Reproductive strategy of the invasive Oxalis pes-caprae: distribution patterns of floral morphs, ploidy levels and sexual reproduction

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Reproductive strategy of the invasive *Oxalis pes-caprae*: distribution patterns of floral morphs, ploidy levels and sexual reproduction

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Abstract *Oxalis pes-caprae,* a tristylous flowering plant native to South Africa, is described in the western Mediterranean basin as an asexual—only 5*x* short-styled morph (5*x* S-morph) invasive weed losing all mating partners after introduction. The objective of this study was to reassess the patterns of floral morph and cytotype distribution and the sexual reproduction ability in this invaded range. For that, floral morph and cytotype composition were evaluated in 39 populations of *O. pes-caprae* in a methodical sampling. The reproductive success of natural populations was assessed as fruit and seed production and seed germination for all floral morphs and cytotypes detected. Self- and morph-incompatibility were also studied with controlled hand pollinations. A remarkable diversity in floral morph and cytotype composition was observed. Furthermore, we observed successful sexual reproduction in several localities across the surveyed area. The S-morph is still dominant in this invaded area, and although it was mostly 5*x*, an additional cytotype (4*x*) was also recorded. Records of both a mid-styled morph (M-morph) and an area with trimorphic populations of this species are reported here for the first time in the invasive range of the Mediterranean basin. The long-styled morph appears to occur randomly across the surveyed area, while the M-morph is concentrated mainly in Estremadura province (Portugal), where a breakdown in the incompatibility system was observed. These distribution patterns may result from events of sexual reproduction after incompatibility breakdown and/or from multiple introduction events from the native area. The ability to reproduce sexually, undetected so far, may have important impacts in the population dynamics and major consequences for the adaptation and selection potential of *O. pes-caprae* in this invaded area.

Keywords Cytotypes · Floral morphs · Incompatibility system · Invasive plant · Sexual reproductive success · Tristyly

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Introduction

Biological invasions comprise long-distance dispersal of individuals (or their propagules), followed by successful establishment, rapid multiplication and range expansion (Richardson et al. 2000). Biological

invasions have ecological and evolutionary consequences, both for the species and communities being invaded and for the invasive species themselves (reviewed in Pyšek and Richardson 2010). Consequently, it is essential to understand the on-going evolutionary processes during invasion and their contribution to invasion success (Sakai et al. 2001; Stockwell et al. 2003).

In order to become invasive, exotic species must overcome a series of barriers. One of the most important barrier after introduction is reproduction, i.e. the introduced individuals must be able to regularly reproduce (sexually and/or asexually) to establish populations in the new range and spread to new areas (Richardson et al. 2000). Due to the huge diversity of reproductive strategies in plants, reproductive modes (e.g. relative importance of sexual vs. asexual reproduction or self-fertilization vs. cross-pollination and dependence on pollination vectors) will affect several aspects of the invasion process, including population genetic structure, demography, biogeography and ultimately determine the opportunities for the evolution of local adaptation (Barrett 2011). Thus, reproduction is a key factor for the successful establishment and spread of invasive species. Still, the roles played by reproductive traits in the successful invasion of exotic species are largely unknown (but see reviews by Barrett et al. 2008; Barrett 2011).

Oxalis is a very large genus, including more than 800 species distributed globally, with the main centres of diversity being in Central and South America and South Africa (Salter 1944; Lourteig 2000). Heterostyly is a common feature in the genus and, considering the weedy character of some species, has been shown to have important reproductive consequences for the invasive populations (e.g. Luo et al. 2006; Castro et al. 2007). *Oxalis pes-caprae* L., a native species from western South Africa, introduced as an ornamental plant in Mediterranean climate regions of the world, ranks among the most troublesome widespread invasive weeds (Rappa 1911; Baker 1965; Ornduff 1986, 1987). *Oxalis pes-caprae* is a heterostylous species with trimorphic flowers (long-, L-morph; mid-, M-morph; and short-styled floral morphs, S-morph) and a self- and morph-incompatibility system (Ornduff 1987; Castro et al. 2007). Additionally, this species is a polyploid complex with diploid ($2n = 2x = 14$ chromosomes), tetraploid ($2n = 4x = 28$ chromosomes) and pentaploid individuals ($2n = 5x = 35$

chromosomes). In the native range, trimorphic populations with variable morph proportions have been observed (with both isoplethic and anisoplethic populations, Turketti 2010). Tetraploidy is the most common ploidy level (te Beest et al. 2012), and in some regions diploids and tetraploids are found to grow in parapatry (Krejčíková et al. 2013). Pentaploid individuals are considered rare and restricted to Cape Town (Michael 1964; te Beest et al. 2012). In the invaded area of the Mediterranean region, a shift to obligate asexuality through clonal propagation was observed as a result of founder events with the introduction of the $5x$ S-morph only (Baker 1965; Ornduff 1987). However, recent field observations in this range reported the occurrence of two floral morphs ($5x$ S-morph and $4x$ L-morph, Castro et al. 2007). Despite the occurrence of two morphs, according to Castro et al. (2007), the plant was not known to reproduce sexually due to a self- and morph-incompatibility system and to different ploidy levels between the two reported floral morphs.

The objective of this work was to reassess the patterns of floral morph and cytotype distribution in the western Mediterranean basin through a longitudinal transect from La Coruña province (Spain) to Essaouira province (Morocco), by sampling new areas in addition to those areas covered in the study of Castro et al. (2007). In doing so, we aimed to answer the following questions (1) what are the current distribution patterns of floral morphs and cytotypes in the western Mediterranean basin? (2) Is *O. pes-caprae,* in this invaded area, capable of sexual reproduction? (3) Does natural sexual reproduction of this invasive species actually occur in this range and if so, how successfuly? (4) Could the breakdown of the incompatibility system of the $5x$ S-morph be one of the mechanisms involved in sexual reproduction causing the emergence of new forms?

Materials and methods

Plant species

Oxalis pes-caprae (Oxalidaceae) is a geophyte, up to 40 cm high, with a true bulb, borne at the apex of a vertical rhizome (Young 1968; Oberlander et al. 2009). The bulb annually produces a contractile root, which pulls the bulb deeper each year, and bulbils are produced in the axillary buds of the vertical rhizome

(Salter 1944; Pütz 1994). Each year a rosette of leaves and umbellate cymes with 4–10 yellow flowers are produced (Young 1968). The flowers are actinomorphic and tristylous, with a sympetalous corolla composed of five petals and two whorls of five stamens and one whorl of five stigmas, arranged at three levels according to the floral morph of each individual (S-, M- or L-morph; for a figure see Castro et al. 2007). In the invaded area of the Mediterranean region, *O. pes-caprae* flowers in late winter to early spring (January–March).

Study area and general sampling procedure

The study was undertaken during September 2009 and February and March of 2010 in the western Mediterranean region. During the flowering period, 39 populations were sampled every 80–100 km along a latitudinal transect from La Coruña province (Spain) to Essaouira province (Morocco). All the populations were located in highly-invaded areas, mainly distributed along the coast. Populations were defined as a group of individuals confined by anthropogenic or natural barriers. A higher number of populations were sampled in Estremadura province (Portugal), the area where all three floral morphs were frequently detected. In this region, the distance between populations ranged from 1 to 6 km. This sampling procedure enabled a complete and methodical screening along the Atlantic coast.

The following information was gathered in all the populations: proportion of floral morphs, cytotype composition and sexual reproductive success. For every population, after determining floral morph proportions (see section *Floral morph proportion*), whenever possible, 10 plants from each detected morph were marked for fruit and seed set assessment (see section *Natural reproductive success*) and one leaf of each individual was collected for ploidy level analysis (see section *Cytotype composition*). All the sampled plants were separated at least 5-m apart to avoid re-sampling clones of the same individual.

Floral morph proportion

The proportion of floral morphs in each population was assessed in 2–3 longitudinal transects across the entire population, where the floral morph of at least 100 individuals was recorded with a 5-m distance between observations. Floral morphs were identified as S-, M- and L-morph. The occurrence of the double-flowered sterile form (St; for details on flower morphology see Castro et al. 2007) was also recorded.

Every population was characterized for the proportion of sexual and sterile forms, and for the proportion of sexual floral morphs excluding the sterile form. A G test was applied to determine whether sexual floral morph ratios differed significantly from isoplethy, i.e. 1:1:1 ratio in trimorphic populations and 1:1 in dimorphic populations (the latter with Yates correction for continuity; Zar 1984). A G test was also applied to assess differences between the proportions of sexual and sterile forms.

Spatial autocorrelation analyses were carried out to establish if closer populations would be more similar in the proportion of floral morphs (i.e. percentage of M- or L-morphs in the populations) than populations spaced further apart (Legendre and Fortin 1989) and thus, if the proportion of M- and L-morphs would follow any pattern. The presence of spatial autocorrelation was approached using Moran's *I* correlogram with the PASSAGE 2 program (Rosenberg and Anderson 2011). A geographic distance matrix based on spherical distances was calculated from longitude and latitude coordinates. Sturge's rule was used to decide the number of classes as follows: number of classes = $1 + 3.3*\log(m)$, where m is the number of distances in the matrix without the diagonal (Legendre and Legendre 1998). Thus, we chose 10 distance classes all of this with equal number of observations. A global test was undertaken to check whether the correlogram contains at least one significant value at $\alpha' = \alpha/$number of distance classes, according to the Bonferroni method of correcting multiple tests (Legendre and Fortin 1989). Additionally, each independent Moran's *I* coefficient from the correlogram was tested with the null hypothesis that the coefficient is not significantly different from zero, i.e. that there is a random spatial pattern.

Cytotype composition

The ploidy level of all the sampled individuals (see section Study area and general sampling procedure) was assessed using flow cytometry. In the field, each collected leaf was identified with the plant and population code, and stored in plastic bags at 4 °C until analysis. In up to 3 days after collection, samples were prepared following the chopping procedure of Galbraith et al. (1983) and the two-step nuclei isolation method with Otto's buffer (Otto 1992; improved by Doležel and

Göhde 1995), following recommendation (Emshwiller 2002; Loureiro et al. 2006). Briefly, nuclei were released after chopping 1 cm^2 of leaf tissue of *O. pes-caprae* and 1 cm^2 of leaf tissue of *Bellis perennis* (internal reference standard with 2C = 3.38 pg, Schönswetter et al. 2007) in 0.5 ml of Otto I buffer (0.1 M citric acid, 0.5 % Tween 20). After filtration with a 50 µm nylon mesh, the nuclear suspension was stained with a solution containing 1 ml of Otto II buffer (0.4 M Na$_2$HPO$_4$·12H$_2$O), 50 µg ml^{-1} of propidium iodide and 50 µg ml^{-1} of RNAse. Samples were analysed in a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) equipped with a green solid state laser (Cobolt Samba 532 nm, 100 mW; Cobolt, Stockholm, Sweden) for PI excitation. At least 3,000 nuclei per sample were analysed. The pooled sample strategy was followed, with leaflets from 5 individuals being analysed simultaneously (Kolář et al. 2009); when several peaks were obtained individual samples were prepared to assign the ploidy levels to each sampled plant. Only histograms with a coefficient of variation (CV) below 5 % for both sample's and standard's G$_1$ peaks were accepted as a quality standard. The DNA index was calculated for all the samples by dividing the *O. pes-caprae* G$_0$/G$_1$ peak mean by that of *B. perennis*. The plants were identified as tetraploids for genome size values of 1.37 ± 0.056 (mean ± SD) and as pentaploids for genome size values of 1.66 ± 0.030 (mean ± SD) (Castro et al. 2007).

Natural reproductive success

The sexual reproductive success was evaluated by assessing fruit set, seed production and seed germination in all the sampled populations. For this, all populations were re-visited before seed dispersal (approximately 1 month later) and one infructescence per marked plant was collected and stored in a paper bag identified with the plant and population code. To avoid loss of fruits and seeds due to the explosive dehiscence of the capsules, the infructescences sampled still had some open flowers. Fruit and seed production were assessed in the laboratory by counting the following variables under a binocular stereomicroscope: number of scars in the peduncle (corresponding to the pedicels of flowers that did not form fruits), number of fruits (capsules) and number of morphologically viable and aborted seeds per fruit. Fruit set was calculated as the percentage of flowers

that developed into fruit by dividing the number of fruits by the sum of the number of scars and capsules.

To assess seed viability seeds from each plant were placed in 6 × 6 cm pots filled with common garden substrate to germinate during September 2010. Each pot was marked with the plant and population code. Pots were placed under natural conditions in the experimental garden of the Botanical Garden of the University of Coimbra. Seedling emergence was recorded every week and seed germination rates were calculated after 3 months (after seedling emergence had stopped). Whenever possible, the ploidy level of up to five seedlings per pot was analysed through flow cytometry (following the procedure described above).

Descriptive statistics (mean and standard error of the mean) were calculated for fruit set, seed production, seed abortion and seed germination. For the latter three variables, zeros due to non-fruit development were removed from descriptive statistics and subsequent analyses. A mixed linear model approach was used to assess differences in the reproductive success between floral morphs and cytotypes; morph and cytotype combined was defined as fixed effect (4x S-, 5x S-, 4x M- and 4x L-morph), population as random effect and fruit set, seed production, seed abortion and seed germination as dependent variables. Fruit set and seed germination were transformed with arcsine (square root (dependent variable)) before the analyses, to achieve normality and homoscedasticity. The reproductive success of each population was also calculated by summing the fruit set of each floral morph present in the population weighted by its proportion in the population. Differences in the reproductive success between monomorphic and polymorphic populations were assessed with a Mann–Whitney Rank Sum test (because normality was not achieved).

Incompatibility system

The breakdown in the incompatibility system was investigated by assessing the ability of the 5x S-morph (supposedly the only morph introduced in the region studied) and of the 4x M- and L-morphs to produce offspring after legitimate and illegitimate crosses. Legitimate pollinations consisted in pollinations between morphs using the anthers of the same level of the style being pollinated; illegitimate pollinations were self-pollinations and within morph pollinations using pollen from both anther whorls equally. In

September 2009, bulbs from up to 64 plants per floral morph (S-, M- and L-morph) from the Colares population (Estremadura province, Portugal) were planted into 2 L plastic pots (11 × 11 cm wide and 21 cm depth) filled with common garden substrate and maintained in the nurseries of the Botanical Garden of the University of Coimbra under natural conditions. At the start of the next flowering period, plants were covered with a nylon mesh to prevent natural pollinations and remained bagged until fruiting. The following treatments were applied to the three floral morphs: self-pollinations, within morph pollinations and between morph legitimate pollinations. All recipient flowers were emasculated to prevent self-pollination, except for the selfing treatment. Up to 64 pollinations per treatment were done in distinct individuals. Cross-pollinations were performed by gently rubbing anthers from 3 to 5 distinct individuals against the recipient stigmas. The fruit and seed production were recorded when mature.

Fruit set was calculated for each pollination treatment and morph as the percentage of treated flowers that developed into fruit. Differences in fruit set among pollination treatments (both overall and within morph with cytotype combined) were analysed using a GLZ with a binomial distribution and logit link function. Differences in the mean number of seeds produced per fruit among pollination treatment and morph were analysed using GLM without interactions (interactions were not allowed because of the empty cells generated by the lack of fruits by S-morph after selfing). All the analyses were carried out in STATISTICA 7.0 (Stat Soft. Inc., Tulsa, OK, USA).

Results

Floral morph proportion

The floral morph ratios of the 39 sampled populations are summarized in Fig. 1 and Appendix 1 of Electronic Supplementary Material. The majority of the populations (87.2 %) comprised of sexual morphs only. The sterile form (St) was present in 5 populations (12.8 %), and was always present in significantly low proportions ($G \geq 10.38$, $P < 0.005$; Appendix 1 of ESM). With respect to sexual morphs composition, half of the populations were monomorphic (51.3 %), being composed either of the S-morph (46.2 % of the total

populations) or by the L-morph (5.1 % of the total populations). As previously described for this region, dimorphic populations composed by the S- and L-morphs were also observed (25.6 % of the total populations). In all dimorphic populations one morph prevailed in relation to the other ($G \geq 5.477$, $P < 0.05$); in most cases the S-morph was dominant, while the L-morph was only dominant in population 15 (Appendix 1 of ESM). Surprisingly, 23.1 % of populations were found to be trimorphic. This is the first record of both the M-morph and of trimorphic populations in this invaded range. The M-morph was always found growing in trimorphic populations. These populations were all anisoplethic ($G \geq 18.63$, $P < 0.001$), usually with dominance of the S-morph. In one population (population 19) the S- and L-morphs were equally dominant, and in two populations (populations 21 and 20) the L-morph dominated (Fig. 1 and Appendix 1 of ESM).

Spatial autocorrelation of floral morphs

A variable is said to be autocorrelated when it is possible to predict its values in space from known values at other sampling points (Legendre and Fortin 1989). M- and L-morph Moran's I correlograms were globally significant, indicating that the overall pattern of spatial distribution of floral morphs ratios is not random. For the M-morph, only the first distance class showed a significant positive value of I ($P < 0.001$; Fig. 1C). For the L-morph a significant positive value of I ($P < 0.001$) was observed for the first class, but negative values were obtained for the second and fourth distance classes ($P = 0.04$ and $P = 0.007$, respectively) (Fig. 1C).

Cytotype composition

Flow cytometric analyses of the ploidy level of each individual (Fig. 1 and Appendix 1 of ESM) revealed novel results in the invaded area of the Mediterranean basin. Besides the previously reported $5x$ S-morph individuals, $4x$ S-morph individuals were found for the first time. These plants were only found in trimorphic populations. The $5x$ S-morph individuals are dominant along the entire range, as well as within the trimorphic populations (Fig. 1). All the M-morph individuals described here for the first time were $4x$. The analyses of the L-morph and St individuals confirmed that, as previously reported, all were $4x$.
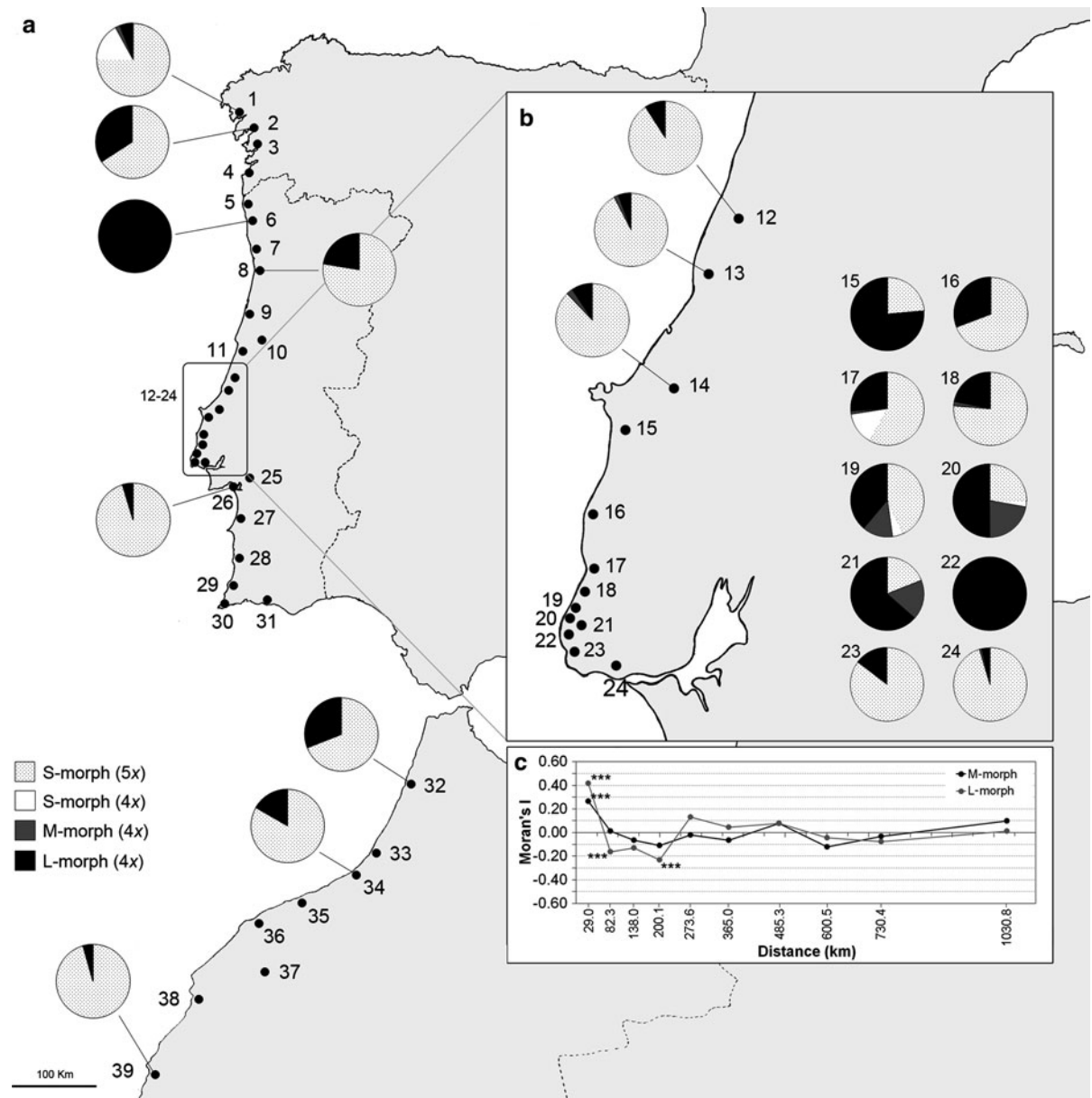
Fig. 1 *Oxalis pes-caprae* floral morph and cytotype composition: **a** populations surveyed in the western Mediterranean basin; **b** detail of Estremadura region, Portugal; **c** spatial autocorrelation of M- and L-morphs (significant values of Moran's I at $P \leq 0.01$ after Bonferroni correction are marked with *asterisks*). Population localities correspond to Appendix 1 of ESM. Populations without diagrams are monomorphic containing the 5x S-morph only. *Floral morphs*: S, short-styled; M, mid-styled; L, long-styled. *Cytotypes*: 4x, tetraploid; 5x, pentaploid

Natural reproductive success

Fruit set, seed production (both viable and aborted) and seed germination were variable across the surveyed populations and were significantly different among the tested groups (morph and cytotype combined) (Fig. 2, and Appendices 2, 3 and 4 of ESM). Overall, the reproductive variables measured revealed a general pattern of variation: the 5x S-morph had significantly lower fruit set, seed production (both viable and aborted) and seed germination than those obtained for 4x M-morph and 4x L-morph ($P < 0.05$;

Table 1), the latter morphs having the highest reproductive success values. The plants sampled of 4x S-morph did not differ significantly from the other groups for any of the reproductive variables, due to the high variability observed in the few available individuals (Table 1). Still, despite the overall low reproductive success recorded for the 5x S-morph, it is worth noticing that: (1) in 7 out of 17 monomorphic population of S-morph, production of fruits was recorded; and (2) in 6 out of 24 populations where this morph produces fruit, 5x S-morph had a fruit set higher than 25 % (Fig. 2 and Appendix 2 of ESM). Finally, it is also worth noticing the particularly high seed abortion recorded for the 4x L-morph (Table 1 and Appendix 3 of ESM). The FCM analysis of the seedlings enabled us to assess the cytotypes that were produced under natural conditions. The 5x S-morph and 4x L-morph produced both 5x and 4x individuals, while the few seedlings obtained from 4x S-morph and 4x M-morph were all 4x (Table 1 and Appendix 4 of ESM).

The reproductive success of the populations ranged from 0 to 58.4 % (Fig. 2 and Appendix 2 of ESM). Significant differences were observed between monomorphic and polymorphic populations ($T = 485.0$, $P < 0.001$; $n_{\text{polymorphic}} = 18$, $n_{\text{monomorphic}} = 20$), with the former ($30.8 \pm 1.8$ %) presenting a higher fruit set than the latter (13.3 ± 1.0 %). Still, in seven monomorphic populations of the S-morph (as referred above) and in all L-morph monomorphic populations, production of fruits was observed with variable rates of fruit set (Fig. 2 and Appendix 2 of ESM). The monomorphic populations where fruit set was recorded under natural conditions were concentrated mainly in south Portugal.

Incompatibility system

All pollination treatments produced fruits, except the self-pollination of the 5x S-morph. Significant differences in fruit set were obtained between pollination treatments ($\chi_2^2 = 55.79$, $P < 0.0001$). However, these were mainly due to differences between treatments within the S-morph ($\chi_2^2 = 83.62$, $P < 0.0001$). The S-morph did not yield any fruit after selfing and had significantly lower fruit set after within morph pollinations than between morph legitimate pollinations (Fig. 3a), while the M- and L-morphs had similar fruit set among all pollinations treatments ($\chi_2^2 = 1.71$,

$P = 0.425$ and $\chi_2^2 = 2.12$, $P = 0.346$, respectively) (Fig. 3a). These results show that the S-morph maintains the self-incompatibility, but the morph-incompatibility broke down to some extent. In the case of the M- and L-morphs a complete breakdown of the incompatibility system was observed. No significant differences were observed in the mean number of seeds per fruit among pollination treatments and morphs ($F_{2,116} = 0.94$, $P = 0.393$ and $F_{2,116} = 0.31$, $P = 0.736$, respectively) (Fig. 3b).

Discussion

Reproduction is a key factor for the successful establishment and spread of invasive species and their reproductive strategies will affect population genetic structure, demography and biogeography and, ultimately determine the opportunities for the evolution of local adaptation and the invasion process itself (Barrett 2011). In the particular case of heterostylous species, founder events have major ecological and evolutionary consequences through the loss of morphs during introduction (Barrett and Forno 1982; Morgan and Barrett 1988; Eckert and Barrett 1992; Wang et al. 2005) and this scenario can be preserved over long periods (Ornduff 1987; Eckert and Barrett 1995).

Oxalis pes-caprae, a noxious weed throughout the Mediterranean regions of the world, has been described to reproduce solely through asexual means in the invaded regions, as the result of loss of mating partners after the introduction of the 5x S-morph only (Symon 1961; Michael 1964; Baker 1965; Ornduff 1987; Castro et al. 2007). However, the L-morph has recently been detected in the western Mediterranean basin (Castro et al. 2007). Indeed, our reassessment and methodical sampling of floral morph and cytotype distribution patterns and natural reproductive success revealed surprising results. The 5x S-morph remains the dominant morph in this invaded area, as previously described (Castro et al. 2007); but 4x S-morph individuals (previously described as 5x, only, for most invaded regions), M-morph and trimorphic populations were detected for the first time, as well as a high number of dimorphic populations composed of the S- and L-morphs, randomly scattered across the western Mediterranean basin. Furthermore, contrary to the results obtained by Castro et al. (2007) where no fruit and seed production was detected in a large scale
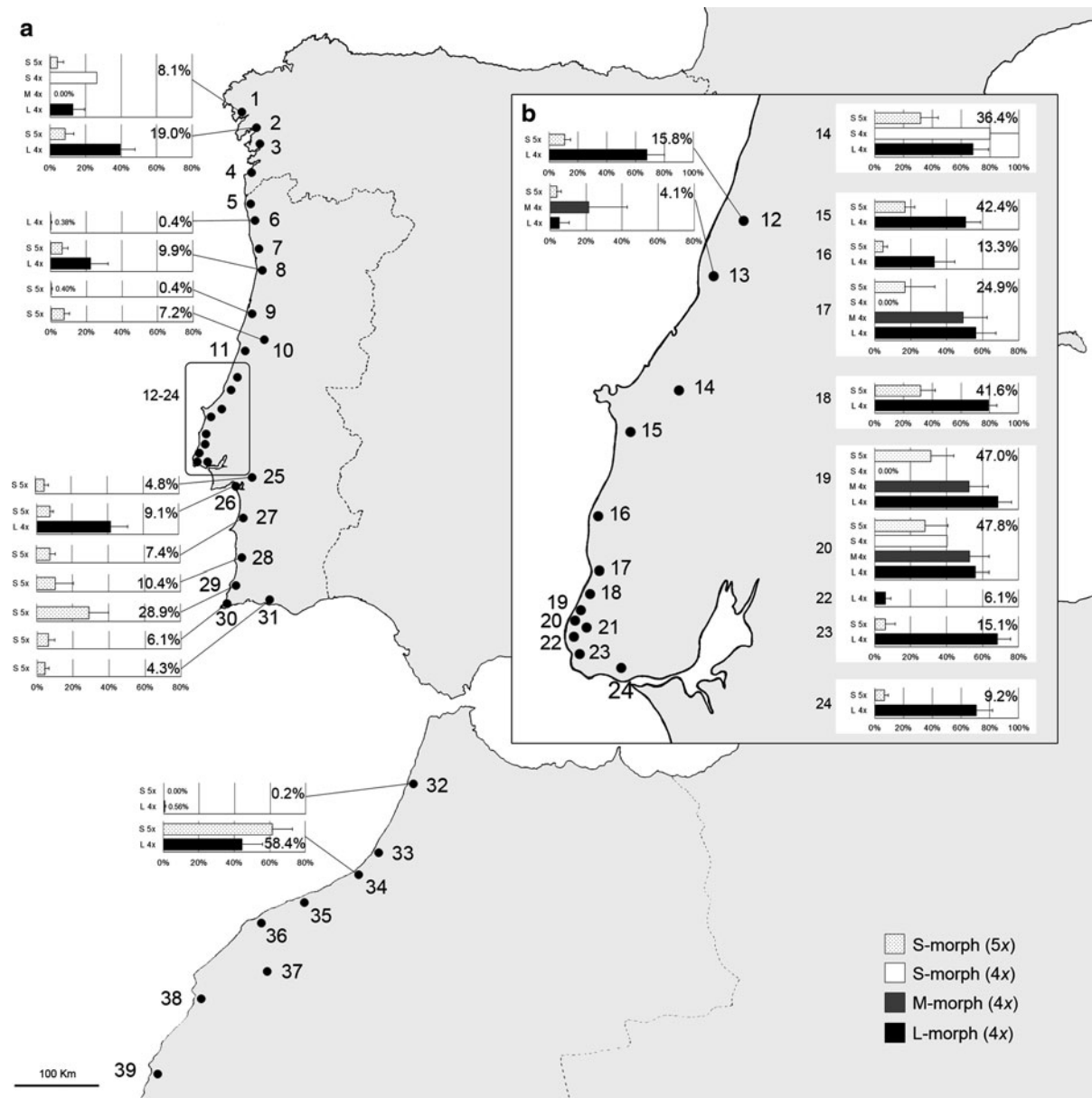
Fig. 2 *Oxalis pes-caprae* sexual reproductive success in the invaded range of the western Mediterranean basin: **a** fruit set in the populations surveyed; **b** detail of the Estremadura region, Portugal. Population localities are according to Appendix 1 of ESM. Populations without graphs had no fruit set (except for population 21 that was lost due to anthropogenic activities). Fruit set (percentage of flowers that developed in fruit) is given as mean and standard error of the mean for each morph and cytotype combined. The reproductive success of the population (sum of the fruit set of each floral morph weighted with its proportion within the population) is presented in the right upper corner of each graph. *Floral morphs*: S, short-styled; M, mid-styled; L, long-styled. *Cytotypes*: 4*x*, tetraploid; 5*x*, pentaploid

screening encompassing 55 populations through the same latitudinal transect, natural fruit and seed production and viable seedlings in variable proportions were observed in 69 % of the populations sampled in the present study. This constitutes the first report of sexual reproduction of *O. pes-caprae* in this invaded range.

The complex distribution patterns of floral morphs and cytotypes observed in this study may result from different processes, not mutually exclusive. The new

Table 1 Summary of the reproductive success of each floral form and cytotype in the invaded range of the western Mediterranean basin

Plant	Fruit set (%)	Seed production	Seed abortion	Seed germination (%)	Ploidy of the offspring
5x S-morph	9.1 ± 1.1 % (343)[a]	1.5 ± 0.1 (97)[a]	1.7 ± 0.2 (97)[a]	25.3 ± 4.3 % (70)[a]	4x (11), 5x (4)
4x S-morph	37.8 ± 15.7 % (6)[a,b]	2.5 ± 0.7 (4)[a,b]	2.6 ± 1.1 (4)[a,b]	37.0 ± 21.4 % (4)[a,b]	4x (6)
4x M-morph	47.7 ± 6.4 % (25)[b]	4.7 ± 0.7 (21)[b]	4.3 ± 0.9 (21)[b]	60.2 ± 8.3 % (21)[b]	4x (1)
4x L-morph	42.5 ± 2.8 % (163)[b]	3.3 ± 0.3 (121)[b]	7.6 ± 0.5 (121)[c]	44.7 ± 3.5 % (98)[b]	4x (22), 5x (14), an. (1)
Statistical test	$F_{3,537} = 32.40$, $P < 0.001$	$F_{3,243} = 10.54$, $P < 0.001$	$F_{3,243} = 27.32$, $P < 0.001$	$F_{3,191} = 5.38$, $P = 0.002$	–

Floral morphs: S-morph, short-styled morph; M-morph, mid-styled morph; L-morph, long-styled morph. Cytotypes: 4x, tetraploid; 5x, pentaploid, an. aneuploid. Values are given as mean and standard error of the mean followed by the sample size in parenthesis. Fruit set and seed germination are given in percentage. Different letters reveal significant differences among cytotype and floral morphs combined
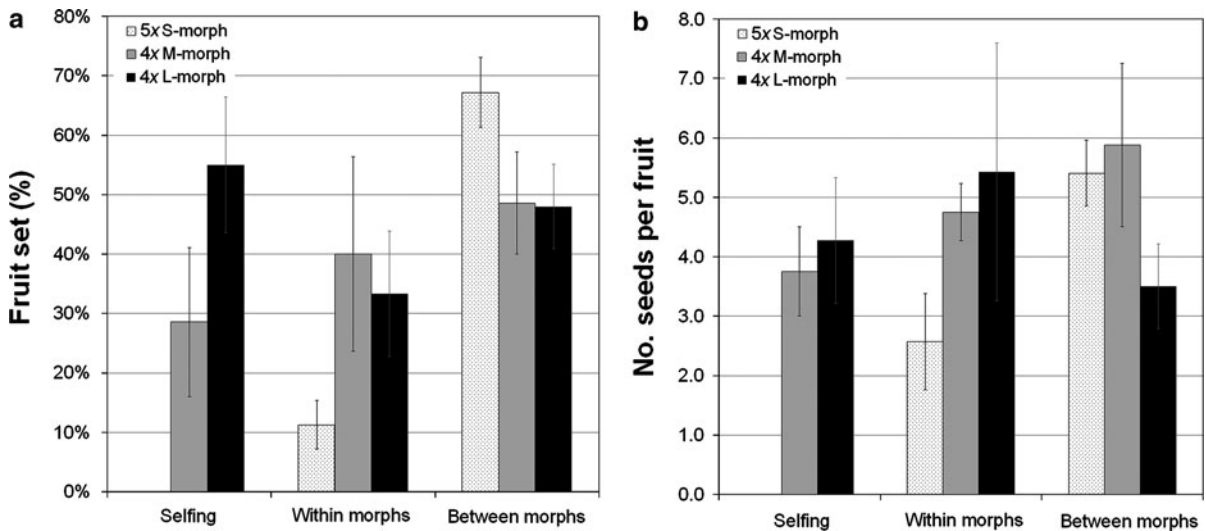
Fig. 3 *Oxalis pes-caprae* incompatibility system: **a** fruit set (percentage of flowers that developed into fruit) and **b** mean number of viable seeds per fruit after selfing, within morph and between morph legitimate pollinations for the three floral forms may be the result of multiple introductions in space and time and/or of newly arisen forms in the invasive range after sporadic reproductive events of the 5x S-morph (supposedly, the only morph introduced in the region studied until very recently). Also, one cannot exclude the possibility that this variability has passed undetected in previous samplings, although it is the most unlikely hypothesis.

morphs (S-, M- and L-morph). S-morph were all pentaploid (5x), M- and L-morph were all tetraploid (4x). Values are given as mean and standard error of the mean

The dominance of the 5x S-morph may be the result of the introduction history of *O. pes-caprae* in this region combined with its reproductive strategies. The self- and morph-incompatibility system (operating at least until very recently) that prevented sexual reproduction (Ornduff 1987; Castro et al. 2007), combined with the profuse clonal reproduction after human activities (Pütz 1994; Vilà et al. 2006), contributed to the maintenance and spread of the 5x S-morph (possibly, the first and only morph introduced; Michael 1964) across this region.

Concerning the newly observed morphs (L- and M-morphs) the spatial analyses provided useful clues regarding its generating processes. Following the statistical approach of Legendre and Fortin (1989), if the proportion of a given floral morph follows a

gradient, this could be related to a single change in one population with subsequent expansion through the rest of the populations. If, in contrast, no pattern is found, it would point to several independent events of emergence of such a floral morph. The spatial distribution of L- and M-morphs followed different patterns. The L-morph appears to occur randomly across the surveyed area, with no distance-based pattern in its proportion, except in the Estremadura province where trimorphic populations are dominant. The M-morph, in contrast, is concentrated mainly in Estremadura province, where its proportion follows a small scale distance-based pattern.

The observed random distribution patterns of the L-morph together with the occurrence of sexual reproduction in natural populations (especially in monomorphic 5x S-morph populations) and in illegitimate crosses, point to a random breakdown in the incompatibility system of the 5x S-morph at several points across the surveyed range. Despite the presence of a strong sporophytic heteromorphic incompatibility system in the native range of *O. pes-caprae* (Ornduff 1987), in the invaded area sporadic events of sexual reproduction of the 5x S-morph have not been completely excluded (Vignoli 1937; Ornduff 1987; Ater 2005; Castro et al. 2007) and sporadic pollen tube development has been observed in intra-morph pollinations between 5x S-morph plants from this region (Castro et al. 2007). The results from illegitimate crosses obtained in the present study show that the self-incompatibility system is still operating in the S-morph in the population studied, as no fruit and seed production were observed after self-pollinations. However, in contrast, intra-S-morph crosses produced viable offspring, showing a breakdown in the morph-incompatibility system of *O. pes-caprae* in populations from central Portugal (the epicentre of trimorphic populations), that may actually be occurring randomly over a wider range. The remaining two morphs showed a complete disruption of the incompatibility system, resulting in similar offspring production after legitimate and illegitimate pollinations. Contrarily to homomorphic systems where incompatibility occurs always at the same level, in heterostylous species, pollen tube inhibition after illegitimate crosses frequently differ between morphs (Dulberger 1992). The breakdown of incompatibility systems has been documented in some heterostylous species (e.g. Ornduff 1972; Barrett 1979; Colautti et al. 2010) and in some

cases, differences in self-compatibility between morphs have also been observed (Charlesworth 1979; Kohn and Barrett 1992; Colautti et al. 2010). The studies performed so far in tristylous species indicate that the M-morph is more susceptible to self-pollination and displays higher selfing rates than the L- and S-morphs (Charlesworth 1979; Kohn and Barrett 1992; Colautti et al. 2010). In *Oxalis*, a different pattern was observed with L-morph tending to be more self-compatible then the M-morph. However, the genetic and physiological mechanisms involved in changes of trimorphic incompatibility system are yet poorly understood. The acquisition of compatibility increases the number of mating partners within the population and has major implications for the population dynamics (Ray and Chisaki 1957; Ganders 1979; O'Brien and Calder 1989).

In most of the *Oxalis* species studied, the modes of inheritance of tristyly consists in two diallelic factors in which the S-morph is dominant for the *S* loci independently of the *M* loci (*Ssmm*, *SsMm*, *SsMM*), whereas the L-morph is recessive for both loci (*ssmm*; M-morph: *ssMm*, *ssMM*) (Lewis and Jones 1992). Under this scenario, events of sexual reproduction involving a 5x S-morph heterozygous at the *M* locus could be one of the mechanisms by which L- and M-morphs are generated in this region. In addition, as the 5x S-morph is able to produce viable gametes (results herein and Costa et al. 2013) it can also be the origin of 4x cytotypes after the fusion of two 2x gametes. Indeed, in the literature, despite of the frequent meiotic abnormalities, plants with odd ploidy levels (e.g. 3x, 5x) were shown to produce viable gametes with 1x, 2x, 3x, 4x and/or 5x ploidy levels (Ramsey and Schemske 1998; Risso-Pascotto et al. 2003). Still, multiple introduction events of the tetraploid cytotype (considered weedy in most regions of the native range; Krejčíková et al. 2013) from the native area, and possibly followed by soil movements in some invaded regions (e.g. across the side of the roads) cannot be completely ruled out. To address these hypotheses, the patterns of colonization are currently being investigated using molecular markers.

The different rates of reproductive success along the surveyed area also revealed a complex pattern. We identified monomorphic 5x S-morph populations unable to produce fruits, monomorphic 5x S-morph populations with variable degrees of natural fruit set (most probably related with different degrees of

incompatibility) and dimorphic and trimorphic populations with significantly higher reproductive success. The occurrence of new floral morphs and cytotypes may have important consequences for the sexual reproductive success, and thus, for the dynamics and genetic structure of invasive populations of *O. pes-caprae*. The unbalanced morph proportion observed in di- and trimorphic populations most likely result from the combined influence of founder effects, clonal growth (Mulcahy 1964; Barrett 1980; Morgan and Barrett 1988; Barrett 1992; Eckert and Barrett 1993), differences in the reproductive success among the floral morphs (Heuch 1979; O'Neil 1992; Arroyo et al. 2002; Hodgins and Barrett 2008) and tristyly inheritance patterns. In particular, the S-morph might be in disadvantaged due to the predominance of 5x plants with lower sexual reproductive success, if its asexual reproduction is similar or lower than the other morphs. Differences in sexual and asexual reproductive success are currently being investigated to evaluate the overall success of each morph.

Concluding remarks

The present study reports a remarkable diversity in floral morph and cytotype distribution patterns of *O. pes-caprae* in the western Mediterranean basin, with successful sexual reproduction in several localities across the surveyed area. The distribution patterns of floral morphs and cytotypes, and sexual vs. asexual populations were shown to be complex, revealing that populations of this species might be currently highly dynamic. The ability to reproduce sexually (undetected in previous studies) may have important impacts in the biology of this species and major consequences for its adaptation and selection potential in this invasive area. Invasive populations of *O. pes-caprae* are thus excellent systems to address numerous evolutionary questions under a contemporary timescale, namely the study of the evolution of heterostyly and incompatibility and the role of sexual vs. asexual reproduction, and their impacts in species adaptation. These topics are currently being address through pollination experiments, extensive field sampling and genetic analyses in a broader research network.

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