

# Hybridization rates between lettuce (*Lactuca sativa*) and its wild relative (*L. serriola*) under field conditions

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**Hybridization and introgression between crops and wild relatives may have important evolutionary and ecological consequences such as gene swamping or increased invasiveness. In the present study, we investigated hybridization under field conditions between crop lettuce (*Lactuca sativa*) and its wild relative prickly lettuce (*L. serriola*), two cross-compatible, predominantly autogamous and insect pollinated species. In 2003 and 2004, we estimated the rates of hybridization between *L. sativa* and *L. serriola* in close-to-reality field experiments carried out in two locations of Northern Switzerland. Seeds set by the experimental wild plants were collected and sown (44 352 in 2003 and 252 345 in 2004). Progeny was screened morphologically for detecting natural hybrids. Prior to the experiment, specific RAPD markers were used to confirm that morphological characters were reliable for hybrid identification. Hybridization occurred up to the maximal distance tested (40 m), and hybridization rates varied between 0 to 26%, decreasing with distance. More than 80% of the wild plants produced at least one hybrid (incidence of hybridization, IH) at 0 m and 1 m. It equaled 4 to 5% at 40 m. In sympatric crop-wild populations, cross-pollination between cultivated lettuce and its wild relative has to be seen as the rule rather than the exception for short distances.**

**Keywords:** autogamy / hybridization / introgression / *Lactuca sativa* / *Lactuca serriola* / pollen flow / RAPD

## INTRODUCTION

In the last decade, the extensive cultivation and commercialization of genetically engineered (GE) crops has exacerbated the arguments about crop-to-wild gene flow and its subsequent environmental effects. A major concern is the potential changes in the ecology of crop-related wild species, due to the introgression of transgenes into wild populations living in agricultural areas (Ellstrand, 2003; Wolfenbarger and Phifer, 2000). The introgression in a wild relative of a herbicide resistance gene could lead to increased invasiveness and/or weediness of introgressants (Snow and Palma, 1997), with a potentially negative impact for the cropping system (Klinger et al., 1991). Risks of gene swamping or pollution of natural gene pools, leading to the extinction of wild taxa have also been stressed (Ellstrand, 2003). These risks are thought to be higher if the wild plants show already weedy tendencies (Snow and Palma, 1997). In this context, *Lactuca serriola* is already described as an invasive weed in Canada and in Argentina, where it occurs in a variety of crops where no-till or conservation tillage systems are used (Weaver and Downs, 2003), and in many European countries, where it occupies ruderal places (Lebeda et al., 2004). Interestingly enough, herbicide-resistant weedy populations

of prickly lettuce have already been found in the United States and in Australia (International Survey of Herbicide Resistant Weeds, <http://www.weedscience.org/in.asp>).

The likelihood of (trans)gene escape increases when the flowering periods of a sexually reproducing crop and of its wild relatives overlap, and when no pre- or post-zygotic barriers to hybridization exist. Both conditions are met for *L. sativa* and *L. serriola*, since the two species are closely related, and are even often considered as conspecific (De Vries, 1990; Lindqvist, 1960a; Whitaker, 1939). Crop lettuce differs from prickly lettuce only for characters connected to domestication, like rapid growth, short life-cycle, absence of dormancy, and generally absence of prickles on the leaves and on the stem (Frietema de Vries et al., 1994). Both species are diploid, and their chromosome length and genetic background are equivalent (Koopman et al., 2001; Lindqvist, 1960a). Self-fertility prevails in *L. serriola* (Mejias, 1994) and is commonly regarded as being complete in *L. sativa* (Jones, 1927).

However, while the potential weedy character of prickly lettuce (Weaver et al., 2003), and its ability to hybridize with crop lettuce (Frietema de Vries et al., 1994) have been demonstrated, no data exist on the likelihood of such events. Intuitively, the chances for hybridization are low for such predominantly autogamous species.

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However, hybridization and introgression with locally adapted lettuce varieties could be one of the possible explanations of the rapid expansion of prickly lettuce towards northern Europe in the last century (Frietema de Vries, 1992). Moreover, recent studies have shown that low outcrossing rates do not prevent cultivated genes from moving in sympatric wild populations. These studies include both, autogamous and/or predominantly insect-pollinated species like soybean, bean or cotton (Ibarra Perez et al., 1997; Nakamaya and Yamaguchi, 2002; Van Deynze et al., 2005), as well as wind-pollinated autogamous species as rice or foxtail millet (Chen et al., 2004; Song et al., 2003; Wang et al., 1997; Zhang et al., 2003).

Plant breeders have long been concerned by crop-to-crop and wild-to-crop hybridizations. In particular, studies on seed purity produced considerable data on hybridization rates, pollen dispersal, and on isolation distances required to minimize it (Bateman, 1947). Durst (1930) found that cross-fertilization in *L. sativa* is a rare event. Thompson et al. (1958) observed about 1% cross-pollination between different lettuce varieties grown in adjacent rows (spaced of 1.2 m) and an average amount of cross-pollination of 2.9% for one variety surrounded by plants of another one. Watts (1958) found that *L. sativa* plants flowering late in the season exhibited a significant increase in the percentage of cross-pollination, and explained this observation by a seasonal increase in the hover-fly population. Pollinators contribute substantially to the effective transfer of pollen to the stigma, within, and between capitula (Watts, 1958). Moreover, they have an influence on seed set (Mejias, 1994). Not surprisingly, distance and pollinator availability have been found to be the most relevant factors for pollen migration between different *L. sativa* varieties (Goubara and Takasaki, 2003; 2004; Thompson et al., 1958). While wild  $\times$  crop hybridization cannot be inferred directly from the results presented above, we can assume that the main factors influencing the pollination of prickly lettuce by the crop are the same, since the species are very closely related. F1 hybrids are easily produced and completely fertile, irrespective of the species acting as the female parent (De Vries, 1990; Lindqvist, 1960a). Yet, in order to assess the risk of gene transfer from GE or conventional crop to wild relative, quantifying potential hybridization is a key issue.

Therefore, our study aims (1) to estimate the rate of hybridization and the incidence of hybridization between *L. sativa* and *L. serriola* in field conditions; (2) to evaluate the frequency of wild individuals setting at least one hybrid seed (incidence of hybridization); and (3) to evaluate the influence of distance on these parameters. Relevance of our results for the cultivation of GE lettuce and for lettuce breeding is highlighted.

## RESULTS

### Hybridization rate (HR)

In 2003, the highest individual hybridization rate (HR) was observed at 1 m (26%) and 0 m (16%) in Bevaix, and at 1 m in Séprais (12% and 14%). As expected, the mean HR also reached high levels: 7.4% at 1 m, or 3% at 5 m. In 2004, the HR values were lower than those obtained in 2003. The HR decreased exponentially as a function of the logarithm of the distance (Fig. 1). Individual HR varied considerably between plants, ranging from 0 to 12% at 0 m, from 0 to 3.15% at 1 m, from 0 to 0.56% at 5 m, from 0 to 0.29% at 15 m, from 0 to 1.33% at 25 m and from 0 to 0.11% at 40 m. Overall, the mean HR varied between 1.9% and 2.75% within the cultivated plot, and between 0.69 to 0.74% at 1 m. It was lower than 0.15% at longer distances. Finally, mean HR was greater than zero at all distances and in both field sites (Tab. 1).

### Incidence of hybridization (IH)

In 2004, the IH decreased as a function of the distance (Fig. 2), but not as drastically as the HR. In 2004, most of the wild plants produced at least one hybrid at 0 m in trials 1 and 2 (87% and 94% respectively) and at 1 m (82% and 97%) (Tab. 2). At 5 m, the IH was 32% in trial 1 and 62% in trial 2, while it was 5 and 21% respectively at 15 m. Surprisingly, the IH was higher at 25 m than at 15 m (except for trial 2 South). Finally, 4 and 5% of the mother plants produced hybrids at 40 m.

### Statistical results

For trial 1, the IH was globally significantly higher in 2003 than in 2004 ( $P < 0.05$ ), while for trial 2, the HR was significantly higher in 2003 than in 2004 ( $P < 0.05$ ). However, because of the lack of data, no within- or between-sites comparisons could be done for the 2003 experiment.

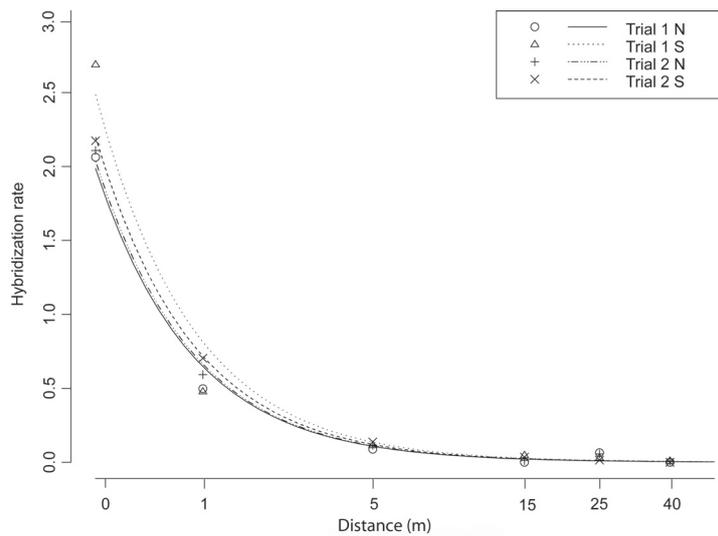
In 2004, the HR did not differ significantly between field sites, but a significant difference was observed between North vs. South lines in field trial 1 (Bevaix,  $P < 0.0019$ ). On the contrary, the number of plants possessing at least one hybrid in their progeny (IH) was significantly higher in trial 1 than in trial 2 ( $P < 0.0083$ ), while no differences due to the orientation were observed within field sites (Tab. II).

Finally, no effect of the prickly lettuce population was observed, neither on HR, nor on IH ( $P > 0.05$ , results not shown).

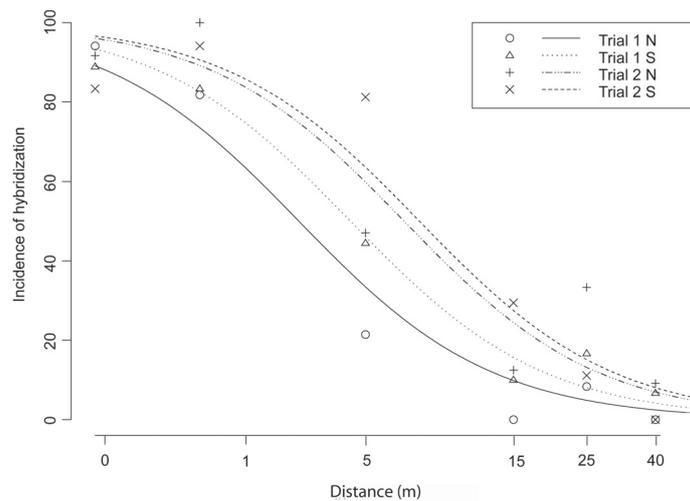
## Hybridization between cultivated lettuce and its wild relative

**Table 1.** Survival, mean hybridization rates (MHR, in %) and incidence of hybridization (IH, in %) of the experimental wild plants, in the two field sites and orientations (North-South), as a function of the distance from the crop pollen source (data of the 2004 experiment). D: distance from pollen source (m); S: number of surviving plants; MHR: average hybridization rate (%); SD: standard deviation.

Field site (orientation)	D	S	Grown progeny (N ind.)	Detected hybrids	MHR	Individual hybridization rate range	SD	IH
Trial 2 North (2004)	0	12	9300	196	2.75	(0–9.83)	3.1	92
	1	18	16 875	100	0.69	(0.1–3.11)	0.69	100
	5	16	16 425	19	0.12	(0–0.56)	0.17	44
	15	16	16 800	2	0.01	(0–0.1)	0.03	13
	25	12	12 450	7	0.06	(0–0.44)	0.13	33
	40	12	11 700	1	0.009	(0–0.1)	0.029	8
Trial 2 South (2004)	0	12	10 395	226	1.9	(0–5.11)	1.6	75
	1	17	15 487	109	0.72	(0–3)	0.68	94
	5	16	14 587	20	0.14	(0–0.36)	0.11	81
	15	17	17 250	6	0.03	(0–0.19)	0.056	29
	25	18	13 155	2	0.02	(0–0.25)	0.06	11
	40	18	5820	0	0	0	0	
Trial 2 overall (%) (2004)	0	67			2.33			87
	1	97			0.71		97	
	5	89			0.13			62
	15	92			0.02			21
	25	83			0.04			22
	40	83			0.0045			4
Overall survival		85						
Trial 1 North (2004)	0	16	14 400	297	2.21	(0.17–12.27)	2.82	100
	1	11	7237	36	0.7	(0–3.15)	0.91	82
	5	14	6750	6	0.04	(0–0.22)	0.09	21
	15	1	375	0	0	0	0	
	25	12	3075	2	0.11	(0–1.33)	0.39	8
	40	12	5550	0	0	0	0	
Trial 1 South (2004)	0	18	13 350	359	2.56	(0–8.67)	2.72	89
	1	12	8775	42	0.74	(0–2.67)	0.93	83
	5	9	6075	7	0.11	(0–0.38)	0.15	44
	15	10	6225	3	0.03	(0–0.29)	0.09	10
	25	12	9563	3	0.03	(0–0.27)	0.08	16
	40	15	10 725	1	0.007	(0–0.11)	0.03	6
Trial 1 overall (%) (2004)	0	94			2.39			94
	1	64			0.72			82
	5	64			0.08			32
	15	31			0			5
	25	67			0.07			12
	40	75			0.0035			3
Overall survival		6						



**Figure 1.** Mean hybridization rates (HR) (%), represented as a function of the distance. The HR is the percent hybrid seeds.



**Figure 2.** Incidence of hybridization (IH) (%), represented as a function of the distance. The IH is the percent plants bearing at least one hybrid seed.

### Pollinators

A total of 12 insect species were captured on *Lactuca* flowers (Tab. 3). All of them were generalist pollinators and belonged to Hymenoptera or Diptera. Temporary foraging as well as continual pollen gathering behaviors were observed. Generally speaking, insects tended to move from one capitula to another on a single plant, before moving to a different plant. In trial 2, where more flowering plants were present, more pollinators were observed, but in both trials, the highest density of pollinators was found in the crop plot.

### DISCUSSION

Crop and prickly lettuce can hybridize at a higher than expected rate, considering that the species possess mechanisms favoring self-pollination. Moreover, hybridization can reach substantial levels, even at a relatively long distance. The importance of crop-wild lettuce hybridization has consequences for the environmental risk assessment of the cultivation of GE crops. Hence, the results and the experimental design need to be discussed and compared, relative to the context of current cultivation of crop lettuce.

**Table 2.** Analysis of maximum likelihood estimates.

Parameter	DF	Estimate error	Standard Chi-Square	Wald	Pr > Chi-Square
<b>Hybridization rate</b>					
Intercept	1	-3.8352	0.0432	7868	< 0.0001
Site	1	0.0511	0.0545	0.8820	0.35
logdist	1	-1.6350	0.0530	952.1	< 0.0001
Bevaix					
North/South	1	0.1145	0.0369	9.612	0.0019
Seprais					
North/South	1	0.0394	0.0385	1.047	0.31
<b>Incidence of hybridization</b>					
Intercept	1	3.2691	0.3976	67.61	< 0.0001
Site	1	-0.8994	0.3409	6.963	0.0083
logdist	1	-1.5580	0.1531	103.6	< 0.0001
Bevaix					
North/South	1	0.2662	0.2539	1.100	0.29
Seprais					
North/South	1	0.0792	0.2057	0.1483	0.70

**Table 3.** *Lactuca* pollinator.

Order	Family	Genus	Species
Hymenoptera	Apidae	<i>Apis</i>	<i>mellifera</i>
Hymenoptera	Apidae	<i>Bombus</i>	<i>lapidarius</i>
Hymenoptera	Andrenidae	<i>Andrena</i>	sp.
Hymenoptera	Halictidae	<i>Halictus</i>	sp.
Hymenoptera	Halictidae	<i>Lasioglossum</i>	sp.
Hymenoptera	Vespidae	<i>Vespula</i>	<i>vulgaris</i>
Hymenoptera	Sphecidae	<i>Ectemnius</i>	<i>continuus</i>
Diptera	Syrphidae	<i>Episyrphus</i>	<i>balteatus</i>
Diptera	Syrphidae	<i>Chrysotoxum</i>	<i>elegans</i>
Diptera	Syrphidae	<i>Sphaerophoria</i>	<i>scripta</i>
Diptera	Syrphidae	<i>Scaeva</i>	<i>pyrastris</i>
Diptera	Syrphidae	<i>Syrphus</i>	<i>vitripennis</i>

In most parts of Europe, crop fields and private vegetable gardens are often surrounded by ruderal habitats, where prickly lettuce commonly occurs at close distances from the crop. Moreover, both species have long blooming periods, which naturally overlap for many weeks. In 2005, 10% of the Swiss production of the two most cultivated cultivars (mainly head type) was not harvested, due to the low prices of lettuce on the market (information gathered at the Swiss central of gardening market, Bern).

In those cases, cultivated plants are left in the field, and can bolt and flower close to wild populations of *L. serriola*. This represents 140 ha in Switzerland that could potentially hybridize with the wild species. It seems realistic then to consider our experimental conditions as intermediate between that of few cultivated lettuce bolting in private gardens and that of large-scale lettuce cultivation, where some plants are not harvested and left in the field. Therefore, suitable conditions for hybridization in agro-ecosystems seem to be frequently met in central and southern European countries.

### Natural hybridization

Significant levels of hybridization between *L. sativa* and *L. serriola* were found. It clearly indicates crop-to-wild hybridization in the lettuce complex despite mechanisms ensuring a high degree of self-fertilization (Mejias, 1994). The mean HR values were comparable to out-crossing frequencies of lettuce varieties observed by Thompson et al. (1958) and Watts (1958). HR varied greatly between plants; however no evidence of a population effect could be detected. More likely, between-plant differences in HR depend on environmental factors such as climatic condition and pollinator abundance or on genetic traits influenced by environmental conditions like the induction of flowering (Prince et al., 1978).

Not surprisingly, the mean HR per row decreased with the distance from the crop plot. Higher mean HRs were detected within the crop plot (0 m distance) or at short distances. Individual HR followed the same trend, with values lower than 0.6% at distances farther than 5 m, but hybridization was still detected at 40 m. These results are not surprising, since the crop pollen source was the most attractive sector of the field trials for the pollinators, because of the high density of flowers.

Results of both field locations were consistent; no influence of the location was found on the HR values, which could indicate that hybridization between the two species is possible when the two species occur in sympatry. The observation of generalist insect species all over the Swiss Plateau and Jura mountains foraging on the crop and its wild relative, supports this view.

Most of the plants that possessed extremely high HR values in 2003 and 2004 suffered from drought or from competition. They presented a scrawny phenotype, produced few flowers and consequently few seeds. These plants possibly also produced a lower amount of pollen. Environmental stresses can influence pollen performance (Snow and Spira, 1996), and temperature significantly influences positively *in vivo* pollen germination in cultivated lettuce (Eenink, 1983). Therefore, the lower amount of pollen produced by some wild plants, combined with the availability of crop pollen, could have facilitated the formation of hybrids. However, this latter point must be assessed.

### Incidence of hybridization

Our results indicated that the IH decreased with distance, but less drastically than observed for the HR values. The IH values were significantly higher in trial 2, compared to trial 1; while the HR values were similar. The HR is probably influenced by the amount of crop pollen carried by insects, while the IH is more influenced by their abundance. Our observations on the abundance of pollinators and their activity are purely qualitative and addressed only trial 2. However, it is likely that the higher floristic diversity of this latter location, and consequently the higher number of flowers surrounding the experimental plants, were more attractive for generalist pollinators, and could explain the differences observed between sites for IH.

For both field trials, the IH ranged between 80 and 100% for plants growing at distance up to 1 m. These results are much higher than those found in the self-fertilizing and insect pollinated crop-wild soybean complex (Nakamaya et al., 2002). Interestingly, our results are only slightly lower than those found in the outcrossing and insect pollinated crop-weed radish complex (Klinger

et al., 1992), and higher than those found between mixed-mating and insect-pollinated tobacco cultivars (Paul et al., 1995). At farther distances, the IH varied greatly, but frequencies as high as 81% at 5 m, 29% at 15 m or 33% at 25 m were found. These high values indicate that a single wild plant growing in proximity of blooming cultivated lettuce has high chances to produce hybrids.

Yet, our empirical data are clearly related to specific experimental conditions, like environmental year effect and experimental design. The fact that the recipient plants were placed in rows produced a shielding effect that probably underestimates the HR at far distances. However, we think that these data are relevant for Europe. First, the general picture arising from the results of the two sites and of the two years are congruent. Second, the pollinators captured are generalist European species, and their abundance was influenced experimentally neither by growing attractive flowers, nor by placing beehives. Third, the distances tested in our experiment are the most frequently observed in central European agroecosystems, where prickly lettuce grows along roads and railways close to or at the edge of cultivated fields.

### Importance of pollinators for gene flow in lettuce

The importance of pollinators for the effective pollination of *Lactuca* flowers has already been demonstrated (Goubara et al., 2003; 2004). Jones (1927), showed that bagged flowers of *L. sativa* had significantly less or no pollen grains on their inner stigmatic surfaces, compared to open-pollinated flowers. He concluded that cross-pollination between plants may be much more frequent than was formerly supposed. Mejiias (1994) showed that freely pollinated flower heads always had a higher seed set than flower heads bagged before anthesis. Since pollinators ensure both self- and cross-pollinations, especially if crop and wild plants are visited alternately, their pollen gathering behavior can largely affect hybridization in lettuce.

Although this aspect could not be tested in our experiment, the distance to which pollen can be transferred between plants, and thus HR and IH, seems to be highly dependent on the factors that can affect pollinator guilds and their foraging strategies. These factors include the number of flowers produced per plant, the seasonal or annual changes in meteorological conditions, the spatial patterns of distribution of the wild population, and their position relative to the crop pollen source (Schmitt, 1983; Van Deynze et al., 2005). Insect visitation rates and therefore possible hybridization rates might depend on the amount of flowers available from *Lactuca*, but surely also on the availability of other plant species, since all observed visitors were generalists. The exceptionally high individual HR, as well as the higher HR in trial 2 and the

higher IH in trial 1 observed in 2003, might be due to the absence of other food sources for pollinators. It can also be explained by an increased activity of the pollinators, due to particularly warm and dry conditions observed in 2003.

### Hybrid characteristics and relevance for GE lettuce cultivation

We clearly showed that the two species can hybridize easily, which represents the first step toward the understanding of the mechanisms of gene flow. In addition, even if no transgenic variety has been commercialized yet, *L. sativa* has already been engineered for many traits such as herbicide resistance (McCabe et al., 1999; Nagata et al., 2000), pathogen resistance (Okubara et al., 1997) and other agronomic traits (Curtis et al., 1999; Goto et al., 2000; Pileggi et al., 2001). Although we did not investigate crop-wild lettuce hybridization using transgenic varieties, our results have clear implications for the risks assessment of their cultivation.

While F1 hybrids can be easily distinguished morphologically from their wild parents at the rosette stage, differences at the adult stage are less evident. Natural hybrids and introgressants could then be more frequent than previously thought, based on their morphology only. Indeed, only a few old reports exist on the hybridization between *L. serriola* and *L. sativa* (McCollum (1953) in Lindqvist, 1960c; Ownbey and Andersson, 1949; Pammel, 1918). However, hybridization is an ongoing process, and natural hybrids between these two species can be found (personal observation, data not shown). Even rare hybridization events could have important consequences on the introgression of conventional and/or GE traits. One plant of *L. serriola* can easily produce from 10 000 to 100 000 seeds (personal observation). Consequently, a hybridization rate of 0.1%, as observed for one plant located at 40 m from the crop, corresponds to 10 to 100 viable hybrid seeds produced. The F1 hybrids could combine the adaptations to wild habitats of the wild parent and those to the agro-ecosystems of the cultivated parent and be positively selected (Ladizinsky, 1984). The hybrids perform well in ruderal habitats and show heterosis in size and seed-set. Moreover, second-generation hybrids are as fit as the wild plants (Hooftman et al., 2005; 2007; Lindqvist, 1960a) which leads to successful introgression even in absence of continued hybridization. This situation relates to that of the maize-teosinte complex, where the pollen of the hybrid is as fit as that of the parents, and can easily pollinate the wild parent (Guadagnuolo et al., 2006).

Since the hybrid involucre bracts reflex at maturity and seeds scatter similarly to *L. serriola*, seed dispersal is not expected to differ from that of the wild parent.

Seed dispersal by human activities, which is mainly by vehicles, should not differ either (Lebeda et al., 2001, see also Snow et al., 1997). Finally, in case of commercialization of transgenic lettuce varieties, the containment of transgenes will be very difficult if not impossible, unless measures are taken to prevent bolting.

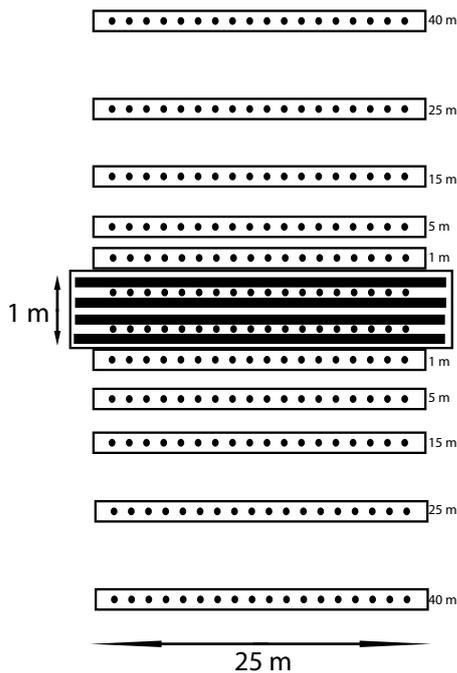
## MATERIALS AND METHODS

### Field trials

Our hybridization experiments were performed in field conditions using the same experimental design in 2003 and 2004, in two locations of northern Switzerland. Field trial 1 was located in Bevaix (canton Neuchâtel) (46° 56' N, 6° 49' E) and field trial 2 in Séprais (canton Jura) (47° 20' N, 7° 12' E). Both experimental plots were oriented North-South and possessed similar climatic conditions. They presented different topography and ecology. In trial 1, a steep slope separated the northern-upper part from the southern flat part; vegetation was typical grassland. Trial 2 was located on a homogenous slope in a typical meagre pasture. The floristic richness was greater in trial 2 than in trial 1 (data not shown).

### Experimental design

In the middle of each field trial, a 25 m × 1 m plot was used as crop pollen source and was planted with 400 *L. sativa* plants, distributed in four rows (Fig. 3). One-hundred individuals of the four different varieties were planted alternately. Spacing between plants was 25 cm. *L. serriola* individuals were planted in 12 parallel rows, 6 North and 6 South at respectively 0 m, 1 m, 5 m, 15 m, 25 m and 40 m of the pollen source. A two compass direction experiment was chosen, due to space limitation. Each row consisted of six plants of each of three populations at 1 m distance. Position of the populations along the row was random. The maximal distance of 40 m was chosen because (1) the aim of the study was to detect changes in the hybridization rates and incidence of hybridization at close distances from the crop; (2) the species were supposed to present a high degree of autogamy; therefore the hybridization rate at greater distances was expected to be too low for a reliable test; and (3) up to now, the only field trials carried out on *L. sativa* varieties tested the maximal distance of 1.2 m. The experimental setup corresponds to the situation most frequently met in agro-ecosystems, where prickly lettuce grows at the edge of cultivated fields or along railways or roads that run parallel to them.



**Figure 3.** Experimental design used in both field trials. (●) represent an individual of *L. serriola*; 18 wild plants from three different populations randomly distributed at 1 m intervals in a row. Twelve rows were planted, 6 North and 6 South (at 0 m, 1 m, 5 m, 15 m, 25 m, 40 m) of a central 1 m wide, 25 m long source of crop pollen. Thick black lines in the middle represent the 4 rows of *L. sativa* (100 plants per row).

### Plant material

We planted 400 *L. sativa* plants of four different conventional non-transgenic cultivars as local pollen source: Reine de mai (butterhead lettuce with green leaves), Laitue de Morges (romaine lettuce, with dark leaves), Laitue de St-Blaise (romaine lettuce, with green leaves), Feuille de chêne rouge (leaf lettuce with red leaves). Three different wild populations of *L. serriola* collected in southern Germany (LBM5: 9° 08' E, 47° 83' N; D20: 8° 61' E, 48° 18' N) and northern Switzerland (CH18: 8°76' E, 47° 50' N) were used as pollen sink. All the plants were sown and grown to a rosette stage in the Botanical Garden of Neuchâtel and then planted in the field.

In both trials, in 2003, *L. serriola* were planted in the field three weeks after *L. sativa* cultivars. In 2004, in order to optimize the overlapping of the flowering period, wild plants were planted three weeks sooner than the cultivated, except for those located at 0 m of trial 2, which were planted simultaneously with the crop.

Seeds of each individual of the wild species were collected separately every two days, except during

rainy days. Due to rather different climatic conditions, the harvesting period was the whole month of July in 2003, while it started in August in 2004.

### Plant survival, overlap in flowering time and seed collection

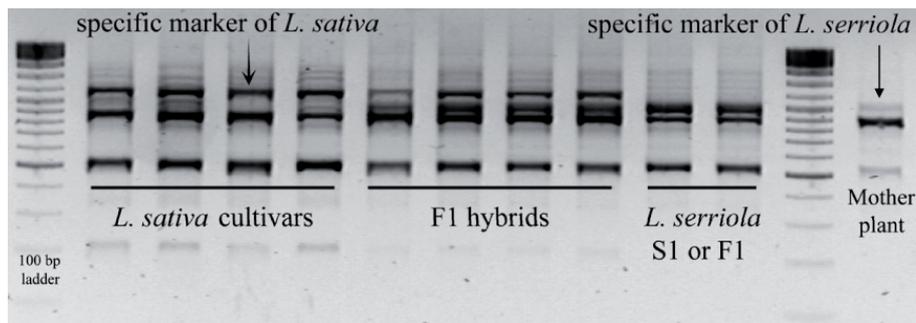
The average plant survival was much higher in 2004 than in 2003. In 2003, the summer was exceptionally warm and dry in Europe, including Switzerland. A very intensive watering of the plants could only be done in Bevaix, but despite this effort, only 37% of the plants reached maturity (80 plants). In Séprais, where the conditions were even more severe and watering was impossible, only 7% of the wild plants reached the adult stage (16 plants). Overall, flowering of the two species overlapped poorly.

In 2004, the climatic conditions were optimal for plant growth. However, *L. serriola* plants in trial 1 were slender, because of the competition by perennial grasses, although nearby surrounding grasses were cut in June. Moreover, the plants located 15 m north of the crop pollen source in the same field trial suffered from drought and almost all of them died. In total, 65% of the wild plants reached maturity in Bevaix (144 plants) and 85% in Séprais (184 plants). Flowering of crop and wild lettuce overlapped completely in both field trials.

### Data scoring

#### Morphological screening

At the rosette stage (4 to 5 leaves), *L. serriola* × *L. sativa* hybrids look like the paternal *L. sativa* variety and are easily recognizable. At the end of the rosette stage, they are more similar to *L. serriola*, with thicker leaves and a row of spines on the underside of the midrib of the leaves. When hybrid plants start to set seeds, it becomes very difficult to distinguish them from pure wild plants. Their leaves possess a row of spines on the midrib of the underside, and are oriented vertically; the stem becomes prickly. The inflorescence as well is *L. serriola*-like, and the involucre bracts reflex at maturity. Based on this evidence, we morphologically screened the progeny of the wild mother plants at the rosette stage. In 2003, only 96 plants survived in the two field trials, and all their seeds were sown. The total number of seeds sown for 2003 was 44 352, representing an average of 462 seeds/mother plant. In 2004, for the mother plants that set enough seeds, up to 1050 seeds were sown individually, which roughly represents the seed set of 70 capitula (15 florets/capitula), while all the seeds were sown for the plants that set less than 1050 seeds. The total number of seeds sown for that year was 252 345, representing an average of 769 seeds/mother plant (Tab. 1).



**Figure 4.** RAPD amplification with primer OPT-06 in the four *L. sativa* cultivars used in the experiment (pollen source), 4 F<sub>1</sub> hybrids, 2 S<sub>1</sub> or F<sub>1</sub> *L. serriola*, and 1 *L. serriola* mother plants (pollen sink). The amplification reveals the presence of specific markers of both, *L. sativa* and *L. serriola* in F<sub>1</sub> hybrids, while mother plants and S<sub>1</sub> *L. serriola* possess only the fragments specific to the wild species.

Individual hybridization rates (HR) were defined as the percentage of hybrids in the sown progeny of each mother plant, while mean HRs were defined as the average of individual HRs of all the plants in the same row. The incidence of hybridization (IH) was measured as the proportion of *L. serriola*

mother plants producing at least one hybrid. The standard deviation was calculated for each distance respective to the crop pollen source, in each field site and orientation (North and South).

#### Molecular confirmation of morphological screening

In order to confirm our hybrid morphological screening, we tested 23 RAPD primers to find specific cultivar markers absent in wild populations of *L. serriola*. Eleven primers, OPB-04, OPB-08, OPB-11, OPC-02, OPC-04, OPC-13, OPC-18, OPP-02, OPP-19, OPT-06 and OPT-07 (Operon technologies, CA-Alameda, USA) produced a total of 17 reliable markers present in the cultivars used and absent from the wild populations (Fig. 4), and were selected for further analysis. Total DNA was extracted from fresh young leaves using QIAGEN<sup>®</sup> DNeasy Plant Mini Kit. RAPDs amplifications were performed in 25  $\mu$ L final volume, with the following final concentrations: 1 X PCR buffer, 0.2 mM dNTP, 0.4  $\mu$ M primer, 0.75 U Taq polymerase (Qiagen AG, Basel) and 20–40 ng template DNA. Amplifications were carried out in a Biometra T3 thermocycler as follow: 4 min initial denaturation at 94 °C, then 37 cycles of 93 °C: 60 s; 41 °C: 60 s and 72 °C: 60 s. Final extension was 5 min at 72 °C. PCR products were mixed with 1/3 volume loading buffer and loaded onto 1.5% (w/v) agarose gels stained with ethidium bromide. Electrophoreses were carried out at 100 V.

Fifty plants scored morphologically as wild progenies of *L. serriola* and 50 plants scored as hybrids from 10 dif-

ferent mother plants were used to verify the morphological screening. All 50 F<sub>1</sub> hybrids scored morphologically amplified the *L. sativa* cultivar-specific RAPD markers, while none of the crop markers were amplified in wild S<sub>1</sub> or F<sub>1</sub> *L. serriola* (Fig. 4). Thus, we considered morphological screening as reliable, and used it for further detection of hybrids. In order to avoid overestimation of the hybridization rate, all plants whose identification was too doubtful were scored as *L. serriola*.

#### Statistical analyses

As many plants died the first year, HR and IH were compared between 2003 and 2004 for both sites with Mann-Whitney U tests with SPSS (SPSS Inc., Chicago, Illinois). Bonferroni's correction (Rice, 1989) was applied in order to calculate significance levels adjusted for multiple comparisons performed on the same data set.

Because of the too different sizes of the 2003 and 2004 datasets, further analyses were carried out for 2004 only. As the data did not fit a normal distribution, we used logistic regression analyses on a logarithmic scale to compare HR and IH between sites and as a function of the distance from the pollen source.

In order to test if the origin of the populations of *L. serriola* influenced the HR or IH, three logistic regressions were successively carried out while removing one population each time. All the comparisons were not significant. Therefore, the origin of the populations of *L. serriola* was removed from further analyses. Logistic regression analyses were carried out with SAS software (SAS 9.1 Institute Inc., Cary, NC, USA) and graphical representation with S-Plus<sup>®</sup> (Insightful Corp.) software.

## Pollinators

While the survey of *Lactuca* pollinators was not the goal of the study, the most frequent insects active on the flowers were captured in trial 2 and identified. Captures were performed during three sunny days in the morning when pollinator activity was intense.

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