

Extensive introgressive hybridization between cultivated lucerne and the native sickle medic (*Medicago sativa* ssp. *falcata*) in Estonia

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We studied the occurrence of the crop-to-wild introgressive hybridization in the *Medicago sativa-falcata* complex in the distribution area of the native *M. sativa* ssp. *falcata* (hereafter *falcata*) in western and northern Estonia. Flower colour and pod shape were used as the diagnostic genetic characters to assess the extent of hybridization from cultivated lucerne to populations of *falcata*. Among the 106 populations examined, only 15 were pure yellow-flowered *falcata* populations. In the remaining 91 populations, 1%–90% of the plants had variegated flowers typical of hybrid plants, indicating widespread but highly variable introgression. Hybrid plants were detected most frequently in disturbed man-made habitats, mostly roadsides, wastelands and fallow fields. Hybrid plants with variegated flowers were found to have not only pod shapes characteristic of *falcata* but also coiled pods characteristic of cultivars, indicating that due to introgression hybrid populations have become morphologically more diverse.

Introduction

Hybridization between domesticated taxa and their wild relatives is quite common under natural conditions in areas of their sympatric distribution and it has been surveyed in many papers (e.g. De Wet & Harlan 1975, Small 1984, Raybould & Gray 1993, Rieseberg & Wendel 1993, Ellstrand *et al.* 1999, Jarvis & Hodgkin 1999, Ellstrand 2003, Hails & Morley 2005, Lane 2005). Introgressive hybridization can negatively affect wild populations in various ways, e.g. by promoting development of aggressive weeds (Ellstrand *et al.* 2010), by genetic modification and assimilation of native wild forms

(Rieseberg & Ellstrand 1993, Levin *et al.* 1996, Rhymer & Simberloff 1996, Ellstrand *et al.* 1999, Lane 2005), through outbreeding depression (Hails & Morley 2005) and through possible escape of crop transgenes to natural populations (Lavigne *et al.* 2002, Chapman & Burke 2006, Chèvre *et al.* 2007, Warwick *et al.* 2008).

Likelihood of the gene flow for each crop-wild or crop-wild relative combination is different and depends on many factors (Lane 2005). Therefore, for successful hybrid formation some important assumptions must be fulfilled. The crop and its wild relative should grow in close proximity, they should share the same pollinators, their flowering times should overlap and

their hybrids should be viable and fertile (Chapman & Burke 2006). As the gene flow from cultivated plants to natural ones is mainly pollen-mediated, it can range over long distances (Raybould & Gray 1993, St. Amand *et al.* 2000, Lane 2005). In addition, cultivated alfalfa has been shown to disperse by seeds and to be able to establish itself in roadside habitats (Bagavathiannan *et al.* 2010) where it is able to hybridize with the native sickle medic (e.g. Jenczewski *et al.* 1999a, 1999b).

The *Medicago sativa-falcata* complex is an excellent example for examining hybridization between a crop and its wild form. *Medicago sativa* ssp. *falcata* (= *M. falcata*, sickle medic, hereafter *falcata*) is characterised by yellow flowers and sickle-shaped pods, while *M. sativa* ssp. *sativa* (hereafter *sativa*) has purple flowers and highly coiled pods (Lesins & Lesins 1979). Their hybrid is called *M. sativa* ssp. *varia* (hereafter *varia*), and it also has coiled pods but its flowers are variegated. The flower colour can be mixed purple-yellow, whitish, and greenish, even brown (Small & Brookes 1984). At the same ploidy level ($2n = 32$), the three taxa mate freely producing fertile hybrids and are therefore treated as subspecies of *M. sativa* s. *lato* (Small & Brookes 1984, Quiros & Bauchan 1988). Although *falcata* and *sativa* have also diploid forms ($2n = 16$), diploid *sativa* is considered a separate subspecies, *M. sativa* ssp. *caerulea*, while the diploid forms of *falcata* have not been recognized as a separate taxon (Small 1984). Whereas crossing is more difficult between ploidy levels, formation of unreduced gametes in diploids is frequent (Barcaccia *et al.* 2003, Small 2011) thus making hybridization possible between diploid and tetraploid taxa.

Extensive hybridization between the native and cultivated forms of *sativa* has been observed in Spain (Jenczewski *et al.* 1999a, 1999b, Muller *et al.* 2001, Prospero *et al.* 2006) and Kazakhstan (Greene *et al.* 2008), whereas in Switzerland (Rufener Al Mazyad & Ammann 1999) and Germany (Bleeker *et al.* 2007) frequent hybridization between native *falcata* and cultivated *sativa* and *varia* has been recorded. It was concluded that natural populations of *falcata* in Europe are under strong introgression pressure from cultivated *sativa* and *varia* (Muller *et al.* 2003).

Medicago sativa ssp. *falcata* is native to Estonia, being mostly distributed on calcareous grasslands, alvars (calcareous grasslands on limestone plain with thin soil), and also along roadsides and on wastelands (Kukk 1999) in western and northern Estonia. *Medicago sativa* ssp. *sativa* was introduced to Estonia in 1830 (Miljan 1932). At present, *sativa* has escaped from cultivation and has become partly naturalized, growing all over the country, but more often in western Estonia (Kukk & Kull 2005). Cultivated *varia* is widely distributed throughout Estonia, being well adapted to the local natural conditions (Kukk 1999, Kukk & Kull 2005). Different introduced cultivars of *sativa* are not as resistant to the Estonian climate as are cultivars of *varia*, which have become more readily naturalized. During the 150-year cultivation period, ample diverse plant material has been introduced to Estonia from western Europe and North America, and even from the surroundings of the Caspian Sea and the Black Sea (Bender & Tamm 1998). Thus, highly different genetic material of alfalfa cultivars, able to cross with the local native sickle medic, has been accumulated in Estonia. The first notes about such hybrid swarms were published in the 1970s and 1990s (Bender & Tamm 1998).

The general goal of this study was to assess the occurrence of crop-to-wild gene flow in the *M. sativa-falcata* complex in Estonia. Our specific objectives were: (1) to describe the distribution of hybrid plants among Estonian natural *falcata* populations, (2) to find out how hybridization has influenced two morphological characteristics (flower colour, pod shape) in *falcata* mixed populations, (3) to clarify if habitat type has any influence on the occurrence of hybrid plants, (4) to elucidate if changes in land use have affected the abundance of hybrid swarms.

Material and methods

Studied taxa

The subspecies *falcata*, *sativa* and *varia* are all perennial, autotetraploid ($2n = 32$), cross-pollinated (Lesins & Lesins 1979) and partially self-incompatible (Viands *et al.* 1988). Although



Fig. 1. Distribution map of the studied 106 *Medicago sativa* ssp. *falcata* populations in Estonia. Black triangles denote mixed populations and black circles denote pure populations of

falcata and *sativa* have also diploid forms ($2n = 16$), in the Estonian native populations so far only tetraploid *falcata* has been found (Kaljund & Leht 2010). Their primary pollinators are bumblebees (*Bombus* spp.) and solitary bees (*Megachile* spp., *Andrena* spp. and others) (Martin *et al.* 1998). The growing conditions for the three taxa are quite similar: they prefer calcareous light soils on road sides, meadows, and wastelands (Lesins & Lesins 1979). In Estonia, *falcata* is also growing in such natural habitats as dry calcareous grasslands. The native sickle medic is more tolerant of winter and grazing than *sativa* or *varia* (Lesins & Lesins 1979), while its forage yield is poorer (Riday & Brummer 2004). In Estonia, cultivated *varia* is well adapted to the natural conditions and is therefore also widely naturalized, while *sativa* persists in natural conditions near cultivated fields only for a short time.

Study sites

The study was carried out in 2008 and 2009 in the distribution area of the native sickle medic in western and northern Estonia. A total of 106 populations were sampled (Fig. 1). The populations studied were growing mostly in open habitats under dry or semi-dry conditions. Of the 106 populations studied 37% grew on roadsides, 27% were settled on grasslands, 16% on alvars,

13% on fallow fields, and 7% were growing on wastelands.

To describe how hybridization has influenced the morphology of the plants in the studied populations, two diagnostic morphological characteristics were recorded: flower colour and pod shape. As noticed earlier, these characters are the most informative for detecting hybrids in the *M. sativa-falcata* complex (Small & Brookes 1984). Flower colour and pod shape are genetically-determined characters (Barnes & Hanson 1967). The purple flower colour pigments of *sativa* are determined by one gene with tetrasomic inheritance. The yellow flower colour of tetraploid *falcata* is caused by at least two genes with accumulative effects (Barnes 1966). Yellow flower colour, sickle-shaped pods, straight pods and corkscrew-twisted pods were assigned to *falcata* and variegated flowers (pale-yellow, greenish-yellow, mixed purple-yellow and brownish) and coiled pods were attributed to plants of hybrid origin.

To estimate how habitat type and changes in the land use may have favoured the distribution of hybrid plants, in each population the mean coverage percentage of *Medicago* plants was estimated separately for the yellow-flowered sickle medic and for plants with variegated flowers. The changes in the land use, caused by human activity, were recorded based on aerial photographs from the 1950s, which were com-

pared with the current situation as described for 67 populations during our fieldwork.

Data analyses

The data were analysed with the statistical software STATISTICA (StatSoft Inc.). One-way ANOVA followed by Tukey's post hoc test was used to test whether the differences between the population characteristics (habitat type and land-use changes in the studied populations) and percentage of hybrid plants in populations are significant. The relationship between flower colour and pod shape was tested using the χ^2 -test.

Landscape analysis was applied to estimate changes in habitat types. Land use changes in the studied populations were described by interpretation of aerial photos (large-scale orthophoto maps 1:10 000) from 1951–1957 using the mapping package MapInfo Professional (MapInfo Corporation 2004).

Results

Among the 106 populations studied only 15 were pure *falcata* populations without *varia* or *sativa* plants (Fig. 1). In the remaining 91 populations, 1%–90% of the plants had variegated flowers, indicating widespread but highly variable introgression. Pure populations occurred on alvars (six), on dry grasslands (four), along roadsides (three), on a fallow field (one) and one inhabited an old sandpit.

In the studied populations, the flower colour and the pod shape varied on a large scale, and the flower colour varied independently of the pod shape. According to the χ^2 -test, yellow-flowered *falcata* plants never had coiled pods. However, plants with variegated or pale yellow flowers may have pods characteristic of *falcata*, or coiled pods ($\chi^2_1 = 9.1$, $p = 0.002$). Some plants had intermediate morphological characters as compared with those of their parental taxa. Those plants had greenish-yellow flowers and pod shapes characteristic of *falcata* or *varia*. The greenish-yellow flower colour indicates first-generation hybrids between *falcata* and *varia* (Small & Brookes 1984). Comparison

of hybrids with greenish-yellow flowers and yellow-flowered *falcata* revealed that plants with yellow flowers had never coiled pods, whereas hybrids had coiled pods or pods characteristic of *falcata* equally frequently ($\chi^2_1 = 9.3$, $p = 0.002$). Comparison of hybrids with greenish-yellow flowers with hybrids that had variegated flowers revealed that flower colour and pod shape were not associated. Hybrid plants with variegated or greenish-yellow flowers had coiled pods or pods characteristic of *falcata* equally frequently ($\chi^2_1 = 0.68$, $p = 0.4$). Occurrence of hybrid plants was not dependent on the habitat type ($F_{4,101} = 0.8$, $p = 0.53$). However, on roadsides and wastelands the percentage of hybrid plants in a population was on the average 20–25, which is higher than that in the other habitat types.

Land-use changes had no influence on the abundance of hybrid plants ($F_{1,65} = 1.98$, $p = 0.16$). In populations where land use has changed, the average percentage of hybrid plants was higher, but that difference was not statistically significant. In populations where land use has remained the same, the average percentage of hybrid plants was 14%; in populations with changed land use it was on average 22%. Land use changes took place in 64% of the studied populations. According to the maps from 1951–1957, the investigated populations inhabited the following habitats: pastures, grasslands, forest, fields, shrubs, and fallow fields. The observations made during the fieldwork showed that only 9% of these pastures are nowadays still used for grazing, 38% of the former pastures are now grasslands which are still mowed, and 48% are abandoned. Of the former pastures 5% are wastelands (Fig. 2). Two former grasslands are used nowadays as grasslands. One former grassland and one former field are now abandoned sandpits. Of the former fields, 57% are nowadays fallow fields and 28% are managed grasslands. Wastelands, overgrown fields and unmanaged grasslands account for 5% each (Fig. 2). The area of two populations was in the years 1951–1957 overgrown with shrubs. Today one of these populations is an abandoned grassland and the other is a wasteland. One former fallow field is used today as a grassland. In 36% of the populations, land use is the same as it was 60 years ago. They are mostly roadsides (84%), grasslands (8%) and pastures (8%).

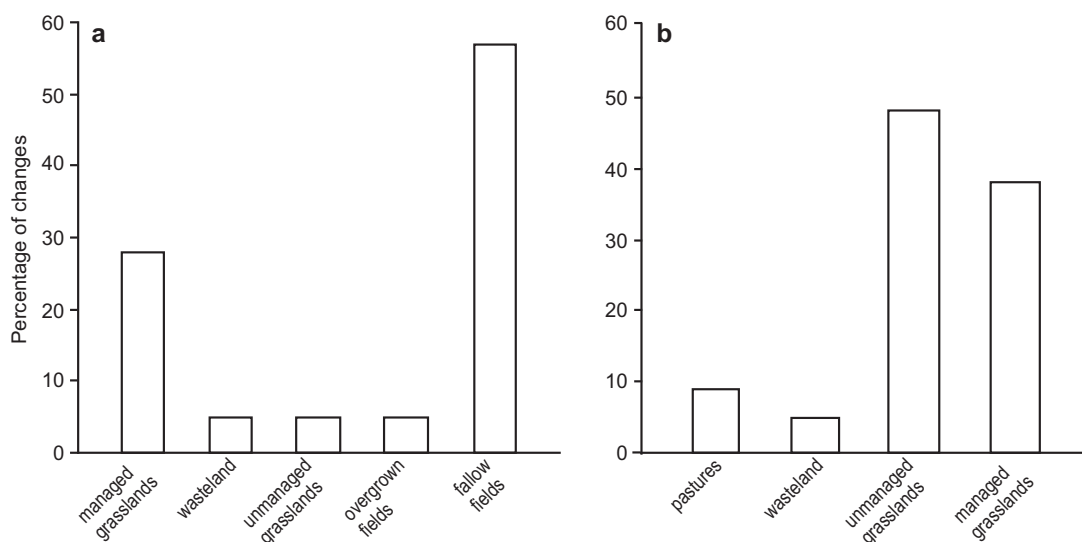


Fig. 2. Land-use changes in (a) fields and (b) pastures during the past 60 years.

Discussion

Variation in diagnostic morphological traits

Hybrid plants in the *M. sativa-falcata* complex have traditionally been identified on the basis of flower colour and pod shape (Small & Brookes 1984, Jenczewski *et al.* 1998). We also used flower colour and fruit characteristics to identify hybrid plants. To distinguish hybrid plants, one should examine both flower colour and pod shape, because of their independent variation among individuals. A confusing and limiting circumstance in the interpretation of the data obtained was that hybrid plants are sometimes hard to distinguish on the basis of morphological characters. Moreover, two Estonian *varia* cultivars bred for pastures have pale-yellow flowers and their pods may vary from sickle-shaped to spirally-coiled (Bender 2006). In addition, the pod characteristics of *falcata* are variable: pods can be occasionally twisted like a corkscrew (Lesins & Lesins 1979, Quiros & Bauchan 1988). In Estonia, owing to morphological similarities between natural *falcata* and pale yellow-flowered *varia*, the latter has sometimes been misidentified as *falcata*. According to the latest distribution map (Kukk & Kull 2005), the distribution area of *falcata* has expanded to south-

ern and southeastern Estonia, but these more southeastern localities are evidently inhabited by yellow-flowered *varia*.

Earlier studies have indicated a continuum of variation among *falcata*, *sativa* and *varia*, but intermediacy between *falcata* and *varia* is smaller. More complete intergradation occurs between *sativa* and *falcata* (Small & Brookes 1984, Quiros & Bauchan 1988). We also found that flower colour and pod shape varied in the observed populations on a large scale, however, there was no strong association between them. We found that yellow-flowered *falcata* plants never had highly-coiled pods but they were quite often corkscrew-twisted. Plants with variegated flowers may have pods characteristic of *falcata*, or even coiled pods characteristic of *sativa*. It has been reported (Small & Brookes 1984) that stabilized recombinant populations with odd character associations, for example, yellow-flowered plants with coiled fruits are frequent in some parts of the world. Obviously, the Estonian plants with pale yellow flowers and coiled pods belong to *varia* and plants with variegated flowers are hybrids with introgressed traits of *falcata*.

Yet no specific molecular markers were found to distinguish between cultivated and wild pools, but the populations of wild and cultivated plants were genetically distinguished by allele frequencies (Jenczewski 1998, Jenczewski *et al.* 1999a,

1999b, Muller *et al.* 2001, Greene *et al.* 2008). On the other hand, diploid forms of ssp. *falcata* and ssp. *sativa* were well distinguishable using SSR markers (Şakiroğlu *et al.* 2010) and cpDNA sequences (Havananda *et al.* 2010). Because of the large overlapping of morphological characteristics and molecular data in the *M. sativa-falcata* complex, both selected molecular markers and morphological characters are needed to study introgressive hybridization in more detail.

Distribution of hybrids in populations depending on the habitat type and changes in land use

Human activities often give rise to ruderal land which can easily be colonized by numerous taxa, mostly species with a high colonization ability. Habitat disturbance creates suitable habitats for hybrid taxa and increases opportunities for spontaneous hybridization. In disturbed habitats, formerly separated taxa may come into contact as human activities have destroyed ecological and spatial barriers (De Wet & Harlan 1975, Levin *et al.* 1996). It is also known that hybrid taxa are most successful in disturbed habitats but usually not in habitats preferred by their parental taxa (De Wet & Harlan 1975).

In breeding programs, *falcata* has been intensively used for improving winter hardiness and grazing tolerance of *varia* cultivars. In Estonia, two grazing-tolerant yellow-flowered *varia* cultivars have been bred. As a result, differences in the requirements for habitat and environmental conditions between *falcata* and *varia* have diminished, and domesticated plants of *varia* are able to survive and spread under natural conditions. This is well evident in Canada where roadside feral populations of escaped cultivars with high parentage from *falcata* are very viable (Bagavathiannan & Van Acker 2009, Bagavathiannan *et al.* 2010). These populations seem to be under natural selection for winter survival and clonal growth. It was assumed that these traits of *falcata* may support persistence of roadside hybrid populations (Bagavathiannan & Van Acker 2009, Bagavathiannan *et al.* 2010). Even more first-generation hybrids between *sativa* and *falcata* show the effects of heterosis i.e., vigor-

ous growth, larger plants and a more erect habit (Riday & Brummer 2004). In Estonia, the traits of *falcata* may support the establishment of hybrid plants in natural populations on alvars and dry grasslands in addition to roadside habitats, as only six populations growing on alvars appeared to be pure.

Besides alvars and dry grasslands, three pure *falcata* populations were growing at roadsides, one pure population occurred on a fallow field and one pure population in an old sandpit. Presumably, these populations were recently established by the initial seedling recruitment from *falcata* seeds. It may be expected that in the course of time, hybrid plants will appear also in these populations from the nearby cultivated fields or as a consequence of human activity (e.g. road-construction works). It has been shown that gene flow by pollen from cultivated *sativa* and *varia* fields may cover long distances (St. Amand *et al.* 2000). Furthermore, also gene flow via seeds can be essential and may increase gene exchange between natural and cultivated populations (Muller *et al.* 2001). We suppose that there may be three main reasons why hybrid plants are missing from some populations: (1) unsuitable environments for dispersal of hybrid plants, (2) lack of cultivated fields nearby natural populations of *falcata*, (3) populations in disturbed habitats that have been established by *falcata* seeds are now densely occupied by plants of *falcata* and other competitive species. As the results of this study show that habitat type had no influence on frequency of hybrid plants, the impact of other factors, e.g. those mentioned above, which do not support spreading of hybrid plants, may play an important role.

Prevalent processes in the Estonian agricultural landscapes are forestation, abandonment and overgrowing of grasslands with shrubs and trees, and expansion of fallow fields in formerly arable land (Kana *et al.* 2008). This is in accordance with our observations which also confirm that former grasslands, pastures and arable fields are often abandoned and overgrown by shrubs. In these unmanaged areas the dispersal of competitive and large-sized invasive species, for example *Galega orientalis* and *Lupinus polyphyllus*, is promoted (Eek & Kukk 2008).

In many cases, it is not known if *falcata* and *varia* are growing on pastures and grasslands nat-

urally or have been sown there during the period of intensified agricultural practices since 1950 until 1990. At many sites, plants sown in that period have persisted until now (Bender 2006). One can claim that cultivation of grasslands and pastures has historically contributed to better distribution of hybrid plants, making co-occurrence of the two taxa possible. Also abandonment of sandpits and former arable fields has enabled both *falcata* and *varia* to colonize new open areas. However, we found no significant difference in the occurrence of hybrid plants at sites with changed land-use as compared with sites where the land use had remained the same, although escaped cultivars obviously have more favourable conditions for spreading at roadsides. It is evident that *varia* has adopted traits of *falcata* which support persistence of hybrid plants in man-made as well as in natural habitats, largely owing to the abandonment of former agricultural areas.

Conclusions

In summary, the present study on hybridization in the *Medicago sativa-falcata* complex showed that there have remained only a few pure *falcata* populations in Estonia; out of the 106 populations studied by us only 15 were pure *falcata* populations, while the remaining 91 populations contained hybrid plants. These results indicate ongoing and variable hybridization in natural populations of *falcata*. The still preserved pure populations are of high conservation value because introgression from cultivated alfalfa is reported throughout Europe (e.g. Jenczewski *et al.* 1999a, Rufener Al Mazyad & Ammann 1999, Bleeker *et al.* 2007).

The occurrence of hybrid plants in the populations does not depend significantly on the habitat type and is not strongly influenced by changes in land use. However, it can be argued that the most favourable habitats for the growth and dispersal of hybrid lucerne plants are disturbed man-made habitats, mostly roadsides, but also wastelands and fallow or abandoned fields. Pure populations of *falcata* contain only individuals with yellow flowers and sickle-shaped pods, while hybrid plants in mixed populations have individuals with a pod shape and flower colour char-

acteristic of *falcata*, *sativa* or *varia*. The habitat preferences and morphological characteristics of *varia* and *falcata* have become more diverse in hybrid plants through introgressive hybridization in mixed populations, favouring their invasion to disturbed and natural habitats. In addition, pure *falcata* populations on calcareous grasslands and alvars of northern and western Estonia, growing on the northern margin of the distribution range, should deserve special attention as a valuable genetic resource adapted to cold winters and frequent summer droughts, and because they can spread vegetatively. *Ex situ* conservation of pure *falcata* gene pool is carried out in Estonia by the Jõgeva Plant Breeding. In 2002–2009, expeditions were carried out in north and west Estonia to collect seeds from natural populations of *falcata* and other legumes and grasses. Our previous study on the genetic diversity in small and fragmented pure populations of the sickle medic in Estonia showed that it still has remained high and deserves protection (Kaljund & Jaaska 2010). Therefore, *in situ* conservation measures of these populations should be considered, such as a ban of cultivation of *sativa* and *varia* in the radius of possible gene flow distance, and preserving remained populations from destruction through human activities. But even then, the populations are not fully protected against gene flow from cultivated crops due to pollinator-mediated gene flow or occasional long-distance seed dispersal of cultivars. Thus, the conservation measures of *falcata* in Estonia are still moderate and in the future *falcata* deserves more attention as a valuable genetic resource.

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