko loisinnassa eroja partenogeneettisesti ja seksuaalisesti lisääntyvien pussikehrääjäperhoslajien välillä ja sisällä sekä tutkia eroaako loisittujen ja loisimattomien yksilöiden geneettinen vaihtelu toisistaan. Pussikehrääjätoukat kerättiin kolmesta populaatiosta Jyväskylän seudulta keväällä 2005. AFLP markkereita käytettiin geneettisen vaihtelun havaitsemiseen sekä lajintunnistukseen. Tämän tutkimuksen mukaan lähes kaikki yksilöt (~94 %) olivat partenogeneettisesti lisääntyvää *Dahlica fennicella* lajia ja loput seksuaalisesti lisääntyvää *Siederia rupicolella* lajia. 5.0 % Sippulanniemi-, 5.0 % Pihta- ja 15.0 % Laajavuori-populaation pussikehrääjistä oli loisittuja. Tulosten mukaan parasitoidit eivät olleet lajispesifisiä eikä loisinnassa ollut merkitsevää eroa seksuaalisesti lisääntyvän *S. rupicolella* ja partenogeneettisesti lisääntyvän *D. fennicella* lajin välillä. Geneettisen vaihtelun mittareina käytettiin keskimääräistä heterotsygotia-astetta, joka vaihteli 0.095-0.119 välillä sekä polymorfisten lokusten osuuksia; noin 30 % lokuksista oli polymorfisia partenogeneettisellä *D. fennicella* lajilla. AFLP analyysin mukaan partenogeneettisten alapopulaatioiden välillä oli havaittavissa geneettistä eriytymistä, mikä vahvistaa myös pussikehrääjäperhosten huonoa levittäytymiskykyä. AMOVA:n mukaan näillä kahdella tutkimuspopulaatiolla ei ollut havaittavissa selvää alarakennetta. Kaikki havaittu vaihtelu oli yksilöiden (~89 %) ja populaatioiden välistä (~9 %), mikä on huomattavan suurta. Loisimattomat ja loisitut *D. fennicella* yksilöt olivat geneettiseltä vaihtelevuudeltaan samankaltaisia (~93 %:n samankaltaisuus) eikä mikään viitannut siihen, että geneettisesti tietynlaiset taikka tietyt kloonit kärsisivät enemmän loisinnasta. Loisituilla yksilöillä oli kuitenkin enemmän alleeleja verrattuna loisimattomiin yksilöihin. Johtuuko tulos valinnasta, satunnaisajautumisesta tai mahdollisesta parasiittikontaminaatiosta, jää tulevan tutkimuksen ratkaistavaksi.

Contents

1. INTRODUCTION 5
2. MATERIAL AND METHODS 8
2.1. Study species 8
2.2. Sample collection 8
2.3. Laboratory work 9
2.3.1. Parasitoids 9
2.3.2. DNA extraction 10
2.3.3. AFLP markers 10
2.3.4. AFLP procedure 10
2.3.5. Species definition with restriction enzymes 11
2.4. Data analysis 12
3. RESULTS 13
3.1. Parasitoids 13
3.1.1. Parasitoid prevalence by populations 13
3.1.2. Parasitoid prevalence by species 13
3.2. Genetic variation 17
3.2.1. Population genetic structure 17
3.2.2. Genetic variation between non-parasitized and parasitized individuals 20
4. DISCUSSION 21
4.1. Parasitoids 21
4.2. Genetic variation 23
Acknowledgements 24
Literature 25

1. INTRODUCTION

Parasites are usually considered to be a burden to the host organisms, which is true as they often are harmful or even lethal to the hosts (Beckage 1997). Parasites may, for example, alter the growth rate, behaviour or reproductive success of the host (Price 1980). Thus, parasitism is costly and easily leads to reduced host fitness. However, on the population level parasites may sometimes provide benefits to the host population (Buckling *et al.* 2006). Parasites are shown, for example, to control the host density (Albon *et al.* 2002). In particular, many evolutionary biologists have suggested that parasites might be important for maintaining genetic variation and even for maintaining sexual reproduction in host populations (Jaenike 1978, Hamilton 1980, Bell 1982, Jokela *et al*. 2003). This idea is better known as the Red Queen hypothesis (Jaenike 1978, Hamilton 1980).

According to the Red Queen hypothesis, coevolution between hosts and parasites maintains genetic variation in the host population (Jaenike 1978, Hamilton 1980). The main idea of this hypothesis is that parasites are expected to infect the most common genotypes of the local host population (Lively & Howard 1994, Lively & Dybdahl 2000). While parasites are under strong selection to infect the common genotypes, the rarer ones could avoid the parasite infection. This kind of frequency-dependent selection could give a selection advantage to a rarer genotype and it should spread quickly in the population (Lively 1996). However, after a time-lag the parasites switch their target host and start to infect the newly arisen most common host genotype. This interaction easily leads to oscillations in the host and parasite gene frequencies, because parasites are never perfectly adapted to their hosts (Bell 1982, Hamilton *et al.* 1990). Tracking of common host genotypes should also lead to local adaptation by the parasite population. Thus, parasites should be more infective to hosts from sympatric (local) locations than hosts from allopatric (distant) locations (Dybdahl & Storfer 2003).

Under the Red Queen hypothesis, tracking of the common host genotypes could give an advantage to those individuals that produce genetically variable offspring (Van Valen 1979). This is the case with sexually reproducing species that recombine their genes in cross-fertilization. As a result, genetic variation is increased in the population and adaptation to changing environments is enhanced (Dybdahl & Krist 2004). As an opposite, parthenogenetic species usually lack the ability of recombination and therefore new genetic material is often produced only through mutations (Maynard Smith 1998), which could also lead to the accumulation of deleterious recessive mutations (Muller 1964). Therefore, parthenogenetic lineages are expected to have limited evolutionary potential. Indeed, parthenogenetic reproduction is expected to be more common in stable environments, where parthenogenetic individuals could be safe from parasites and pathogens (Bell 1982). Thus, coevolutionary interactions with parasites may select for sexual reproduction in hosts as a way to evolve new genetic defences against rapidly co-evolving parasites (Hamilton *et al.* 1990). This may also give an explanation to the widespread occurrence of sexual reproduction (Hamilton *et al.* 1990, Howard & Lively 1994).

On the other hand, a limitation to the Red Queen hypothesis brings up the fact that a diverse parthenogenetic clone population could also have the same benefit, because sex is not selected for per se, but diversity is (Lively & Howard 1994, Lively 1996). For example, Lively *et al.* (1990) found out that the infection rate by trematoda (*Microphallus* sp.) parasites was greater in parthenogenetic mosquito fish (*Poeciliopsis monacha*) clone populations compared to sexual subpopulations except for a case where the sexual population was highly inbred due to a bottleneck-effect. Therefore, also sexual species can suffer from increased parasitism, if the amount of genetic variation decreases in the population. Clonal diversity, in turn, may be gained through repeated mutation of sexual

individuals to parthenogenetic reproduction (Delmotte *et al.* 2001a), hybridization from sexual lineages (West *et al.* 1999) or occasional recombination with rare males produced by parthenogenetic females (Butlin *et al.* 1998). Clonal diversity may also be maintained by ecological differences among clones since each clone is adapted to specific environment (frozen niche variation hypothesis; Vrijenhoek 1979, Vorburger 2006). Moreover, the all-else-equal assumption (e.g. production of equal number and equal quality of offspring) is expected to be true by the Red Queen hypothesis (Maynard Smith 1978), which may be affected by the type of parthenogenesis, origin of asexuality and ploidy level (Johnson & Leefe 1999, Delmotte *et al.* 2001).

Empiric testing of the Red Queen hypothesis for genetic diversity is limited, because of the lack of suitable natural study systems (Jokela *et al.* 2003). In order to meet the testing conditions, closely related parthenogenetic and sexual species need to coexist and compete for the same resources. They also need to be exposed to the same highly virulent parasites (May & Anderson 1983). Therefore, most of the studies made in this field are either theoretical or computer modelling, which also need empiric research to support them (Dybdahl & Lively 1998). However, empiric support for the Red Queen hypothesis is given especially by studies made with the New Zealand freshwater snail (*Potamopyrgus antipodarum*) populations which fulfil the assumptions. As an example, Lively & Dybdahl (2000) showed that highly virulent trematoda (*Microphallus* sp.) parasites infected mostly the most common freshwater snail host genotype of the population rather than the rarer genotypes. They also found out in a reciprocal cross-infection experiment that trematoda parasites were more infective to local host snails than to distant snails. In addition, common freshwater snail clones were found to be more infectible than rare clonal genotypes (Dybdahl & Lively 1998, Dybdahl & Krist 2004). Moreover, Lively *et al.* (2004) found out in meta-analysis studies that triploid snail populations were more resistant to distant parasite populations than sexual populations. This finding supports the recent idea that parasite resistance is affected by the host ploidy (Osnas & Lively 2006). There is also some evidence of parasite-mediated clonal selection in the cyclical parthenogen water flea (*Daphnia magna*) (Little & Ebert 1999, Carius *et al.* 2001). All these results support the idea that parasites track specific host genotypes and adapt to local populations under natural conditions.

Also, bag worm moths (Lepidoptera: Psychidae) offer an opportunity to study the rare coexistence of closely related species with different reproductive modes that are also vulnerable to the same parasitoids (Hymenoptera: Ichneumonidae). Parthenogenesis occurs rarely in butterflies (Lepidoptera), but among bag worm moths, parthenogenetic reproduction is very common alongside with sexual reproduction (Suomalainen 1962). According to Kumpulainen (2004), parthenogenetic lineages, especially in the *Dahlica* genus, arose independently from sexual bag worm moth ancestors at least three times. Therefore, parthenogenesis is most likely not a result of interspecific hydridization events (Kumpulainen 2004, Grapputo *et al.* 2005a). Because of the continuous arising of parthenogenetic lineages it is probable that parthenogenetic reproduction has a selective advantage in this group (Kumpulainen 2004, Grapputo *et al.* 2005a).

Parthenogenetic and sexually reproducing bag worm moths of *Dahlica* and *Siederia* share the same habitat in few regions in Finland. Strictly parthenogenetic *Dahlica fennicella* and strictly sexually reproducing *Siederia rupicolella* are the most common species in the region of Central Finland, but also the sexually reproducing *Dahlica charlottae* species is found in the same area (Kumpulainen *et al.* 2004). The proportion of sexual and parthenogenetic species varies depending on the area, and in some locations one of either species can be absent (Kumpulainen *et al.* 2004). Parthenogenetic females start laying eggs immediately after hatching, while sexually reproducing species must first attract males by secreting pheromones and waiting for the males to arrive in order to

copulate (Suomalainen 1980). It has been shown that a male can effectively fertilize one female; a male's multiple mating decreases female's fertility drastically (Kumpulainen 2004). To date, no males are known to exist for parthenogenetic *D. fennicella* species. Female moths are always wingless and sessile, whereas sexual males can fly short distances from ten to 100 metres. However, it is possible that small bag worm moth larvae use wind as an aerial dispersal force (also called ballooning) (Hättenschwiler 1997). According to Kumpulainen *et al.* (2004) comparisons between female sizes, offspring quantity and quality have showed no significant differences between the sexual and parthenogenetic forms; therefore the all-else-equal assumption (Maynard Smith 1978) is expected to be upheld. These bag worm moths are extremely similar in morphology and ecology, therefore species definition is difficult without genetic markers or if the reproductive mode is not known (Kumpulainen *et al.* 2004).

Dahlica fennicella moths are tetraploids and reproduce through automictic parthenogenesis (Suomalainen 1962). This kind of parthenogenesis is quite rare, but it is relatively common in insects (Suomalainen 1962). The first stages of automictic parthenogenesis are reminiscent of the normal meiosis, but in the end the female's two central haploid polar nuclei fuse (also called central fusion) and form a diploid egg cell (Suomalainen 1962). This fusion gives rise to offspring that are not completely clones of each other (Maynard Smith 1998). Many automictic organisms regulate this process cytologically, which could allow a high degree of heterozygosity but could also lead to complete homozygosity in one generation (Hood & Antonovics 2004). Sexual *S. rupicolella* and sexual *D. charlottae* species are both diploid. Recently, Grapputo *et al.* (2005b) studied genetic diversity and population structure in bag worm moths using allozymes. They noticed that parthenogenetic *D. fennicella* populations were genetically very diverse despite their reproduction mode: 65 different genotypes were detected among 86 individuals. However, sexually reproducing *S. rupicolella* and *D. charlottae* populations were genetically more diverse than parthenogenetic populations, even though the populations were isolated and inbred because of limited gene flow due to weak dispersal ability. Grapputo *et al.* (2005b) also noticed that all species suffered from high heterozygote deficiency, which could be due to presence of null alleles.

Dahlica and *Siederia* moths are vulnerable to the same lethal *Orthizema* (Hymenoptera: Ichneumonidae) parasitoids, and are infected by at least two species of this genus. Parasitoids usually lay only one egg per host larva. The egg stays dormant until the larva has grown and is ready to pupate. Then the parasitoid starts to develop and uses the larva as a food resource, having an extreme effect by killing its host (Kumpulainen *et al.* 2004). Kumpulainen *et al*. (2004) found out that sexual bag worm moth species were common in the areas where the frequency of parasitoids was high, whereas parthenogenetic species dominated in the areas that had few parasites or none. This supports the hypothesis that continuous arising of new gene combinations in the host species is required to resist the parasites. However, in previous studies reproductive mode and genetic background of parasitized individuals could not be determined because parasitized moths always die and there is not enough suitable tissue left for genetic analyses.

The aim of this study was to investigate the importance of genetic variation to the risk of Hymenoptera parasitism in bag worm moths. The objectives were (1) to find out, if there are any differences in the parasitoid infection rate between and among parthenogenetic and sexual bag worm moth species and (2) to examine, if the genetic variation of parasitized individuals differs from non-parasitized ones. In addition, population genetic structure and genetic differentiation between populations were also studied. Under the Red Queen hypothesis, parasitoids were expected to infect more the parthenogenetic species, if they are genetically less diverse than sexual species, and that

infected individuals should express the most common genotypes. In this study, AFLP (amplified fragment length polymorphism) markers were used as a molecular tool for assessing genetic variation and also for the identification of the species.

2. MATERIAL AND METHODS

2.1. Study species

Bag worm moths (Lepidoptera: Psychidae) are quite small butterflies that carry a case when they are at larval stage of development. The case is mostly made of plant materials and it includes sand and also silk weaved by the larva. The case is used for hiding from predators and parasites, and is cryptic, blending well into the trunk of the trees. The length of the larva is about three to six millimetres and the case is about the same size as the larva. Bag worm moths spend most of their lifespan, from one to two years, as larva whereas the adult stage lasts only a few days (Suomalainen 1980). The final instar larvae climb up trees during early spring to pupate and the adult moths hatch after a few weeks (Kumpulainen 2004). Bag worm moths live in warm forest edges and in sparsely populated forests with not too dense undergrowth (Kumpulainen 2005). They consume e.g. mosses, lichens and algae, which are found on the ground, on the roots and on the bark of the trees (Suomalainen 1980).

2.2. Sample collection

Fieldwork was carried out in spring 2005 in the area of Jyväskylä, in Central Finland (Fig. 1). Bag worm moth larvae were collected from Pihta, Sippulanniemi and Laajavuori populations (Table 1). The study areas were located several kilometres apart, and therefore each area represents a separate population (Fig. 1). The study areas were chosen based on the previous knowledge of the study sites (Kumpulainen *et al*. 2004). The aim was to choose the populations so that they would contain both parthenogenetic and sexual bag worm species in the same proportion. The Pihta population was the most representative in this ratio, whereas in Sippulanniemi parthenogenetic species and in Laajavuori parthenogenetic and sexual species were the most common, respectively (Kumpulainen *et al*. 2004). The habitat in these areas was formed by warm and open forest edges that were sparsely populated, though Sippulanniemi and Laajavuori were denser than Pihta. Study areas consisted mostly of Norwegian spruces (*Picea abies*), Scotch pines (*Pinus sylvestris*) and silver birches (*Betula bendula*). Also, a few Siberian fir (*Abies sibirica*) were found in Pihta. All study areas were located near lakes or other sizeable bodies of water.

The final instar bag worm moth larvae were caught by setting tape traps around tree trunks at about one metre from the ground. Traps were set in 12–16 trees in each of the areas (Table 1). The tape used was a sticky brown tape approximately six centimetres wide. Traps were set on March 30th when the weather seemed warm enough for the climbing to start. Most of the traps were set on Norwegian spruces (*Picea abies*), some on Silver birches (*Betula bendula*) and few on Siberian fir (*Abies sibirica*). The distance between the trees varied between three metres to 30 metres depending on the area and the location of the trees.

Sample collection was performed from the first to the 28th April. Traps were checked once or twice a week depending on the weather conditions. Bag worm moth larvae were carefully collected from the tapes using tweezers. All individuals collected from one tree were kept in a separate box (height 6.7 cm; diameter 3.7 cm). Some of the larvae were transferred from the tapes to the upper part of the tree, so that the population would not

significantly suffer from the experiment. In the laboratory, cases of the larvae were removed using tweezers and individual larvae were put in separate Eppendorf tubes (1.5 ml). The collection date, area name, tree number and individual larva number were marked for each of the larvae. Bag worm moth larvae were preserved at -70 ˚C for later examination of the parasitoid presence and extraction of the DNA.

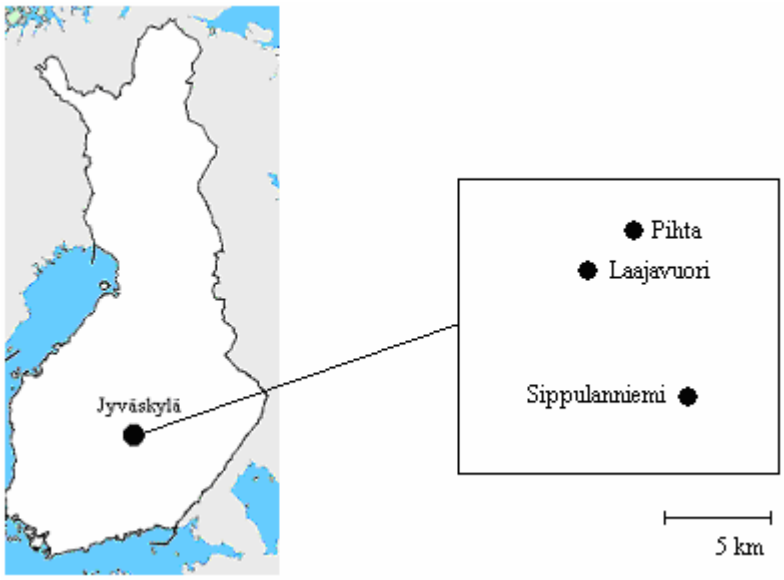

Figure 1. Locations of the study areas.

Table 1. Basic information of the study areas.

Study area	Habitat size	Number of traps	Coordinates
Pihta	30 m × 100 m	16	62°16’53’’, 25°42’46’’
Sippulanniemi	25 m × 40 m	12	62°11’56’’, 25°45’07’’
Laajavuori	30 m × 60 m	13	62°15’33’’, 25°41’59’’

2.3. Laboratory work

2.3.1. Parasitoids

Laboratory work was performed in the Ecology DNA laboratory of the Department of Biological and Environmental Sciences at the University of Jyväskylä. To determine the presence of parasitoids, frozen samples were transferred from the freezer to the laboratory keeping the samples on ice. Approximately one millilitre ethanol (97%) was added to the Eppendorf tubes, so that the larvae would soften before preparation. After a few minutes the larva was ready to be transferred under the preparation microscope. A drop of alcohol was added to the preparation plate in order to keep the larva moist enough. The rear end of the larva was removed using sterilized tweezers and preparation knife. The inside of the larva was carefully squeezed out so that observation of the parasitoid larva would be possible. Parasitoids (white larva about 3 millimetres in length) were put into separate Eppendorf tubes and parasitized larvae were marked. Moth larvae were put back into the Eppendorf tubes and preserved in ethanol (97%) until DNA extraction.

2.3.2. DNA extraction

Genomic DNA was extracted from the larval tissue by the following protocol. First, the larva was cut in a proportion of one third including the head using sterilized preparation knife and tweezers. The piece of larva was placed in a sterilized Eppendorf tube (1.5 ml). Then 60 µl extraction buffer (10 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, pH = 8) was added and larva was homogenized with a sterilised pipette tip. After homogenizing, 5 µl Proteinase K (Qiagen Proteinase 7.5 AU) was added. The tubes were vortexed and incubated at 37 °C overnight and after that at 90 °C for five minutes. Finally, samples were centrifuged at 13 000 r.p.m for three minutes.

2.3.3. AFLP markers

The AFLP analysis has been applied especially to detecting genetic variation and diversity between closely related species or individuals of a species (Blears *et al.* 1998). AFLPs are powerful molecular markers that combine the repeatability of restriction fragment analysis and the power of the polymerase chain reaction (PCR) (Vos *et al.* 1995). The AFLP analysis generates hundreds of high-resolution markers from DNA of any origin or complexity without the knowledge of prior sequence information (Mueller & Woelfenbarker 1999). Analysis consists of four steps: (1) digestion of the genomic DNA with two restriction enzymes and ligation of specific double-stranded oligonucleotide adaptors to the ends of the DNA fragments, (2) preselective PCR amplification by primers with a single nucleotide extension and (3) selective amplification with specific primers that have three selective nucleotides at their 3' end and (4) gel electrophoresis of the PCR fragments (Vos *et al.* 1995).

AFLP method is fast and cost efficient technique compared to, for example, RAPD (randomly amplified polymorphic DNA), RFLP (restriction fragment length polymorphism) and allozymes. AFLP method also provides at least equal power in terms of repeatability and resolution (Mueller & Woelfenbarker 1999). One of the greatest advantages of the AFLP technology is its sensitivity to polymorphism detection at the total-genome level (Robinson & Harris 1999). Furthermore, only small amounts of DNA are needed for the AFLP analysis allowing the use of small samples or organisms (Mueller & Woelfenbarker 1999). AFLP methods generate dominant rather than co-dominant markers, with polymorphisms detected as either band presence or absence of bands. Therefore, identification of homologous alleles is not allowed, which makes microsatellites more appropriate for that kind of use (Mueller & Woelfenbarker 1999). Nevertheless, AFLP markers have emerged as a major new type of genetic marker with wide applications in plant and animal genetics as well as in microbiology (Savelkoul *et al.* 1999). Recently it has been used in phylogenetics (*Humulus lupus*; Seefelder 2000), genetic diversity studies (*Olea*; Angiolillo *et al.* 1999), DNA fingerprinting (*Porphyra*; Sun *et al.* 2005), species identification (*Acer rubrum*; Bless 2006), paternity analysis (*Luscinia svecica*; Questiau *et al.* 1999), quantitative trait loci (QTL) mapping (rat; Otsen *et al.* 1996) and conservation genetics (*Arborimus pomo*; Blois & Arbogast 2006).

2.3.4. AFLP procedure

AFLP marker profiles were developed following a modified version of the basic procedures from Vos *et al.* (1995). Genomic DNA was digested with MseI and EcoRI restriction enzymes (Fermentas), and ligated with MseI and EcoRI adapters in sterile 1.5 ml Eppendorf tubes. Digestion of DNA and ligation of adapters was performed in a total volume of 25 µl using 7.575 µl H_2O (sterilized water), 1.25 µl DTT (100 mM), 0.25 µl ATP (20 µM), 0.125 µl BSA (10 mg/ml), 0.5 µl MseI adaptor (50 µM), 1 µl EcoRI adaptor

(5 µM), 0.3 µl MseI (10 u/µl), 0.5 µl EcoRI (10 u/µl), 2.5 µl Buffer R+ (10 X), 1 µl T4 ligase (1 u/µl) (Fermentas) and 10 µl of sample DNA. The samples were incubated at 37 °C for three hours and then diluted with 225 µl of H_2O.

Preselective amplification was performed in a total volume of 25 µl using 15.15 µl H_2O, 0.75 µl $MgCl_2$ (50 mM), 1.5 µl dNTPs (2 mM), 1 µl Primer MseI-C (5 µM), 1 µl Primer EcoF-A (5 µM), 2.5 µl Buffer (10 X), 0.1 µl Taq (5 u/µl; Biotools) and 3 µl of the diluted ligation product. Amplifications were performed in MBS satellite thermocyclers. PCR cycling conditions were one cycle at 72 °C for two minutes and one at 94 °C for two minutes, followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at 56 °C and two minutes at 72 °C followed by one cycle at 60 °C for 30 seconds. 10 µl of the pre-amplified PCR products with 2.5 µl Orange –loading dye were resolved in a 1.5 % agarose gel stained with ethidium bromide. The PCR results were checked under UV-light. The remaining 15 µl of the pre-amplified products were diluted with 100 µl of H_2O.

Selective amplification was performed with four sets of selective primer combinations with fluorescent labels: 6-FAM-EcoF-AGA and MseI-CGA, NED-EcoF-ACG and MseI-CGT, VIC-EcoF-ACT and MseI-CGT, and PET-EcoF-AAG and MseI-CGC. PCR was performed in a total volume of 12.5 µl using 6.55 µl H_2O, 0.5 µl $MgCl_2$ (50 mM), 0.75 µl dNTPs (2 mM), 0.1 µl BSA (1 mg/ml), 0.5 µl Primer MseI-NNN (5 µM), 0.25 µl Primer EcoRI-NNN (5 µM), 1.25 µl Buffer (10 X), 0.1 µl Taq (5 u/µl; Biotools) and 2.5 µl of the diluted pre-amplified product. The PCR cycling conditions were one cycle at 94 °C for two minutes, followed by 13 cycles of 30 seconds at 94 °C, 30 seconds at 65 °C and two minutes at 72 °C followed by 24 cycles of 30 seconds at 94 °C, 30 seconds at 56 °C and two minutes at 72 °C and one cycle at 72 °C for ten minutes. 2.5 µl of the amplified PCR products with 2.5 µl H_2O and 2.5 µl Orange –loading dye were resolved in a 1.5 % agarose gel stained with ethidium bromide to check the results as before. Thermo-Fast® 96 non-skirted plates were used for the both preselective and selective amplifications.

Amplified fragments were separated using the ABI 3100 (Applied Biosystems) automated sequencer. For the ABI run, 96-well plate was prepared with five microlitres of amplified PCR products of each primer combination. One microlitre of the primer mix was diluted with 39 µl of H_2O. Loading buffer was prepared using formamide (Applied Biosystems) and size standard (Gene Scan 500 Liz, Applied Biosystems) in the proportion 1:160. For each sample, nine µl of loading buffer was combined with 1 µl of diluted primer mix and the run was performed. AFLP profiles were analyzed using GeneMapper ver 3.7 (Applied Biosystems) software and the number of AFLP bands were estimated for each sample. Generated AFLPs were binary scored either present (1) or absent (0) across all the genotypes, each band being considered as a separate locus. Both polymorphic and monomorphic bands were scored.

2.3.5. Species definition with restriction enzymes

The AFLP profile did not allow a clear distinction between the three species. Therefore, from the mitochondrial sequences of the COI and COII gene published by Grapputo *et al.* (2005a) and available in GenBank (accession numbers: AY449388 – AY449457) I looked for specific restriction sites for the three species in question. Three restriction enzymes, Mnl I, Pac I and Bcl I (New England Biolabs), showed alternative cutting sites in the three species. The restriction enzymes were first tested using samples whose species status and sequences were already known. Thus, the mitochondrial DNA of adult females of *D. fennicella*, *S. rupicolella* and *D. charlottae* for which we earlier obtained larvae through parthenogenesis (*D. fennicella*) and through matings with species specific males (*S. rupicolella* and *D. charlottae*) were analyzed to ensure the method and to be used as

positive controls.

S. rupicolella and *D. charlottae* species had a restriction site for Mnl I which produced from the amplified COI-COII fragment of 574 bp, two bands when resolved in agarose gel of respectively, 129 bp & 445 bp for *S. rupicolella* and 154 bp & 420 bp for *D. charlottae* (Table 2). *D. fennicella* does not have the restriction site for Mnl I, and so an uncut band of 574 bp was expected (Table 2). *D. fennicella* instead had a restriction site for Pac I and the expected bands were 200 bp and 374 bp (Table 2). *S. rupicolella* and *D. charlottae* did not have the restriction site for Pac I and thus the expected band was 574 bp (Table 2). The fragments produced by Mnl I in the two sexual species were not different enough for a clear resolution in agarose gel, and so we tried also a third enzyme Bcl I. Unfortunately, Bcl I did not work out the way it should have worked. Therefore, parthenogenetic *D. fennicella* moths could be separated from sexual moths, but sexual moths could not be separated from each other based on these restriction enzymes.

Table 2. Expected species-specific bands for the restriction enzymes used.

Species	Mnl I Expected bands (bp)	Pac I Expected bands (bp)
D. fennicella	574	200 & 374
S. rupicolella	129 & 445	574
D. charlottae	154 & 420	574

Primers S2792 (Brower 1994) and C2-N-3389 (Simon *et al.* 1994) were used for the amplification of the DNA. PCR was performed in a total volume of 50 µl using 28.3 µl H_2O, 2.5 µl $MgCl_2$ (50 mM), 5 µl dNTP´s (2mM), 2 µl Primer S2792 (5 µM), 2 µl Primer C2-N-3389 (5 µM), 5 µl Buffer (10X), 0.2 µl Taq (5,0 u/µl; Biotools) and 5 µl of the extracted DNA. Amplifications were performed in MBS satellite thermocyclers using the following PCR cyclic conditions: one cycle at 94 °C for four minutes, followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at 45 °C, 45 seconds at 72 °C and one cycle at 72 °C for five minutes. 5 µl of PCR product with 2 µl H_2O and 2 µl Orange –loading dye were resolved in a 1 % agarose gel stained with ethidium bromide.

Digestion with Mnl I was performed in a total volume of 20 µl using 7.6 µl H_2O, 2 µl NE buffer 2 (New England Biolabs), 0.2 µl BSA (New England Biolabs), 0.2 µl Mnl I (500 units) and 10 µl DNA amplification product. Similarly, for Pac I 8.7 µl H_2O, 1 µl NE buffer 1 (New England Biolabs), 0.2 µl BSA (New England Biolabs), 0.1 µl Pac I (250 units) and 10 µl DNA amplification product were used. The enzymes were spun down before adding them to the mix and mixed carefully up and down with a pipette about twenty times. After adding the DNA the Eppendorf tubes were carefully tapped with a finger to mix. Tubes were incubated at 37 °C for two hours. Digested products were resolved in a 1 % agarose gel stained with ethidium bromide and the bands were analysed under UV-light.

2.4. Data analysis

AFLP-SURV 1.0 program (Vekemans *et al.* 2002) was used to estimate the population genetic structure and genetic variation of the AFLP analyzed samples. Descriptive statistics were obtained for the percentage of polymorphic loci and the average expected heterozygosity, which were used as measures of genetic variation in a population. Statistics were also computed on the overall genetic differentiation among populations (F_{st}). 1000 random permutations were performed to test for genetic differentiation. Allele frequencies were computed according to a Bayesan method with non-uniform prior distribution and

assuming Hardy-Weinberg genotypic proportions (Zhivotovsky 1999). Statistics for genetic diversity and population genetic structure were computed following the approach of Lynch & Milligan (1994).

The assumption of Hardy-Weinberg equilibrium may not be true for estimating genetic variation using dominant markers (Keiper & McConchie 2000, Salvato *et al.* 2002), and therefore, the pairwise genetic distances between AFLP profiles also were calculated using Jaccard's similarity coefficient calculations (Jaccard 1908), which measures the proportion of shared bands. The resulting matrix was used to investigate population structure by AMOVA (hierarchical analysis of molecular variance) using Arlequin ver 3.0 software (Excoffier *et al.* 2005). PHYLIP version 3.6 (Felsenstein 1995) was used to build neighbour-joining (NJ) phylogenetic trees first to search for population groupings based on Nei's pairwise genetic distances (Lynch & Milligan 1994) and then to more detailed groupings of individuals using Jaccard's similarity coefficient calculations (Jaccard 1908). 1000 bootstraps were performed for genetic distances using WinBoot software. Other statistical analyses were performed using SPSS for Windows, version 14. Pearson's Chi-Square test and Mann-Whitney's U-test were used as non-parametric tests.

3. RESULTS

3.1. Parasitoids

3.1.1. Parasitoid prevalence by populations

A total of 410 bag worm moth samples were collected from the study populations (Table 3). All collected samples were prepared in order to search for parasitoids. Parasitoids were found from all three study populations. In Pihta 5.0 %, in Sippulanniemi 5.0 % and in Laajavuori 15.0 % of the samples were parasitized (Table 3).

Table 3. Numbers of non-parasitized and parasitized samples from the total sample sizes by population.

Population	N	Non-parasitized samples	Parasitized samples
Pihta	299	284	15
Sippulanniemi	84	80	4
Laajavuori	27	23	4

3.1.2. Parasitoid prevalence by species

DNA extraction was carried out using all samples from Sippulanniemi (N= 84) and over half of the samples from Pihta (N= 185). The DNA extracted samples from Pihta included all samples (N= 19) with parasitoids and other non-parasitized samples were randomly chosen. The Laajavuori population was excluded from the analysis because of the low sample size and therefore no DNA was extracted from these samples.

According to the AFLP markers and restriction enzymes, Pihta and Sippulanniemi populations consisted of parthenogenetic and sexual bag worm moth species. There were some difficulties separating sexual moths (*S. rupicolella* and *D. charlottae*) from each other (see Species definition with restriction enzymes). However, it is more probable that all sexual moths were *S. rupicolella* rather than *D. charlottae* species based on the

phylogenetic tree (Fig. 2). In addition, *S. rupicolella* and *D. fennicella* are locally the most common species (80 % or more of all species of psychid moths) (Kumpulainen *et al.* 2004). Therefore, all sexual moths are considered as being *S. rupicolella*. On the other hand, I found out that most of the specimens were parthenogenetically reproducing *D. fennicella* species, while only few were sexually reproducing *S. rupicolella* species. In Pihta approximately 94 % (N= 170) of the analysed specimens were parthenogenetic *D. fennicella* species, while 6 % (N= 10) of the samples were sexually reproducing *S. rupicolella* species. In Sippulanniemi corresponding numbers were 93 % (N= 79) for parthenogenetic *D. fennicella* and 7 % (N= 5) for sexual *S. rupicolella* species.

Parasitoids infected both parthenogenetic *D. fennicella* and sexual *S. rupicolella* species. In Pihta 13 parthenogenetic *D. fennicella* and two sexual *S. rupicolella* specimens were parasitized (Fig. 3). In Sippulanniemi only one of the four parasitized specimens could be identified as *D. fennicella* and the others were not identified (Fig. 3 a). There were no significant differences in the parasite infection rate between parthenogenetic *D. fennicella* and sexual *S. rupicolella* species in Pihta (Pearson's Chi-Square test; N= 180, χ^2= 1.887, p< 0.170). Also, when Sippulanniemi and Pihta populations were tested together differences between the species and parasitism were not observed (Pearson's Chi-Square test; N= 275, χ^2= 1.384, p< 0.239). However, power of the test remains low due to the small sample sizes of sexual species and thus it cannot be ruled out that parasitoids prefer one species over the other or that one species copes better with the parasitoids than the other one.

In addition, parasitoids were randomly distributed in the populations and no structure was observed between parasitized and non-parasitized samples based on the results of the phylogenetic analysis (Fig. 2). This finding also suggests that samples with parasitoids were not contaminated by the parasite DNA, because otherwise we would have expected some kind of clustering among parasitized samples in the phylogenetic tree.

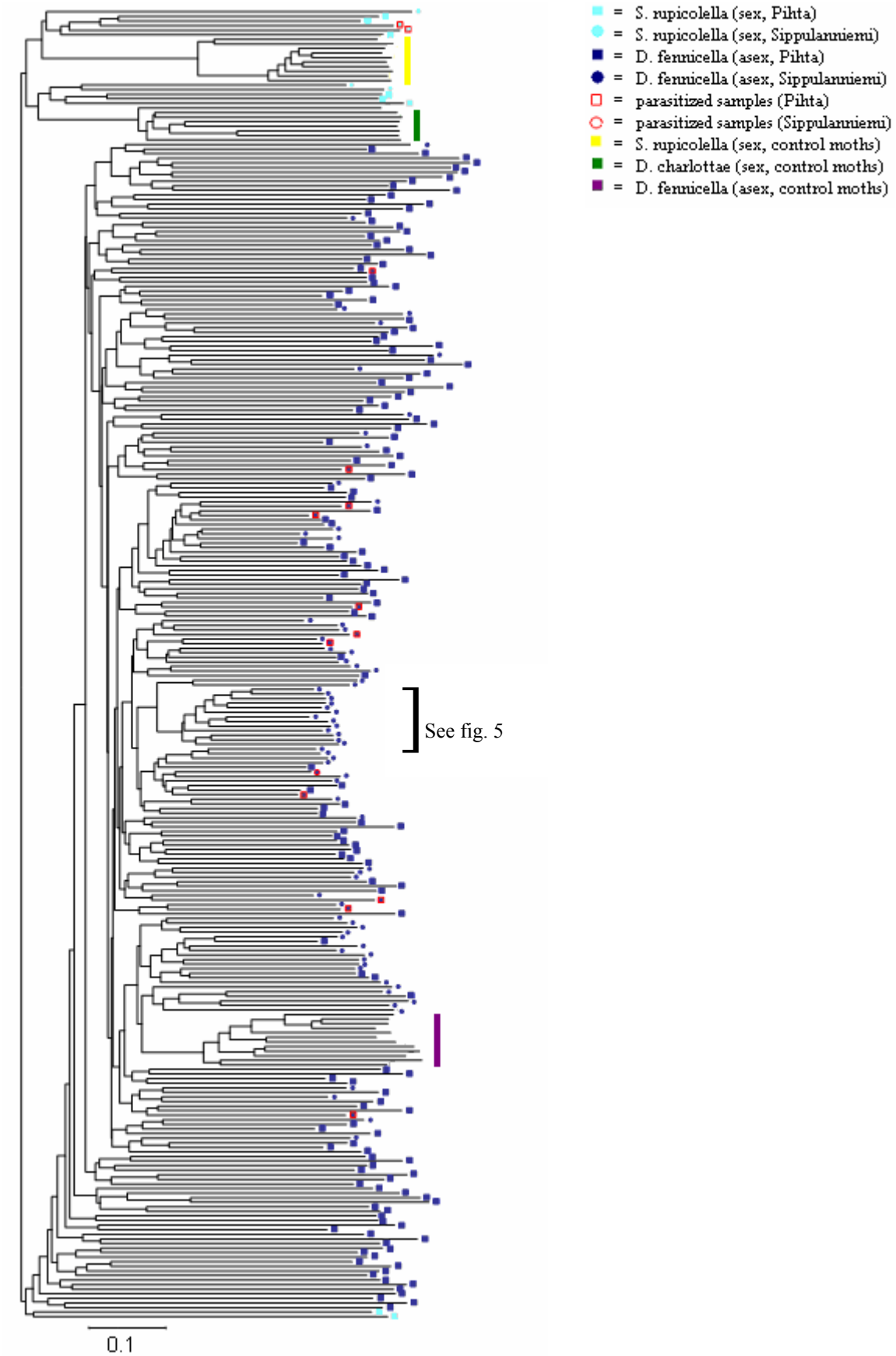

Figure 2. Neighbour-joining tree (Jaccard's similarity coefficient) representing the relationships among parthenogenetic and sexual individuals. Species and populations are marked in different colours and shapes. Individuals with parasitoids are marked in red.

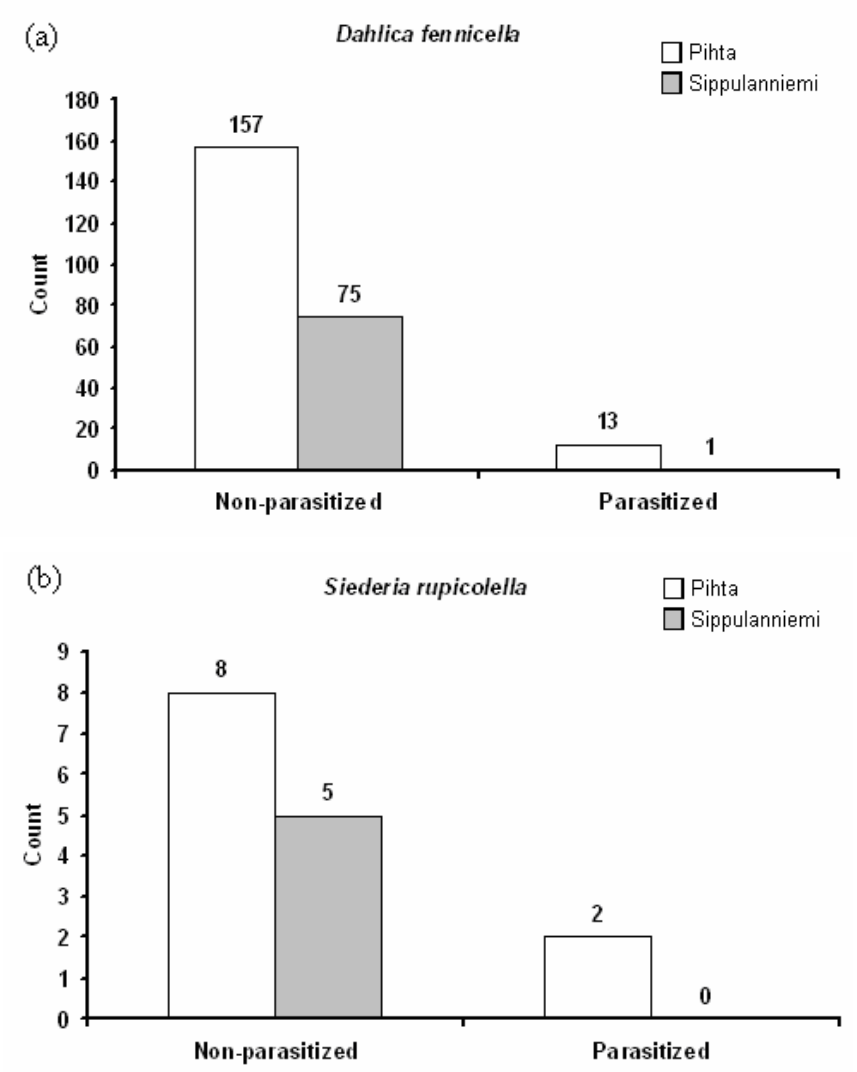

Figure 3. Parasitoid prevalence of (a) *D. fennicella* and (b) *S. rupicolella* species by populations.

3.2. Genetic variation

3.2.1. Population genetic structure

The population genetic variation was compared between the two parthenogenetic *D. fennicella* populations of Pihta and Sippulanniemi. A total of 309 bands, of which approximately 30 % were polymorphic, were obtained with the four primer combinations used (Table 4). The number of bands varied from 15 to 79. Variation as large as here indicates that some part of the variation in band numbers must be due to the quality of DNA, which affects the amplification. Mean number of bands per individual was 40.3 and the mean band size was 155 (kb). Sexual specimens were not included in this population comparison analysis, because of the low sample sizes.

Genetic variation in a population was measured by the average expected heterozygosity and mean percentage of polymorphic loci. Mean expected heterozygosity, averaged across all loci, was 0.097 in Pihta and 0.119 in Sippulanniemi (Table 4). The average heterozygosity was significantly higher in Sippulanniemi compared to Pihta (Mann-Whitney U-test; N_{Pihta}= 9, $N_{Sippulanniemi}$= 7, Z= -3.334, p= 0.001). However, there was no significant difference in the percentage of polymorphic loci between the populations (Mann-Whitney U-test; N_{Pihta}= 9, $N_{Sippulanniemi}$= 7, Z= -1.910, p= 0.056). The overall genetic differentiation among populations, F_{st}, was 0.0764 and significantly differed from zero ($p< 0.01$), which indicates that there is some genetic differentiation among populations.

Table 4. AFLPs generated among parthenogenetic *D. fennicella* individuals using four primer combinations.

Population	N	Total number of loci	Number of polymorphic loci	Polymorphic loci (%)	Average heterozygosity
Pihta	169	309	85	27.5	0.09685
Sippulanniemi	74	309	97	31.4	0.11877

Phylogenetic relationships among trees sampled were estimated with the neighbour joining method and according to Nei's genetic distance (Lynch & Milligan 1994). Only trees with more than three individuals were taken into account. Neighbour-joining analysis of trees showed that trees clustered by population (Fig. 4), which was not seen in the phylogenetic analysis of individuals (Fig. 2). Even though there is structure between the trees, the population structure is very weak. The genetic distance between trees in the Pihta population was lower than in the Sippulanniemi population (Fig. 4), which could be due to population and habitat sizes. Although, the sample size was larger in Pihta, the Sippulanniemi population is larger in total (Kumpulainen *et al.* 2004), which could also explain the higher average heterozygosity in Sippulanniemi. In Sippulanniemi, the physical distance between tree number one and tree number 12 is the longest. Therefore, there could be some isolation by distance in Sippulanniemi. Though the genetic distances are very low in Pihta, the trees number one to three are located nearest together, while the trees number 13 and 14 are located furthest away.

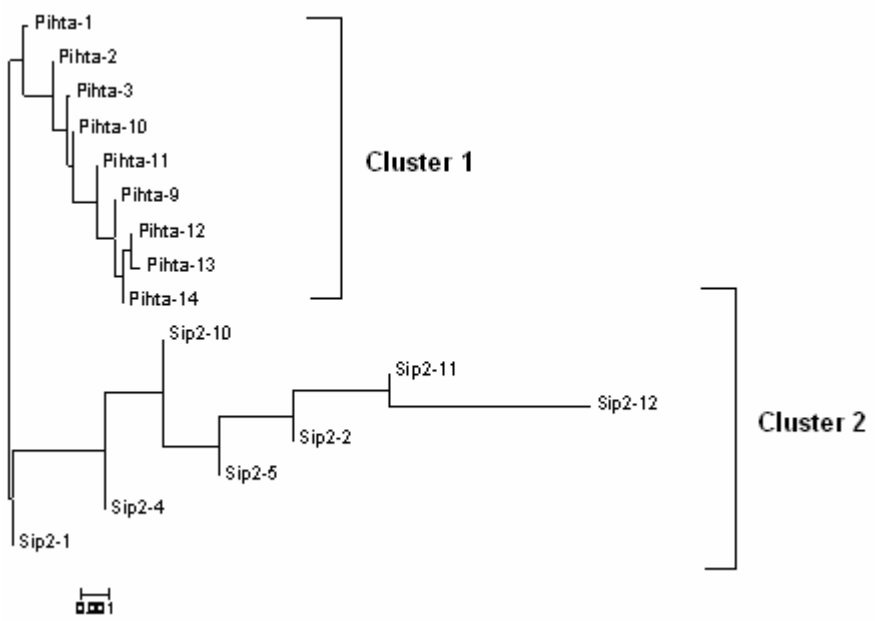

Figure 4. Relationships among trees from were individuals were sampled from two parthenogenetic *D. fennicella* populations based on a neighbour-joining analysis (Nei's genetic distance). Cluster 1 includes nine different trees from Pihta (N= 166) and cluster 2 includes seven trees from Sippulanniemi (N= 69). The tree number is reported after the population name.

Based on the hierarchical analysis of molecular variance (AMOVA), genetic similarity among Pihta and Sippulanniemi populations was high and only 9.42 % of their variation was different ($p < 0.001$) (Table 5). This indicates that there is not a strong genetic differentiation between the populations. Trees within populations showed even less variation, only 1.68 % of their variation was due to the trees within populations (AMOVA; $p < 0.001$) (Table 5). Although the variation among trees within populations was significantly different from zero, it was extremely low indicating that there is not a strong population substructure within the two populations under study. 88.9 % of the variation was caused by individuals within trees (Table 5), which is surprisingly high because the populations are strictly parthenogenetic.

Table 5. AMOVA of the two investigated *D. fennicella* populations of Pihta and Sippulanniemi.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Significance
Among populations	1	230.634	2.10030 Va	9.42	< 0.001
Among trees within populations	14	351.998	0.37541 Vb	1.68	< 0.001
Within populations	219	4340.618	19.82018 Vc	88.90	< 0.001

The neighbour-joining tree of all AFLP analysed individuals from Pihta and Sippulanniemi was constructed based on Jaccard's similarity coefficient calculations. The neighbour-joining tree showed that distances between parthenogenetic and sexual individuals were small and populations were mixed together (Fig. 2), which indicates that these two populations are closely related. There was no clear clustering by species, except for control moths that were more clustered (Fig. 2). On the other hand, clustering of the control moths could be affected by the fact that DNA was extracted from adult moths, not from larva as here. There may be some differences between the quality of larval and adult DNA. In particular, parthenogenetic *D. fennicella* specimens from Pihta and Sippulanniemi

were clearly mixed in the phylogenetic tree. However, there was some structure in some parts of the phylogenetic tree (Fig. 5, see also Fig. 2). Also, sexual *S. rupicolella* specimens clustered quite nicely close to *S. rupicolella* control moths (Fig. 2).

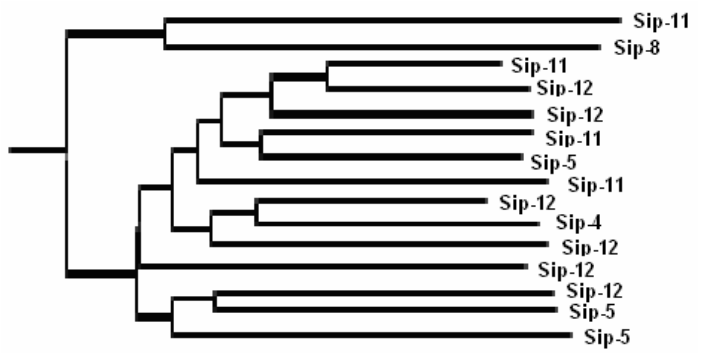

Figure 5. A piece of the phylogenetic tree showing some structure. All individuals in the picture are parthenogenetic *D. fennicella* species from Sippulanniemi. The tree numbers are mentioned after the area name.

In order to further study the differences between the parthenogenetic *D. fennicella* subpopulations, the numbers of bands (alleles) were compared. Sippulanniemi population had significantly more bands compared to the population in Pihta (Mann-Whitney U-test; N_{Pihta} = 166, $N_{Sippulanniemi}$ = 69, Z= -6.734, p< 0.001) (Fig. 6). In Pihta mean number of bands was 36 (95 % CL: 33.9), while minimum number of bands was 15 and maximum was 72. In Sippulanniemi mean number of bands was 51 (95 % CL: 47.59), while minimum number of bands was 22 and maximum was 79. This finding could be due to larger total population size in Sippulanniemi, while population in Pihta is known to be smaller. On the other hand, large variation in band numbers indicates that there have been differences in the quality of DNA, which in turn have affected the outcome of PCR.

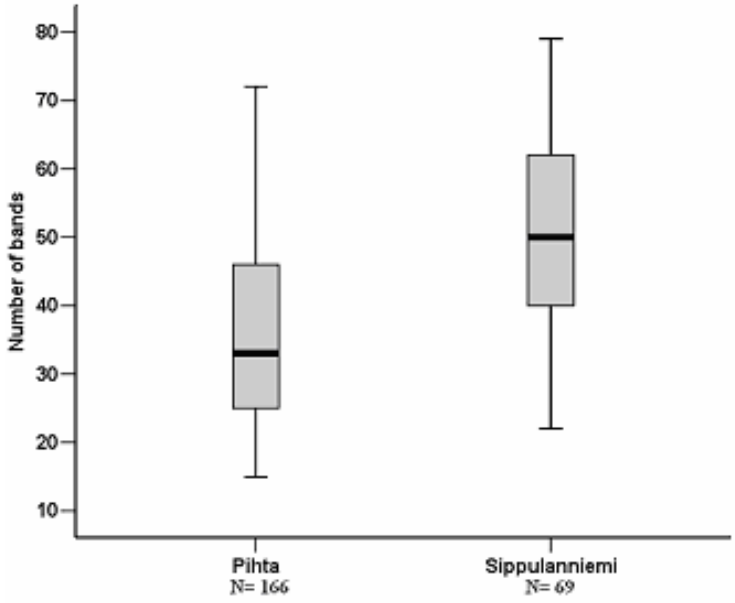

Figure 6. Comparison of the band numbers between parthenogenetic *D. fennicella* populations.

3.2.2. Genetic variation between non-parasitized and parasitized individuals

Within species genetic variation was compared between non-parasitized (N= 157) and parasitized (N= 12) parthenogenetic *D. fennicella* individuals from Pihta. Mean number of fragments per individual was 35.6 and the mean fragment size was 155 (kb). The average heterozygosity for all 309 loci was 0.095 in non-parasitized individuals and 0.118 in parasitized individuals (Table 6). The average heterozygosity and percentages of polymorphic loci between non-parasitized and parasitized individuals was similar.

Table 6. AFLPs generated between non-parasitized and parasitized *D. fennicella* individuals in Pihta.

Population	N	Total number of loci	Number of polymorphic loci	Polymorphic loci (%)	Average heterozygosity
Non-parasitized	157	309	84	27.2	0.09501
Parasitized	12	309	83	26.9	0.11784

According to AMOVA, only 6.67 % of the variation among non-parasitized and parasitized *D. fennicella* individuals was different between them ($p < 0.001$) (Table 7). This indicates that they are genetically very similar and that there are not major genetic differences between them. On the other hand, the comparison of band numbers between non-parasitized and parasitized individuals showed that parasitized individuals had significantly more bands compared to non-parasitized ones in Pihta (Mann-Whitney U-test; $N_{non\text{-}parasitized}$ =154, $N_{parasitized}$= 12, Z= -3.188, p= 0.001) (Fig. 7 a). In Sippulanniemi, comparison between non-parasitized and parasitized individuals was not possible because only one parasitized individual was found but the trend was the same as in Pihta.

Table 7. Hierarchical analysis of molecular variance (AMOVA) of the non-parasitized and parasitized *D. fennicella* individuals in Pihta.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Significance
Among populations	1	51.924	1.43240 va	6.67	< 0.001
Within populations	164	3285.214	20.03179 vb	93.3	< 0.001

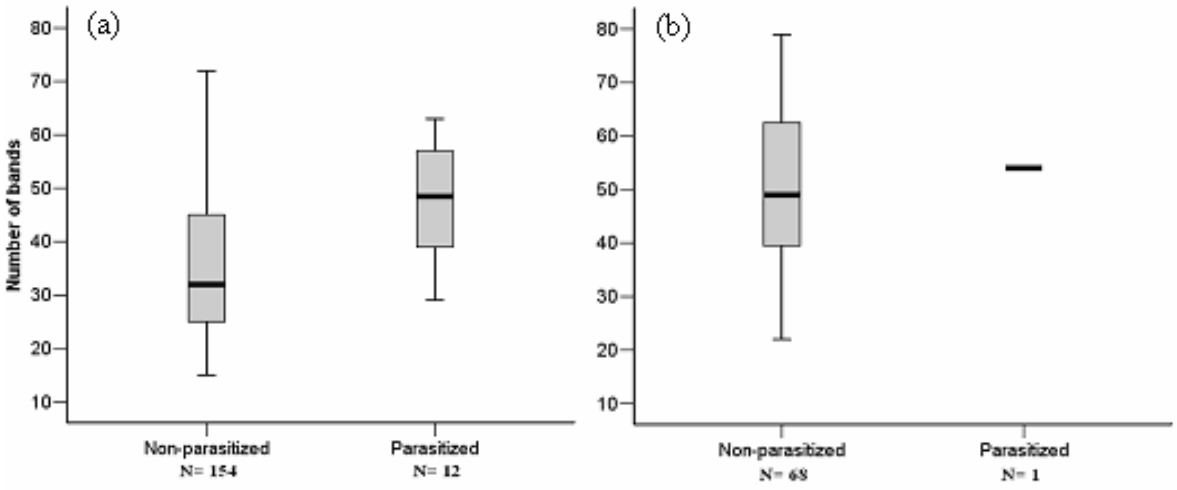

Figure 7. Comparison of the band numbers between non-parasitized and parasitized parthenogenetic *D. fennicella* individuals in (a) Pihta and (b) Sippulanniemi.

4. DISCUSSION

4.1. Parasitoids

The importance of genetic variation to the risk of Hymenoptera parasitism in bag worm moths was studied. According to my results, parasitoids attacked both species and there were not significant differences in the parasite infection rate between sexually reproducing *S. rupicolella* and parthenogenetic *D. fennicella* species. Usually parasitoids are thought to be species-specific rather than generalists and often they only attack a particular life stage of one or several related species (Hoffmann & Frodsham 1993). Since these two bag worm moth species are closely related and morphologically similar, it is likely that the same parasitoid species can infect both of them. This result is in concordance with the earlier finding that parasitoids did not differentiate between host larvae of different *Dahlica* or *Siederia* species as long as the larvae were fully grown (Kumpulainen *et al.* 2004). However, the observed positive correlation between parasitoids and sexual reproduction (Kumpulainen *et al.* 2004) can not be only a result of species-specific parasitism because parasitoids did not favour parthenogenetic individuals over sexual ones as is expected by the Red Queen hypothesis. Even though both species had parasitoids, the fact that there could be differences in susceptibility, meaning that one of the species copes better against parasitoids, can not be ruled out.

I found no evidence that would have suggested that the most common parthenogenetic *D. fennicella* clones would be more vulnerable to parasitism. Individuals with parasitoids were randomly distributed in the phylogenetic tree and no clustering was observed among parasitized samples that would have suggested that they share the same genotype. Furthermore, parasitized and non-parasitized *D. fennicella* individuals were genetically very similar. However, distinguishing between common and rare genotypes was not possible with the AFLP markers, because of the great amount of variation between individuals. In light of the Red Queen hypothesis, common clones generated by parthenogenetic reproduction are expected to be more vulnerable to parasitoids compared to rare genotypes produced by cross-fertilization, however, this is true only if the sexual species are genetically more variable (Lively & Howard 1994). Parthenogenetic *D. fennicella* species were genetically highly diverse despite their reproductive mode. High genetic diversity in parthenogenetic *D. fennicella* species could be due to several reasons: the theletokyous parthenogenesis with central fusion characteristic of the closely related species *Dahlica triquetrella* and presence of recombination (although recombination is thought to be suppressed in Lepidoptera females), and polyploidy (Grapputo *et al.* 2005b). Furthermore, Osnas & Lively (2006) suggested that polyploidy may affect parasite resistance of the host. Therefore, it remains unclear whether or not parthenogenetic *D. fennicella* has an advantage because of polyploidy when it comes to avoiding parasitoids. If so, the all-else-equal assumption, which is one of the conditions of the Red Queen hypothesis, might be violated.

The parasite infection load may also be determined by the population structure between competing parthenogens and sexuals as well as dispersal rates (Johnson & Leefe 1999). Kumpulainen *et al.* (2004) found no ecological differences that would suggest that these coexisting bag worm moth species are not competing for the same resources. However, there could still be some differences in the habitat use or quality, even though it is unlikely that habitat quality alone would explain the parasitoid infection rates. Both of these study species live on the egdes of their distribution area (Suomalainen 1980), but often this area is wider in parthenogenetic species (Suomalainen 1962, Bell 1982). This could be explained by their ability to establish a new population by themselves, while sexual species need to find a mate in order to do so (Bell 1982). According to Johnson &

Leefe (1999), higher dispersal rates of parthenogens into marginal habitats can help them to escape parasites and to persist at the metapopulation level. However, parthenogenetic bag worm moth females are immobile and it is not likely that their dispersal rate exceeds that of their parasitoids. Finally, because species statuses of the parasitoids were not investigated, we can not totally exclude the possibility that there are different Hymenoptera parasitoid species infecting these bag worm moths.

An interesting finding was also that, the parasitoid prevalence was lower in all three study populations (from 5 % to 15 %) compared to previous results. Constant observations during three years, from 1999 to 2001, showed that parasitoid prevalence of these populations was much higher (Kumpulainen *et al.* 2004). Based on Kumpulainen *et al.* (2004), parasitoid prevalence varied between 15 % and 30 % in Pihta, 20 % and 40 % in Sippulanniemi and 30 % and 50 % in Laajavuori during these above mentioned years. Also, the proportion of sexual moths was lower compared to previous results. Based on my results, both Pihta and Sippulanniemi populations consisted mostly of parthenogenetic *D. fennicella* species, while fewer than ten percent were sexually reproducing *S. rupicolella* species. According to Kumpulainen *et al.* (2004), the proportion of sexual moths varied from 30 % to 50 % in Pihta and from 15 % to 20 % in Sippulanniemi during the years 1999-2001. However, there were missing a few years of data between these studies, which do not allow a complete survey of the proportion of sexuals and parthenogens in these populations along all this period of time. Nevertheless, expectations about equal species division and higher parasite prevalence were not fulfilled in this study. On the other hand, changes in prey-predator or host-parasite population dynamics, or so called fluctuations, are common in nature (Korpimäki *et al.* 2002). Based on only a few years of examinations it is very difficult to know what caused a sudden decrease in the numbers of sexual individuals, as well as, the numbers of parasite-infected individuals. Long-term research is needed in order to conclude, whether such fluctuations are common or just random cases.

One possible explanation for the decrease of sexual individuals may be a boom of parthenogenetic *D. fennicella* individuals that have been able to displace sexual species due to the two-fold advantage of parthenogenetic reproduction. Moreover, according to Grapputo *et al.* (2005b) sexual moths are inbred and poor dispersers, which may provide favorable conditions to local extinction of sexual moths. Also, ecological factors need to be considered, for example, temperature or other abiotic conditions could be unfavorable to both parasitoids and sexual reproduction in certain locations where parthenogenetic moths manage to survive (Kumpulainen *et al.* 2004). Indeed, insect population parameters are strongly affected by the weather conditions. For example, Blanford *et al*. (2003) suggested that in pea aphids (*Acyrthosiphon pisum*) temperature may mediate parasite resistance of the host. Most of the bag worm samples in Pihta were found from Siberian fir (*A. sibirica*), which might provide better protection from parasitoids. The branches of these trees were located near the ground, whereas trunks of spruces (*P. sylvestris*) and birches (*B. bendula*) were uncovered by branches at the base, therefore leaving bag worm moths living in these trees more vulnerable to parasitoids and other environmental conditions. Interestingly, Kumpulainen (2004) noticed that the larval case protects bag worm moth larvae from ant predation, while ants predate on the parasitoids that infect bag worm moths. Kumpulainen (2004) suggested that ants might even favour parthenogenetic reproduction of bag worm moths due to reduced parasitism by the hymenopterans, which are expected to favour sexual reproduction among their hosts. In light of the Red Queen hypothesis, these results are in congruence with the hypothesis because parthenogenetic species are expected to dominate in the areas with low numbers of parasitoids.

It is also possible that the rate of parasitism was higher than we estimate and many parasitoids were undetected, which would explain the observed low parasitoid prevalence. However, recent studies with the same populations have confirmed that sexually

reproducing species and parasitoid prevalence is still very low (Elzinga *et al.* unpubl.). Thus, it suggests that I managed to detect parasitoids, but parasitoid prevalence was truly low. At least two species of Hymenoptera parasitoids are known to infect these bag worm moths (Kumpulainen *et al.* 2004), but in this study, species statuses of parasitoids were not defined. Therefore, it is not known whether different parasitoid species have differences in development rates or not. As such, it could be possible that some of the parasitoid species were not developed enough and were not visible through microscope observation. In the future, designing of species-specific primers for PCR amplification could be one solution to detect and distinguish among different parasitoid species.

4.2. Genetic variation

Based on my results, parthenogenetic *D. fennicella* show a considerable genotypic diversity. Also, high genetic diversity has been observed in triploid snails (*P. antipodarum*) (Fox *et al.* 1996). In fact, quite many parthenogenetic organisms harbour relatively high genetic diversity, which could be maintained e.g. through repeated mutations to parthenogenetic reproduction (Vorburger 2006). However, it is not always clear what processes contribute to variability in asexuals, but a role for hybridization and polyploidy has frequently been suggested (Little 2005). Mutations could have increased the genotypic variation in parthenogenetic lineages, but it is not likely that only mutations are responsible for such a great amount of variation as was observed in *D. fennicella* moths. Besides, mutational contributions are expected to increase genetic variation slowly. Recently, Gallini (2007 unpubl.) found out that even *D. fennicella* sister clones differed from each other, therefore it is unlikely that mutations could have generated that high variation in such a short time period. Furthermore, abnormalities caused by mutations have not been spotted in bag worm moths in previous studies (Kumpulainen *et al.* 2004).

Diversity in parthenogenetic species could be maintained by hybridization of closely related species, but bag worm moths are thought to be of non-hybrid origin (Grapputo *et al.* 2005a). Based on mitochondrial sequences, parthenogenetic and sexual females represent different species (Grapputo *et al.* 2005a). On the other hand, existence of cryptic sex could lead to false conclusions on the occurrence of asexuality (Little 2005). However, no males are known for *D. fennicella* species (Kumpulainen 2004). A more likely explanation is that there are some unusual mechanisms going on during the meiosis of automictic reproduction, which could lead to the high genotypic variation observed. Hood & Antonovics (2004) found that the genome of the automictic fungus (*Microbotryum*) was extremely rich in heterozygosity and had substantial variation in chromosome sizes suggesting accumulation of repetitive DNA elements. The variation they observed is not influenced by the relatedness between individuals in the manner assumed by most measures of population genetics. Therefore, Hood & Antonovics (2004) suggest that caution is needed before interpreting within- and among-population heterozygosity in automictic species. This could also explain the higher average expected heterozygosity observed in Sippulanniemi compared to Pihta.

My results suggest that populations of *D. fennicella* are genetically subdivided, which is a reasonable outcome of the fragmentation of their habitat and their inability to fly. Relationships among trees showed that trees are less diverse in Pihta than in Sippulanniemi. One reason to this could be that there is less genetic variation in Pihta, which might be caused by habitat differences, lower population size or there could be more movement (ballooning of larvae) between trees in Pihta. In general, the Sippulanniemi population is larger and denser, while Pihta is a more open area with a smaller population, which may allow more intense dispersion between the trees. In Sippulanniemi trees are located more evenly and the distances between trees do not vary as much as in Pihta. In

Pihta, some trees are located very close together, while others are further away. On the other hand, genetic differentiation based on AMOVA was not large, and this result could be explained by drift or migration. In case of drift, populations that were alike in the first place are expected to loose variation due to fixation of different alleles, while migration evens the genetic differences of the populations.

Comparison of allele numbers showed that parthenogenetic *D. fennicella* individuals in Sippulanniemi had more alleles compared to individuals in Pihta. However, it is likely that the larger total population size in Sippulanniemi probably with more clones have, at least in some part, led up to this result. More interestingly, parasitized individuals had significantly more alleles compared to non-parasitized ones. This result could be due to selection or drift, but further study is needed. Alternatively, this intriguing result could be due to an artefact. It is possible that AFLPs detect also the parasites of the target organisms, and therefore parasite alleles may interfere with the analysis (Butcher *et al.* 2004). However, this seems not to be the case here, because some kind of clustering should be expected, if samples with parasitoids were contaminated by the parasite DNA.

To my knowledge, here the AFLP method was used for the first time to reveal genetic variation in bag worm moths. Unfortunately, distinguishing common and rare genotypes was not possible with the AFLP markers, because of the great amount of variation between individuals. Thus, species-specific bands could not be clearly defined. It is possible that AFLP variation was affected by the ploidy level (amount of variation observed), mutations or the amount and quality of DNA. Also, the choice of restriction enzyme or primer can affect the number of AFLP polymorphisms detected as well as the number of primer combinations used (Robinson & Harris 1999). Here, different primer combinations were carefully tested beforehand and the best combinations were chosen. However, these combinations were first tested on tiny fresh parthenogenetic *D. fennicella* larvae, which may have had better quality DNA. In the phylogenetic tree the adult control moths are separated more clearly from the other samples. This could suggest that the markers might work differently for larval DNA and adult DNA, which may be due to methylation of DNA (Dowling *et al*. 1997). Several studies have shown that AFLPs reveal more polymorphic bands compared to other markers such as RAPDs or allozymes (e.g. Nakajima *et al.* 1998). Therefore, AFLP markers were the best possible choice to be used for bag worm moths at the moment. In the future, use of microsatellites could be one option. The use of codominant markers would also allow the discrimination of heterozygote and homozygote genotypes. Clearly, more studies are needed to reveal how genetic variation is maintained in parthenogenetic *D. fennicella* populations.

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