Patterns of hybridization and hybrid survival in the invasive alien *Fallopia* complex (Polygonaceae)

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Background – Hybridization and polyploidization are considered important driving forces of invasive processes. In the invasive *Fallopia* spp. complex, hybridization between taxa of various ploidy levels has been experimentally demonstrated. Extensive genetic variation has also been observed among hybrids in the field. However, what makes a certain hybridization event of evolutionary importance is still the subject of speculation considering the invasive potential of hybrid *Fallopia*.

Aims – The present study examined landscape scale patterns of interspecific hybridization within the alien invasive *Fallopia* complex, and tested whether cold winter might act as selective agent for hybrid survival.

Methods – Eighty seeds per clone were collected from four taxa (*F. japonica*, *F. sachalinensis*, *F. aubertii*, and *F. × bohemica*) and sown in greenhouse and outdoor conditions. Ploidy levels of the resulting seedlings were determined by flow cytometry at early stages of seedling growth and compared to those of mature clones present in the same landscapes.

Key results – The four studied taxa were involved in interspecific hybridization patterns. Seedlings resulting from hybridization had a large range of ploidy levels, including aneuploid and euploid progeny, and polyploid levels that were not observed in mature clones. Cold winter had a negative effect on germination success. However, a comparison of ploidy levels of seedlings that survived the cold winter with those grown under greenhouse conditions indicated that cold winter was not a significant selection agent for particular ploidy levels.

Conclusions – Our results stress the importance of interspecific hybridization and polyploidization events in generating invasive hybrids in the alien *Fallopia* spp. complex. Cold winter represents a selection agent for the survival of early stages of progeny but does not explain the discrepancies in ploidy levels between produced seedlings and currently occurring mature clones.

Key words – *Fallopia*, flow cytometry, hybridization, alien invasive plant, polyploidy.

INTRODUCTION

Interspecific hybridization involving an alien species has frequently promoted the evolution of plant invasiveness (Abbott 1992, Ellstrand & Schierenbeck 2006, Schierenbeck & Ellstrand 2009). Increased human activities has generated new opportunities for taxa to co-occur and environmental conditions that may better suit hybrids than their parents (Milne & Abbott 2000, Vilà et al. 2000).

Hybridization and polyploidization together induce rapid evolution of newly formed species (Abbott 2000, Ainouche et al. 2003, Ayres et al. 2009, Soltis et al. 2004) and could be the determinants of invasiveness as a result of genotypic and genomic alterations (Ellstrand & Schierenbeck 2000, 2006). Polyploidization is a source of increased genetic variation

and can be one way of overcoming hybrid sterility (Soltis & Soltis 1999). Hybridization often involves crosses between different cytotypes generating odd-ploidy intermediates that subsequently produce new even-ploidy levels (Ramsey & Schemske 1998, 2002). The cytotype frequency and composition of the offspring depend on factors such as the mating system and the degree of pre- and post-zygotic reproductive isolation between cytotypes (Ramsey & Schemske 1998).

Hybridization and gene flow involving alien plant species have frequently been described (Abbott 1992, Abbott et al. 2003, Ellstrand & Schierenbeck 2000, 2006, Preston et al. 2002, Schierenbeck & Ellstrand 2009) and various studies concentrated on the invasive capacities of hybrid taxa (Moody & Les 2002, Pyšek et al. 2003, Thum & Lennon 2006). However, at present, little is known about the selec-

tion processes after the occurrence of crosses between taxa of different ploidy levels and leading to the invasive success of some of the resulting hybrids. The evolutionary importance of particular hybridization events remains to be determined.

Knotweeds, *Fallopia* spp., are among the most troublesome invaders in Belgium (Branquart 2008, Verloove 2006) and more generally in Europe and North America (Barney 2006, Weber 2003), enhancing nutrient cycling rates and topsoil fertility, and affecting native plant and animal communities (Dassonville et al. 2007, Gerber et al. 2008, Vanderhoeven et al. 2005). Originating from Asia (China, Japan, parts of Korea and Taiwan) and introduced in Europe in the mid 1800's as ornamental plants and fodder (Bailey & Conolly 2000), knotweeds are now widely naturalized (Fojcik & Tokarska-Guzik 2000, Forman & Kesseli 2003, Hollingsworth & Bailey 2000, Mandák et al. 2003, Verloove 2006). These plants thrive in various habitats, including riparian ecosystems and disturbed areas such as roadsides and waste places (Weber 2003).

In the invasive *Fallopia* species complex, different ploidy levels resulting from hybridization and clonality are undoubtedly key factors in their invasiveness (Bailey et al. 2009, Gammon & Kesseli 2010, Gammon et al. 2007, Grimsby et al. 2007, Krebs et al. 2010). Although vegetative regeneration is widely recognized as the main mode of reproduction in the adventive regions, in Belgium several taxa (*F. japonica*, octoploid, 2n = 88; *F. sachalinensis*, tetraploid, 2n = 44; *F. aubertii*, diploid, 2n = 20; *F. × bohemica*, octoploid, 2n = 88, and hexaploid, 2n = 66) are involved in the hybridization patterns (Tiébré et al. 2007a, 2007b). *F. sachalinensis*, *F. aubertii*, and the octoploid and hexaploid *F. × bohemica* display clones producing viable pollen and constitute a potential source for hybridization with *F. japonica* (octoploid, 2n = 88), which is male sterile (Tiébré et al. 2007a). The very low fruit set observed in *F. japonica* is balanced against very important total production of seed and potential seed rain. As observed in the UK (Bailey et al. 1995) and the USA (Bram & McNair 2004, Forman & Kesseli 2003), seed germination was shown to be possible in Belgium, although significantly reduced after a cold winter period (Tiébré et al. 2007a). Moreover, RAPD markers indicated that hybrids in this area displayed high levels of genetic diversity (33 genotypes in 36 sampled hybrid clones) and hexaploid or octoploid levels (Tiébré et al. 2007b).

In the Czech Republic, a survey comparing vegetative regeneration showed a rather low number of hybrid genotypes and suggested a possible environmental selection against progeny originating by sexual reproduction (Pyšek et al. 2003). Moreover, Mandák et al. (2005), investigating isoenzyme diversity in the same area, recorded 33 genotypes in 88 sampled hybrid *Fallopia × bohemica* clones. They proposed that only a few hybrid progeny, preferentially hexaploid hybrids, go through the sieve of natural selection. These two studies suggest that natural selection might be a key factor in hybrid cytogenetic patterns, but they do not indicate the diversity of hybrids on which the selection acts, or the selection agent.

The objectives of the present study were to (1) characterize the outcome of hybridization events within the alien inva-

sive *Fallopia* complex, (2) compare ploidy level of seedlings resulting from hybridization processes with those of mature clones observed in some landscapes, and (3) assess the effect of the cold winter on germination and seedling growth for different hybridization patterns.

MATERIAL AND METHODS

Species

In Belgium, the four following exotic taxa have been reported within the genus: three erect rhizomatous perennials, *F. japonica* (Houtt.) Ronse Decr. (2n = 88), *F. sachalinensis* (F.Schmidt) Ronse Decr. (2n = 44), and *F. × bohemica* (Chrtek & Chrtková) J.P.Bailey (2n = 66; 2n = 88), and one climbing horticultural perennial, *F. aubertii* (L.Henry) Holub [synonym *F. baldschuanica* (Regel) Holub (2n = 20)] (Lambinon et al. 2004). *F. × bohemica* is a hybrid between *F. japonica* and *F. sachalinensis* and any backcrosses with the parent species. Based on RAPD markers, no genotypic variation was found for *F. japonica* (forty clones) and *F. aubertii* (two clones) in Belgium (Tiébré et al. 2007b). Two different genotypes were found among six clones of *F. sachalinensis*. Twenty-eight genotypes were observed out of 29 hexaploid hybrid *F. × bohemica* clones and three genotypes out of three octoploid *F. × bohemica* hybrid clones.

Sampling

Two different Belgian landscapes were selected where previous extensive surveys localized all clones of *Fallopia* spp. (Tiébré et al. 2007a, 2008): one semi rural landscape (1300 ha) in the vicinity of Gembloux (50°33'N 4°41'E) and one urban landscape in the Brussels region (50°50'N 4°21'E). The present sampling in the investigated areas included clones in the morpho-genetic study of Tiébré et al. (2007b). In the field, different species were distinguished according to reported morphological vegetative characteristics (Beerling et al. 1994, Lambinon et al. 2004, Meerts & Tiébré 2007). Taxon identification had previously been confirmed by cytogenetic analysis (Tiébré et al. 2007a). Leaves were collected for ploidy level assessment of 27 *F. japonica* clones, two *F. sachalinensis* clones, 21 *F. × bohemica* clones, and two *F. aubertii* clones. Within each landscape, seeds from among these 52 clones were collected in October 2005, including eight *F. japonica* clones, two *F. sachalinensis* clones, one *F. × bohemica* clone, and two *F. aubertii* clones. The small sample size of *F. sachalinensis* and *F. × bohemica* in this study reflects the sparse occurrence of *F. sachalinensis* clones in the landscapes and the absence of seed production, except for the sampled clone. For each clone, the proximity to any other male fertile clone of *F. aubertii* or *F. × bohemica* was recorded. At the landscape level, clones were considered as isolated if the distance to the nearest pollen donor was more than 400 m.

Collected seeds were directly sown in a 2:1 peat:sand mixture. Forty seeds per clone were grown in a greenhouse at 25°C with 16 h light (from 6:00 a.m. until 10:00 p.m.), and forty seeds per clone were placed outside to test the effects of cold winter temperatures on germination and hybrid cytotype survival.

Ploidy level assessment

Ploidy level was measured by flow cytometry, which rapidly determines relative nuclear DNA content by measuring the fluorescence of a specific DNA binding fluorochrome (Galbraith et al. 1983). Small leaf disks were chopped with a razor blade in Petri dishes, after addition of 500 µL 100 mM pH 7 sodium hydrogen phosphate containing 0.5% v/v Tween 20. After filtration through a 30 µm nylon filter, 500 µL of a solution of 5g/L DAPI in 100 mM sodium hydrogen phosphate was added (CyStain® DNA 2 steps, Partec®). Flow cytometry measurements were performed with a Partec® machine (CA3 software, 1995) equipped with a UV lamp. The fluorochrome had excitation and emission peaks respectively at 340 and 470 nm. A tetraploid individual (*F. sachalinensis*) with a known chromosome number was used as an internal standard for each measurement. We used the ratio 'mean fluorescence intensity of seedling / mean fluorescence intensity of internal standard' to assess the ploidy level of seedlings. To interpret our observations more accurately, chromosomes were counted for fresh roots tips (according to Bailey & Stace 1992) of some mature plants that were used as marks (seven nuclei from fresh root tips per clone) (Bailey & Stace 1992, Tiébré et al. 2007a). Ploidy level assessment was performed both on greenhouse and outdoor grown seedlings, as well as on 52 samples collected from mature individuals in the studied landscapes, including the parents of the seed collections. The seedling ploidy level distribution was compared to that of mature clones.

Statistical analyses

We used a Chi-square test to examine differences in mean ploidy levels between seedlings from isolated clones and from clones located close to male fertile clones.

RESULTS

Mean germination rates in the greenhouse were 45% and 27.5% for *F. japonica* and *F. sachalinensis* respectively and decreased to 3.75% and 5% respectively for the seeds that overwintered outside. *F. × bohemica* seeds reached up to 25% germination in the greenhouse but they did not germinate outside. No *F. aubertii* seeds germinated in the greenhouse or outside. Seeds germinated after seven days in the greenhouse and five months when grown outside.

For mature clones, the mean peak ratios in fluorescence intensity were 0.72, 1, 1.59, and 2.12 respectively for *F. aubertii* (2n = 20), *F. sachalinensis* (2n = 44), *F. × bohemica* (2n = 66), and *F. japonica* (2n = 88) (fig. 1). Note that because of the larger size of *F. aubertii* chromosomes (Bailey 1989), the fluorescence peak ratio was not expected to be directly proportional to the number of chromosomes. Substantial variation in the mean peak ratios was observed for *F. japonica* (range = 1.96 to 2.21, SD = 0.08, N = 16) while chromosome counts always revealed 2n = 88 (seven replicates), indicating substantial measurement variability. *F. × bohemica* clones (range = 1.54 to 2.17, SD = 0.20, N = 20) included both hexaploid (N = 17) and octoploid (N = 3) levels. One aneuploid hybrid individual was identified with 44 to 66 chromosomes
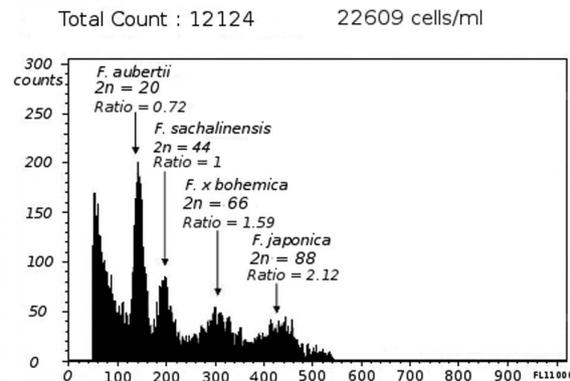

Figure 1 – Flow cytometry histogram showing the fluorescence intensity peaks of four taxa of the *Fallopia* complex.

in the nuclei (seven replicates).

Based on germination success, a total of 142 seedlings grown from seeds collected on eight *F. japonica* clones (115 seedlings), two *F. sachalinensis* clones (fourteen seedlings), and one *F. × bohemica* clone (thirteen seedlings) were used in flow cytometry analyses. *F. japonica* seedlings were cytologically extremely variable, with fluorescence ratios ranging from 1.36 to 3.06. Within this range, three distinct groups of ploidy levels can be distinguished (fig. 2). Forty-nine seedlings (42.6%) exhibited low peak ratios ranging from 1.36 to 1.61. This group covers three potential hybridization outputs. First, it includes the ratio expected for crosses with *F. aubertii* (2n = 20) leading to hybrids that should exhibit 2n = 54. The second group of seedlings had peak ratios corresponding to mature plants exhibiting 2n = 66. These would be typical hexaploid hybrids with *F. sachalinensis* as the paternal parent although no male fertile *F. sachalinensis* were found in the studied landscapes. The third range includes the peak ratio value of the observed mature aneuploid hybrid with 2n between 44 and 66. These seedlings could therefore also be hexaploid hybrids with *F. sachalinensis* that, in addition to the normal chromosome number (2n = 66), suffered from irregularities. Thirty-nine seedlings (33.9%) exhibited peak ratios ranging from 1.81 to 2.30. These seedlings may result from different backcrosses with unreduced or irregular pollen grains from the hexaploid *F. × bohemica* leading to about 77 chromosomes, or from backcrosses with irregular or unreduced gametes of the octoploid *F. × bohemica* leading to about 110 chromosomes. Finally, twenty seedlings (17.4%) exhibited peak ratios ranging from 2.46 and higher, where unreduced maternal gametes could play a role as well as backcrosses with the octoploid *F. × bohemica* (ploidy levels up to 2n = 132).

F. × bohemica seedlings were highly polyploid with ratios ranging from 1.56 to 3.17, which involves crosses with unreduced gametes. Only one of the seedlings from *F. × bohemica* corresponded to mature clones in the field and could be from a normal cross between reduced and regular pollen of the hexaploid *F. × bohemica*.

Most *F. sachalinensis* seedlings showed ratios between 1.28 and 1.52. These seedlings suggest crosses with *F. × bohemica* (2n = 66) leading to hybrids that should exhibit 2n
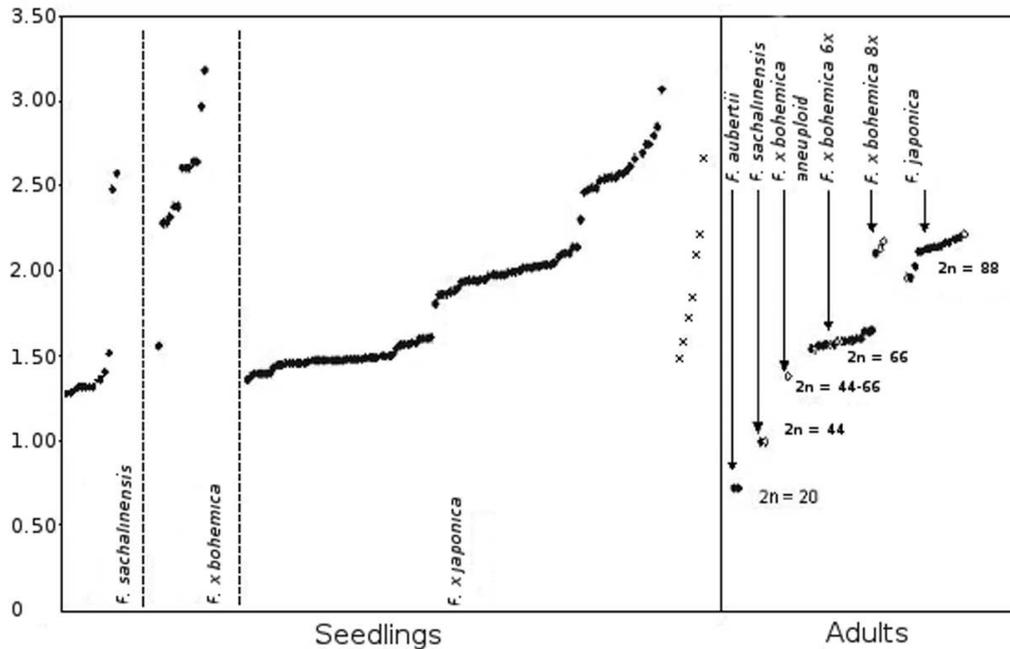
Figure 2 – Fluorescence intensity ratios for seedlings (seeds collected from *F. sachalinensis*, *F. × bohemica*, and *F. japonica*) and mature plants (*F. aubertii*, *F. sachalinensis*, *F. × bohemica*, and *F. japonica*) in the *Fallopia* complex. Cross marks represent seedlings of *F. japonica* that have survived the cold winter. Open circles represent mature plants assessed by chromosome counts.

= 55. Two seedlings exhibited higher ratios (2.48 and 2.57). Ratios for the seven seedlings of *F. japonica* that germinated outside were also variable and ranged between 1.47 and 2.66. When the location of *F. japonica* clones was taken into account, no significant differences in mean ploidy were found between seedlings from isolated clones vs. seedlings from clones close to male fertile *F. × bohemica* (Chi square = 3.13, d.f. = 2, p = 0.209).

DISCUSSION

The restoration of *F. japonica* sexual reproduction through hybridization was previously demonstrated in Belgium (Tiébré et al. 2007a, 2007b). In the present study, our results indicate that four taxa, i.e. *F. japonica*, *F. sachalinensis*, *F. aubertii*, and *F. × bohemica* are involved in interspecific hybrid crosses in the area. A very large range of ploidy levels was observed as a result of these hybridization processes, including aneuploid and euploid progeny, sometimes with very high ploidy levels.

43% of *F. japonica* seedlings originated from crosses with *F. aubertii* and *F. sachalinensis*, while no male fertile clone of the latter was found in the investigated areas. Seedlings resulting from backcrosses between the hybrid *F. × bohemica* and *F. japonica* were also recorded. This second generation hybridization further complicated the picture, as second generation hybrids had significantly higher ploidy levels than first generation hybrids. However, none of them was found among established plants in the investigated sites. Interestingly, only one hybrid collected from *F. japonica* was hexa-

ploid, which could in principle be produced through regular meiosis. Fertilization by pollen grains of *F. × bohemica* with odd-ploidy levels may also be involved as well as unreduced crosses with *F. × bohemica* or *F. sachalinensis*.

Polyploids in flowering plants generate a high frequency of aneuploid gametes (the mean occurrence of aneuploid pollen in allopolyploids is about 45%), which has two possible outcomes as stated by Ramsey & Schemske (2002). First, aneuploidy may be lethal either at the gamete or the embryonic developmental stages. The resulting progeny of polyploids is a limited number of euploids. Second, aneuploid gametes operate as euploids generating viable gametes. The resulting progeny are numerous but comprise a high frequency of aneuploid individuals (the mean occurrence of aneuploid progeny in allopolyploids is about 28%) (Ramsey & Schemske 2002). The extensive variation of seedling ploidy levels shown in the present study indicates the importance of aneuploid gametes in hybridization events occurring in the knotweeds complex. *F. × bohemica* has very irregular meiosis, with large numbers of univalents, and multivalents up to quadrivalents, especially at the hexaploid level (Bailey & Stace 1992). However, in the investigated area, only one mature clone (of 21 examined hybrids) was identified as aneuploid (from 44 to 66 chromosomes). Aneuploidy is often assumed to act as a key step in evolution and speciation, sometimes enabling transitions between euploid chromosome numbers (Burton & Husband 1999, 2001, Ramsey & Schemske 2002). When aneuploid individuals acquire some degree of fertility, they may be involved in crosses allowing higher ploidy levels to occur. Moreover, massive clonal growth may help main-

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tain and increase aneuploid clones, consequently enhancing the phenomenon.

It is worth noting that unreduced gametes of the hexaploid *F.* × *bohemica* may play a role in hybridization patterns. In this case, unreduced gametes might act as a pathway for autopolyploidization within *F.* × *bohemica*. Unreduced gametes are formed as a result of meiotic dysfunction induced by genetic or environmental factors (Bretagnolle & Thompson 1995, Carputo et al. 2003). The formation of allopolyploids by fusion of unreduced gametes is thought to be the main pathway of production because almost every plant species produces a variable but small amount of unreduced gametes via meiotic processes (Chen & Ni 2006). In the present study, the ploidy levels of mature plants in the investigated landscape did not indicate any successful establishment of decaploid seedlings or seedlings with higher ploidy levels resulting from crosses involving unreduced gametes. Unreduced gametes have frequently been evoked in the putative origins of the octoploid *F.* × *bohemica* (Bailey & Wisskirchen 2006, Mandák et al. 2003). In our study, 21.5% of *F. japonica* progeny were octoploid. Very little is known about the future of these produced hybrids. In the study area, these latter putative hybrids might potentially cross with reduced or unreduced pollen of the hexaploid *F.* × *bohemica*, explaining high seedling ploidy patterns (ratio higher than 2.3).

Our methodology did not allow us to monitor seedling fate up to the mature stage. Indeed, flow cytometric analyses were performed on the first leaves available, which was destructive for the seedling. This experimental option was chosen intentionally in order to assess ploidy levels as close to the hybridization event as possible. It allowed assessing seedlings that would suffer from developmental problems hindering their survival in later stages. However, when comparing ploidy levels of seedling and mature clones in the field, a variety of cytotypes did not seem to persist until the mature stage.

Beerling et al. (1994) showed that hybrid progeny probably did not survive in low temperatures at the end of the growing period. Yet, Tiébré et al. (2007a) demonstrated that germination was possible, although significantly reduced, after a cold winter period. Germination rates observed in the present study are comparable to ones previously shown in the same area by Tiébré et al. (2007a), although much lower than those recorded from wild populations in the USA (Forman & Kesseli 2003, Bram & MacNair 2004). In the latter study, neither cold treatment nor dormancy was required for high germination (Forman & Kesseli 2003). In our study, the decreased germination in overwintered individuals seems to differentially affect the three taxa. Little is known about *Fallopia* seed dormancy, but Forman & Kesseli (2003) showed genetic variation in *F. japonica* dormancy in the USA.

In a survey comparing vegetative regeneration at the genotype level, Pyšek et al. (2003) suggested a possible environmental selection against progeny originating by sexual reproduction. In the present study, we observed an important decrease in germination capacities of seeds having experienced a cold winter period. Moreover, no seeds collected from *F. sachalinensis* or *F.* × *bohemica* germinated outdoors. The cold winter period might thus differentially affect hy-brids from different hybridization patterns within the complex. Interestingly, the seven seedlings from seeds collected from *F. japonica* that survived the cold winter period had various ploidy levels (ratio 1.47–2.66). For three of them, the observed ploidy level was not found at the mature stage in the investigated areas. This indicates that the cold winter period would not be the only selection pressure acting against hybrid progeny.

Another hypothesis explaining the difference in ploidy level distribution between seedlings and mature plants is the possibility of multiple introductions of hybrids in the study zones. However, multiple introductions are less likely than local sexual reproduction for attaining such high levels of genetic diversity (33 genotypes from 36 clones) observed for hybrids in Belgium and neighbouring countries (Krebs et al. 2010, Tiébré et al. 2007b). Indeed, it would more likely result from multiple *in situ* hybridization between taxa coupled with the restoration of male fertility in the hybrids (Krebs et al. 2010, Tiébré et al. 2007b).

In conclusion, we stress the importance of interspecific hybridization and polyploidization events in generating invasive hybrids in the alien *Fallopia* spp. complex. Two processes may however limit hybridization events in generating invasive progenies and should be further investigated. First, some crosses may be the result of chromosomal incompatibilities between taxa. Second, selection can act on early stages of seedling growth as well as on mature plant establishment. Even so, interspecific hybridization, the restoration of male fertility, and the massive clonal growth capacities observed in the complex and demonstrated for the hybrids undoubtedly strengthen the striking invasive success of knotweeds in the introduced range.

ACKNOWLEDGEMENTS

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