

Research article

Variation in the incompatibility reactions in tristylous *Oxalis pes-caprae*: large-scale screening in South African native and Mediterranean basin invasive populations



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ABSTRACT

Establishment and spread of organisms after long-distance dispersal often involve changes to the mating systems. Here, we tested for increased compatibility under a mate limitation scenario after long-distance dispersal of the tristylous *Oxalis pes-caprae*, a South African geophyte invasive in regions with Mediterranean climate. We investigated variation in the expression of the trimorphic incompatibility system in plants from 16 native populations covering the entire distribution range in South Africa, and 18 invasive populations from the western Mediterranean basin by performing controlled hand-pollinations. After legitimate pollinations, native plants had higher fruit and seed set than invasives, while after illegitimate pollinations (self- and intramorph pollinations), invasives were more successful than natives. We detected morph-specific differences in the weakening of the incompatibility system and within-population variability in the expression of the incompatibility reactions for invasive plants. Additionally, the offspring obtained after illegitimate pollinations was able to germinate, survive and flower under glasshouse conditions. Our results provide experimental evidence for differences in the expression of the incompatibility system between native and invasive populations of *O. pes-caprae* after introduction and under strong compatible mate's limitation. The weakening in the incompatibility system reported here for the L- and M-morphs might possibly constitute an additional strategy for reproductive assurance in the invaded range of the Mediterranean basin.

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1. Introduction

Colonization after long-distance dispersal often involves the successful establishment of a new population founded by a limited number of individuals. After introduction into an unoccupied habitat by that species, colonizer individuals are subjected to a demographic sieve, i.e., the interplay between a reduced population size, mate limitation and reproductive mode, that will determine if single individuals or small colonies are able to successfully establish in the new environment (Baker, 1955, 1967; Stebbins, 1957; reviewed by Pannell et al., 2015). Species with mating systems that confer some level of reproductive assurance during colonization of novel habitats (e.g., Sutherland, 2004; Pyšek and Richardson, 2007; van Kleunen et al., 2010; Moravcová et al., 2015) are in advantage, as

they will be able to persist and establish a new colony (Baker, 1955, 1967; Stebbins, 1957). Since the reproductive strategy of a species, among others, affects the genetic diversity and differentiation of its populations (Fryxell, 1957; Schoen and Brown, 1991; Hamrick and Godt, 1996), it strongly influences the capacity of colonizers to respond to novel and unpredictable environmental fluctuations, as well as, the opportunities for local adaptation after establishment (García-Ramos and Rodríguez, 2002; Barrett, 2011).

Reproductive assurance under mate limitation during colonization is guaranteed by the predominance of asexual over sexual strategies via apomixis (Amsellem et al., 2001) or clonality (e.g., Hollingsworth and Bailey, 2000; Wang et al., 2005; Ferrero et al., 2015), and by the transition from outcrossing to selfing (Petanidou et al., 2012; Ward et al., 2012). For example, some successful invaders have prolific vegetative reproduction (e.g., *Carpobrotus edulis*, Roiloa et al., 2010; *Nymphoides peltata*, Wang et al., 2005; *Salvinia molesta*, Oliver, 1993), which has been involved in their successful establishment and spread in the non-native ranges.

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Also, several studies describing the mating system of invasive plant species in their non-native ranges provided evidence for the dominance of self-compatibility over self-incompatibility when invasive species were compared with species from the communities being invaded (Rambuda and Johnson, 2004; Sutherland, 2004; van Kleunen and Johnson, 2007; Harmon-Threatt et al., 2009; Hao et al., 2011). Increased self-compatibility was also shown to be higher in introduced naturalized species when compared with related non-naturalized ones (van Kleunen et al., 2008; but see Sutherland, 2004; Burns et al., 2011). To evaluate shifts in the mating systems during colonization, it is necessary to simultaneously compare the reproductive biology of a species in its native and introduced areas (Barrett, 2011). However, this approach has been seldom explored and only a few studies have gathered data from both ranges (Petanidou et al., 2012; Ward et al., 2012).

The Bermuda buttercup (*Oxalis pes-caprae* L., Oxalidaceae) is a polyploid, highly clonal South African geophyte (2x, 4x and 5x cytotypes; Ornduff, 1987; Castro et al., 2007, 2013; Turketti, 2010) that was introduced to regions with Mediterranean climate, where it became a widespread invasive (Michael, 1964; Ornduff, 1987; Vilà et al., 2006; Castro et al., 2007, 2013; Signorini et al., 2013). *Oxalis pes-caprae* is tristylous, and native populations are composed by the three style morphs (long-, mid- and short-styled morphs, hereafter L-, M- and S-morph, respectively) associated with a trimorphic self-incompatibility system (hereafter TSI; Ornduff, 1987; Turketti, 2010) that prevents self- and intramorph fertilizations (Barrett, 1993; Barrett and Shore, 2008). Contrarily, most invaded areas worldwide were colonized by the 5x S-morph, which successfully spread through asexual reproduction (Michael, 1964; Baker, 1965; Ornduff, 1987; Castro et al., 2007, 2013). In the Mediterranean basin, most populations are composed by the 5x S-morph only, and the occurrence of 4x plants of the three style morphs is quite restricted, particularly the 4x S-morph (Castro et al., 2013). The patterns of style morph composition of the populations in this area resulted from multiple introductions from the native range of all three style morphs (Ferrero et al., 2015). However, the timing of introduction of each style morph is unknown (Ferrero et al., 2015), thus raising several questions about the selective pressures that each style morph has been subjected to after its introduction and the mechanisms involved in the current structure of the populations.

The opportunities for sexual reproduction of *O. pes-caprae* in the Mediterranean basin are reduced because of compatible mate's limitation, which is caused by the under-representation of reciprocal style morphs (L- and M-morphs), and by the dominance of a predominantly asexual pentaploid cytotype across the entire region (Castro et al., 2007, 2013). Notwithstanding, *O. pes-caprae* effectively integrated the pollination networks (Ferrero et al., 2013) with successful pollen flow in invasive populations of the western Mediterranean, regardless of morph composition (Costa et al., 2016). In addition, preliminary observations pointed to a weakening in the incompatibility reactions under experimental conditions (Castro et al., 2013; Costa et al., 2014), and some seed production was reported in several invasive populations (Castro et al., 2013; Costa et al., 2016), although it remains unclear how frequent is the recruitment from seed in this area. Altogether, the available data suggests the occurrence of changes in the TSI of *O. pes-caprae* in the invaded area of the Mediterranean basin.

Here, we tested for geographical differences in the TSI between native and invasive populations of *O. pes-caprae*, which might suggest evolutionary transitions in the mating system during invasion. We addressed the following specific questions: (1) Do native and invasive populations differ in the strength of the TSI? We predicted that native populations would have a strong TSI, while invasive populations would show a weakening in the strength of the TSI. This prediction is based on preliminary observations of fruit and

seed production after incompatible hand-pollinations (Castro et al., 2013; Costa et al., 2014), and of natural seed production under a scenario of strong compatible mate limitation (Castro et al., 2013; Costa et al., 2016). (2) Is there evidence of a style morph specific incompatibility weakening in the invaded area? Given the high sterility of the 5x S-morph, we hypothesized that some level of compatibility would be particularly advantageous for the 4x morphs as an additional mechanism for reproductive assurance. (3) Is the strength of the TSI variable among individual plants within native and invaded areas? We expected to detect natural variation in the strength of the incompatibility system. (4) Does the offspring sired after illegitimate pollinations (i.e., intramorph and self-pollinations) reach the adult stage? We hypothesized that the offspring sired after illegitimate pollinations should reach the adult stage and flower in order to be able to contribute to the recruitment from seed in natural populations (even if its frequency is unknown). To answer these questions, we compared the strength of the TSI between native and invasive populations of *O. pes-caprae* by setting up a controlled hand-pollination experiment under common garden conditions with plants from 16 native and 18 invasive populations covering the entire native distribution range and the western Mediterranean region, respectively.

2. Materials and methods

2.1. Study species and populations

Oxalis pes-caprae is a geophyte that can grow up to 40 cm high (Pedraja, 2015), and has a great capacity of asexual reproduction via the production of numerous bulbs associated with the contractile capacities of its roots (Pütz, 1994). Every year, *O. pes-caprae* plants produce a basal rosette of leaves and yellow tristylous flowers arranged in terminal umbellate cymes (Pedraja, 2015). In the native range, it flowers from May to August (Dreyer et al., 2006), while in the invaded area of the Mediterranean basin, flowering occurs from December to April (Castro et al., 2007).

Plants used in the controlled hand-pollinations were obtained by growing bulbs previously collected in South Africa and in the Mediterranean basin between 2010 and 2012. In the field, style morph composition was assessed in each population by recording the floral morph of at least 100 plants along 2–3 longitudinal transects across the entire population. Sampled plants were at least 5 m apart to avoid resampling the same genet (Castro et al., 2007, 2013). Data on style morph composition is provided in Table 1. Natural fruit production was assessed by sampling one infructescence from 10 plants per style morph in the populations (Table 1). Bulbs from 16 native populations (including 4x L-, 4x M- and 4x S-morphs) and 18 invasive populations (including 4x L-, 4x M- and 4x S-morphs and the 5x S-morph; Table 1) were planted in 2 L plastic pots (11 × 11 cm wide, 21 cm depth) filled with common garden substrate during the autumn 2010–2012. Plants were maintained outdoors under uniform conditions at the Botanical Garden of the University of Coimbra and were protected from pollinators with a nylon mesh.

2.2. Ploidy level assessment

We confirmed the ploidy level of each plant used in the pollination experiment by flow cytometric analyses of fresh leaves. Nuclei were isolated following the procedure of Galbraith et al. (1993) by chopping 1 cm² of leaf tissue of *O. pes-caprae* and 1 cm² of leaf tissue of *Solanum lycopersicum* 'Stupické' (internal reference standard with 2C = 1.96 pg; Doležel et al., 1992) in 1 mL of WPB buffer (Loureiro et al., 2007). The nuclear suspension was filtered using a 50 µm nylon mesh and 50 µg ml⁻¹ of propidium iodide (PI, Fluka,

Table 1

Oxalis pes-caprae invasive and native populations studied, number of plants involved in the controlled pollination experiment, number of crosses performed, style morph frequency in natural populations, and natural fruit set. Detailed information on cytotype composition and fruit set for each sampled population from the invaded area is given in Castro et al. (2013). Data on fruit set from native range available from Ferrero et al. (2015), except the ones marked with an asterisk that correspond to unpublished data from the authors.

Population	Geographical coordinates		n		Style morph (%)				Fruit set (%)	
			Plants	Crosses	L	M	S (4x)	S (5x)		
Native range: South Africa										
P1	ZA: Yzerfontein	33°20.979'S	18°09.302'E	5	16	32	22	46	0	85.0
P4	ZA: Lamberts Bay	32°11.517'S	18°19.924'E	10	27	28	26	46	0	84.0
P17	ZA: Worcester	33°33.671'S	19°54.072'E	17	37	40	37	22	0	83.0
P19	ZA: Suurbraak	34°03.469'S	20°35.526'E	5	18	53	16	32	0	47.5*
P21	ZA: Barrydale	33°47.247'S	21°08.652'E	12	34	37	23	40	0	60.0
P22	ZA: Oudtshoorn	33°32.827'S	21°50.612'E	8	18	39	56	5	0	84.0
P23	ZA: Mossel Bay	34°05.665'S	22°03.400'E	11	26	20	51	29	0	83.8*
P24	ZA: Gouritsmond	34°17.703'S	21°49.356'E	11	28	30	38	32	0	73.0
P25	ZA: Stilbaai	34°21.241'S	21°25.003'E	4	16	21	41	38	0	57.1*
P26	ZA: Witsand	34°15.118'S	20°59.556'E	11	33	24	41	35	0	50.5*
P27	ZA: Bredasdorp	34°18.129'S	20°12.213'E	9	29	33	21	46	0	57.9*
P28	ZA: L'Agulhas	34°41.391'S	20°01.198'E	11	49	24	24	52	0	61.0
P30	ZA: Standford	34°27.371'S	19°35.046'E	5	18	36	3	60	0	77.3*
P31	ZA: Caledon	34°10.961'S	19°24.159'E	13	48	8	11	81	0	40.9*
P32	ZA: Botrivier	34°13.400'S	19°11.994'E	9	35	26	70	4	0	63.0*
P33	ZA: Cape Point	34°09.413'S	18°26.100'E	15	38	15	76	8	0	54.0
				156	470					
Invaded range: Western Mediterranean basin										
4.	SP: Baiona	42°06.747'N	8°49.636'W	12	47	0	0	0	100	0.0
6.	PT: Belinhos	41°35.704'N	8°47.130'W	8	23	100	0	0	0	0.4
7.	PT: Lavras	41°14.436'N	8°42.871'W	8	26	0	0	0	100	0.0
8.	PT: São Pedro da Maceda	40°55.264'N	8°39.625'W	17	39	22	0	0	78	9.9
9.	PT: Praia de Mira	40°27.257'N	8°46.756'W	8	23	0	0	0	100	0.4
10.	PT: Coimbra	40°12.363'N	8°25.431'W	13	57	0	0	0	100	7.2
11.	PT: Vieirainhos	40°00.163'N	8°48.341'W	9	26	0	0	0	100	0.0
12.	PT: Marinha Grande	39°44.403'N	8°56.063'W	15	40	9	0	0	91	15.8
16.	PT: Casais da Areia	39°03.651'N	9°23.934'W	15	49	31	0	0	69	13.3
20.	PT: Colares I	38°48.015'N	9°28.061'W	81	254	50	22	3	25	47.0
21.	PT: Colares II	38°47.863'N	9°28.577'W	52	166	63	18	4	15	47.8
19.	PT: Colares III	38°48.752'N	9°28.394'W	38	133	39	13	9	39	NA
26.	PT: Tróia	38°29.495'N	8°54.386'W	10	30	5	0	0	95	9.1
27.	PT: Melides	38°07.843'N	8°46.961'W	12	45	0	0	0	69	7.4
28.	PT: Almogrove	37°38.885'N	8°47.320'W	13	39	0	0	0	90	10.4
31.	PT: Armação de Pêra	37°04.856'N	8°17.201'W	15	48	0	0	0	81	4.3
32.	MA: Moulay-Bousselham	34°52.542'N	6°17.831'W	13	36	31	0	0	69	0.2
39.	MA: Essaouira	31°29.722'N	9°45.638'W	3	14	4	0	0	96	0.0
				342	1095					

Style morphs: L, M and S for long-styled, mid-styled and short-styled morphs, respectively. Cytotypes: 4x, tetraploid; 5x, pentaploid. Countries: ZA, South Africa; SP, Spain; PT, Portugal; MA, Morocco. NA, information not available. Style morph frequency and fruit set in natural populations are given in percentage (%).

Buchs, Switzerland) was added to stain the DNA. To avoid staining of double stranded RNA, 50 µg ml⁻¹ of RNase (Fluka, Buchs, Switzerland) was also added. Samples were analysed in a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec GmbH., Görlitz, Germany) for PI excitation. We followed the pooled sample strategy, and leaflets from 5 individuals were analysed simultaneously (Kolář et al., 2009); when several peaks were obtained, individual samples were prepared to assign the ploidy levels to each sampled plant. As a quality standard, we only considered histograms with a coefficient of variation below 5%. By dividing the *O. pes-caprae* G₁ peak mean by that of *S. lycopersicum*, we obtained the DNA index. For DNA index values (mean ± SD) of 0.70 ± 0.03, plants were scored as tetraploids, while for values of 0.86 ± 0.02, plants were scored as pentaploids (Castro et al., 2007).

2.3. Incompatibility system assessment

Controlled hand-pollinations were carried out between 2011 and 2014, during the winter months corresponding to the species flowering period (December–April) at the latitude of the experimental garden. Before conducting the pollination experiment, plants were grown for one generation to avoid potential mater-

nal effects and, despite some differences in flowering phenology between ranges were detected, the flowering period of native and invasive plants largely overlapped. Details on the number of plants used per population and the total number of pollinations performed are given in Table 1. The following pollination treatments were performed (Fig. 1): intermorph pollinations (i.e., legitimate pollinations), intramorph and self-pollinations (i.e., illegitimate pollinations). An additional non-manipulated flower per plant was used as control for pollen contamination, and no fruit and seed production were obtained in this treatment. Each plant involved in the study received all pollination treatments; in some cases, with several replicates, while in other plants it was not possible to completely follow this approach due to limited pollen availability. Except for the selfing treatment, all recipient flowers were emasculated before hand-pollinations to avoid stigma contamination with self-pollen. Pollinations were done by gently rubbing anthers against the recipient stigmas using fine forceps. Intermorph and intramorph pollinations were performed using anthers from three distinct individuals from the same population. The pollination experiment is illustrated in detail in Fig. 1. However, due to mate limitation in monomorphic invasive populations, intermorph pollinations of the 5x S-morph were performed using reciprocal pollen donors (4x morphs) from other invasive populations. Legit-

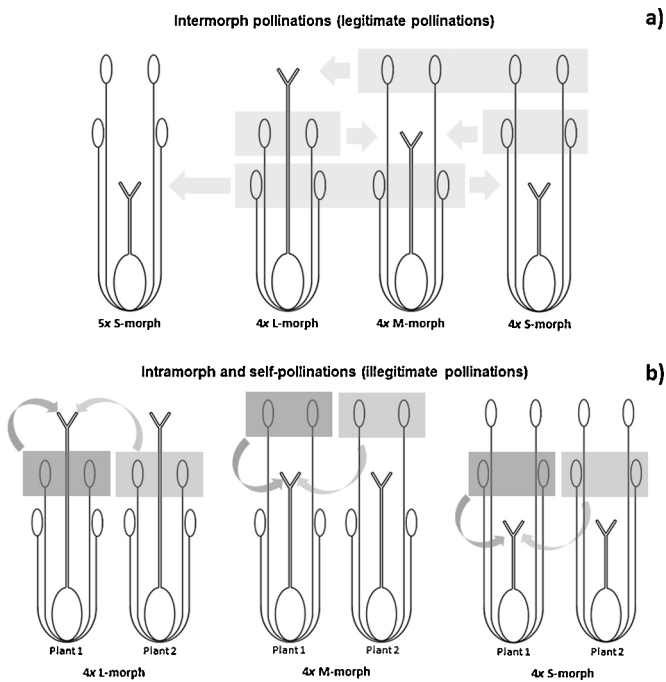


Fig. 1. Illustration of the experimental design followed in this study: (a) intermorph pollinations, i.e., legitimate pollinations – involved 4x reciprocal floral morphs as pollen donors with 4x L-morph flowers crossed with pollen from long-level anthers of the 4x S- and 4x M-morphs, 4x M-morph flowers crossed with pollen from mid-level anthers of the 4x L- and 4x S-morphs, and both 4x and 5x S-morph flowers crossed with pollen from short-level anthers of the 4x L- and 4x M-morphs; (b) intramorph and self-pollinations, i.e., illegitimate pollinations – L- and S-morphs were pollinated with pollen from the closest anther level, i.e., the mid-level, while for the M-morph, pollen from long-level anthers was used (here exemplified for the 4x morphs, but the pollinations of the 5x S-morph followed the same methodology).

imate pollinations always involved 4x reciprocal floral morphs as pollen donors. Specifically, we cross-pollinated 4x L-morph flowers with pollen from long-level anthers of the 4x S- and 4x M-morphs, 4x M-morph flowers with pollen from mid-level anthers of the 4x L- and 4x S-morphs, and both 4x and 5x S-morph flowers with pollen from short-level anthers of the 4x L- and 4x M-morphs (Fig. 1a). For illegitimate pollinations of the L- and S-morphs, we used pollen from the closest anther level, i.e., the mid-level, while for the M-morph, pollen from long-level anthers was used (Fig. 1b). Pollination treatments were labeled with cotton lines of different colors laced around the flower pedicel. Since *O. pes-caprae* capsules are dehiscent, fruits were bagged to prevent seed losses, and fruit and seed production were recorded when mature. Fruit set per pollination treatment was calculated as the percentage of pollinated flowers that developed into fruits. Seed set per pollination treatment was calculated as the mean number of seeds produced per fruit. A measure of reproductive success was calculated per individual and pollination treatment by multiplying fruit set by seed production.

Ten flowers from distinct individuals per floral morph and ploidy level were used to estimate the mean number of ovules for native and invaded areas. Estimations were made under fluorescence microscopy following the procedure described in Dafni et al. (2005). The obtained values were then used to calculate several reproductive indices described below.

2.4. Reproductive indices

Outcrossing (OUT), morph-compatibility (MC) and self-compatibility (SC) measures were calculated. An outcrossing measure was obtained for each plant by calculating the seed/ovule

(S/O) ratio after legitimate pollinations. Morph- and self-compatibility indices were obtained for each plant by dividing the S/O ratio of intramorph and self-pollinations, respectively, by the S/O ratio after intermorph pollinations (modified from Lloyd and Schoen, 1992). The compatibility indices varied between 0 (incompatible) and 1 (full compatible). Three criteria were established before the calculation of the indices: first, for individuals in which no legitimate pollination was made, the average outcrossing S/O ratio of the population was used to calculate the compatibility indices; second, if the S/O ratio of self- and/or intramorph pollinations were larger than the S/O of legitimate pollinations (resulting in indices larger than 1), the plant was scored as full compatible (i.e., 1); lastly, when there were several replicates of a given treatment in one plant, average outcrossing, morph-compatibility and/or self-compatibility measures were calculated per individual and these average values were used in subsequent statistical analysis.

2.5. Fitness of illegitimate offspring

To evaluate if seeds obtained after illegitimate crosses (i.e., intramorph and self-pollinations) were able to germinate and produce viable plants, we studied the fitness of the offspring obtained after legitimate and illegitimate crosses of the invasive populations. Seeds obtained in the first season (2011–2012) were sown in the following season (2012–2013) in 1 L plastic pots (8.6 × 8.6 cm wide, 21 cm depth) filled with standard soil, one seed per pot. Germination was assessed weekly during the first two months and plant survival and flowering assessed in the peak flowering. The 4x S-morph plants did not sire any seeds after illegitimate crosses and thus, these were not included in this experiment.

2.6. Statistical analysis

Descriptive statistics, i.e., mean and standard error, were calculated for the number of ovules, fruit set, seed set, reproductive success and incompatibility indices for each style morph from native and invaded areas. Given that two ploidy levels are described for the S-morph in the invaded area of the Mediterranean basin (Castro et al., 2007, 2013), style morph and cytotype were combined and designated as “form”, as follows: 4x L-morph, 4x M-morph, 4x S-morph and 5x S-morph.

Differences in fruit set, seed set and reproductive success among forms, pollination treatments and areas (i.e., native and invaded) and the interaction between the latter two were investigated by means of mixed-effects models (hereafter GLMM) or generalized linear models (hereafter GLM). In both cases, a binomial distribution with a logit link function was used for fruit set and a Poisson distribution with a log link function was used for seed set and reproductive success. In all statistical analyses, individual and population variability were included in GLMMs as random factors. However, when the estimated variance for these factors was smaller than the residuals, they were removed from the analysis and GLMs were used instead (Bolker et al., 2009). Form, pollination treatment and area were specified as fixed effects in the models. Because the interaction between pollination treatment and area was significant, we explored the differences between areas for each pollination treatment and the differences within areas among pollination treatments separately, accounting with form in both, and following the procedure described above. When significant differences were obtained, post-hoc tests for multiple comparisons were conducted afterwards.

Before calculation of the reproductive measures, we assessed differences in ovule number between regions and among style morphs using one-way ANOVA Type-III analysis of variance. Since no differences in mean ovule number (mean ± SE; species: 39.85 ± 0.87; invaded range: 39.60 ± 0.98, native range:

41.35 ± 1.72) between regions ($F_{1,135} = 0.500$, $P = 0.481$), among style morphs ($F_{2,134} = 0.015$, $P = 0.985$) and between cytotypes in the invaded area ($F_{2,114} = 0.528$, $P = 0.591$) were found, we used the average value for the calculations. The values of the outcrossing measure and of the compatibility indices were transformed with the $\log_{10}(x+2)$ before all statistical analysis (Zar, 2010). Differences in the outcrossing measure and compatibility indices between areas and among forms within areas were investigated using GLMMs or GLMs, with a gamma distribution and a log link function, followed by a Type-III analysis of variance.

Differences in seed germination, plant survival and flowering among forms and between pollination treatments (i.e., legitimate and illegitimate pollinations), and the interaction between the two were investigated with GLMs. We also tested for (a) fitness differences among forms after each pollination treatment separately, and (b) compared offspring fitness for each form between pollination treatments. In all cases, a binomial distribution with a logit link function was used for the three response variables. Form and pollination treatment were specified as fixed factors. When significant differences were obtained, post-hoc tests for multiple comparisons were made afterwards.

All analyses were performed in R software version 3.0.1 (R Core Development Team, 2013), using the packages “car” for Type-III analysis of variance (Fox and Weisberg, 2015), “lme4” for mixed-effects models (Bates et al., 2014), “multcomp” for multiple comparisons after Type-III analysis of variance (Hothorn et al., 2015), and “stats” for generalized linear models (R Core Development Team, 2013).

3. Results

3.1. Effect of the pollination treatment in the reproductive success

Most pollination treatments produced fruits and seeds, even if in low amounts (Fig. 2). Overall, GLMMs analysis revealed a significant effect of pollination treatment in all reproductive variables measured (Fig. 2; Appendix A in Supplementary material). A more detailed analysis within area of origin equally showed a significant effect of pollination treatment in fruit production, seed set and reproductive success (Table 2). Native and invasive plants significantly produced more fruits and seeds after legitimate than after illegitimate pollinations ($P < 0.05$; Fig. 2a–d). Accordingly, reproductive success after intermorph crosses was significantly higher than after intramorph crosses and selfing for plants from both areas of origin ($P < 0.05$; Fig. 2e, f).

3.2. Strength of the incompatibility system in native and invaded ranges

Plants from South Africa produced significantly more fruits after legitimate pollinations than plants from the Mediterranean basin (SA: 0.97 ± 0.01 ; MB: 0.82 ± 0.02 ; Table 3; Fig. 2a, b). The opposite pattern was found for intramorph pollinations (SA: 0.03 ± 0.02 ; MB: 0.19 ± 0.02) and for self-pollinations (SA: 0.04 ± 0.02 ; MB: 0.21 ± 0.03 ; Table 3; Fig. 2a, b), with invasive plants having higher fruit set than natives after illegitimate pollinations. South African plants yielded, on average, more than twice the number of seeds produced by invasive plants after intermorph pollinations (SA: 18.73 ± 0.79 ; MB: 4x plants, 11.13 ± 0.52 and 5x S-morph, 3.95 ± 0.24). No statistically significant differences in seed production were detected between areas for intramorph and self-pollinations (Table 3; Fig. 2c, d).

Overall, native plants had a significantly higher reproductive success after legitimate pollinations than invasive plants (SA: 18.14 ± 0.81 ; MB: 6.60 ± 0.33 ; Table 3; Fig. 2e, f). Contrarily, plants

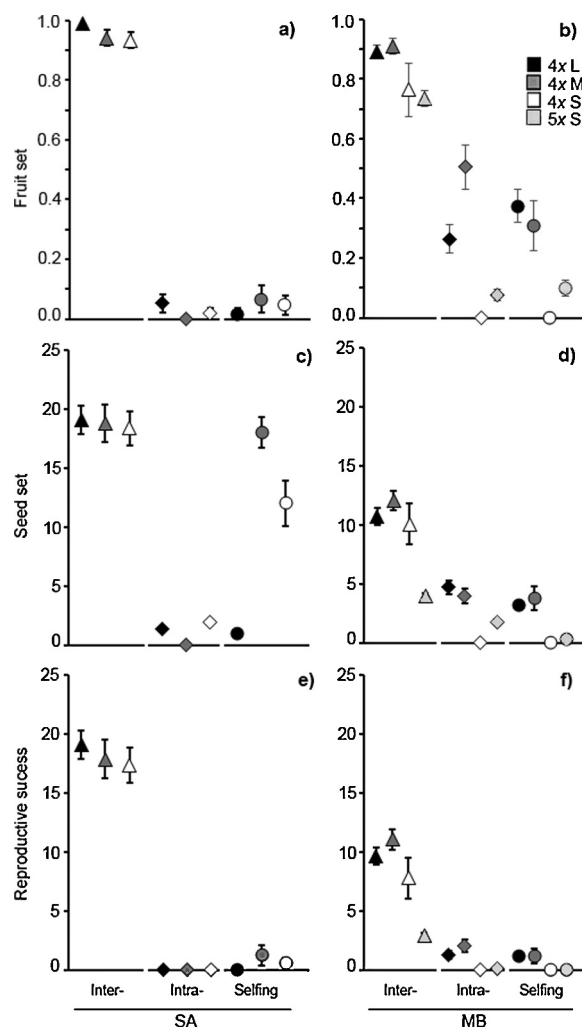


Fig. 2. Reproductive success of *Oxalis pes-caprae* plants from South Africa (SA) and the Mediterranean basin (MB) after hand-pollination treatments: (a–b) fruit set; (c–d) seed set; (e–f) reproductive success. Results are mean and standard error. Triangles, diamonds and circles represent intermorph (inter-) pollinations, intramorph (intra-) pollinations and self-pollinations (selfing), respectively. L, M, and S represent the L-morph, M-morph and S-morph, respectively.

from the Mediterranean basin tended to have a higher reproductive success after intramorph (SA: 0.05 ± 0.02 ; MB: 0.71 ± 0.13) and self-pollinations than natives (SA: 0.48 ± 0.27 ; MB: 0.56 ± 0.121 ; Table 3; Fig. 2e, f).

The patterns of reproductive success described above led to a significant effect of area of origin in all reproductive measures calculated (Table 4). Plants from the native area had a significantly higher OUT measure than plants from the invaded area (SA: 0.58 ± 0.02 ; MB: $0.11 \pm 1E^{-2}$; Fig. 3a), while the MC (SA: $0.002 \pm 1E^{-2}$; MB: 0.09 ± 0.01 ; Fig. 3b) and SC (SA: $0.02 \pm 1E^{-3}$; MB: 0.08 ± 0.01 ; Fig. 3c) indices were found to be significantly higher in plants from invasive populations than from native ones (Table 4).

3.3. Morph-specific differences

A significant effect of form, i.e., the combination of style morph and cytotype as 4x L-, 4x M-, 4x S- and 5x S-morph, was detected in almost all analyses conducted (Tables 2–5). While this factor had no effect in the comparisons within the native range for any of the response variables studied (Tables 3 and 5; Figs. 2 and 3), differences among invasive forms were detected in reproductive variables across all pollination treatments (Table 2; Fig. 2) and

Table 2
Generalized linear model analyses of the effect of pollination treatment, i.e., intermorph, intramorph and self-pollination on the reproductive variables of *Oxalis pes-caprae* for each area separately. Fruit set was analyzed using generalized mixed-effects models. d.f., degrees of freedom. Statistically significant differences are highlighted in bold.

Factors	d.f.	Native area		d.f.	Invaded area		
		Wald χ^2	P		Wald χ^2	P	
		Fruit set					
Pollination treatment	2	11.575	0.003	2	320.679	<0.001	
Form	2	0.008	0.996	3	32.205	<0.001	
		Seed production					
Pollination treatment	2	74.356	<0.001	2	154.038	<0.001	
Form	2	2.396	0.302	3	53.186	<0.001	
		Total reproductive success					
Pollination treatment	2	573.403	<0.001	2	1059.641	<0.001	
Form	2	0.970	0.616	3	52.742	<0.001	

Table 3
Generalized mixed-effects model analyses of the effect of area of origin and form on the reproductive variables of *Oxalis pes-caprae* measured after each pollination treatment separately, i.e., intermorph, intramorph and self-pollinations. ** – a generalized linear model was used instead. d.f., degrees of freedom. Statistically significant differences are highlighted in bold.

Factors	d.f.	Intermorph		d.f.	Intramorph		d.f.	Self	
		Wald χ^2	P		Wald χ^2	P		Wald χ^2	P
		Fruit set							
Area	1	12.563	<0.001	1	11.452	<0.001	1	20.650*	<0.001
Form	3	11.104	0.010	3	25.140	<0.001	3	21.124*	<0.001
		Seed production							
Area	1	420.390*	<0.001	1	2.037	0.153	1	3.596*	0.060
Form	3	633.150*	<0.001	3	7.922	0.048	3	14.161*	<0.010
		Total reproductive success							
Area	1	577.500*	<0.001	1	1.022	0.312	1	0.700	0.403
Form	3	890.360*	<0.001	3	2.163	0.539	3	2.609	0.456

Table 4
Generalized mixed-effects model analyses of the effect of area of origin and form on the reproductive indices calculated for *Oxalis pes-caprae* native (SA, South Africa) and invasive plants (MB, Mediterranean basin) after controlled hand-pollinations. ** – a generalized linear model was used instead. d.f., degrees of freedom. Statistically significant differences are highlighted in bold.

Factors	d.f.	Outcrossing		d.f.	Morph-compatibility		d.f.	Self-compatibility	
		Wald χ^2	P		Wald χ^2	P		Wald χ^2	P
		SA and MB							
Area	1	389.980	<0.001	1	24.913	<0.001	1	16.759	<0.001
Form	3	46.100	<0.001	3	29.671	<0.001	3	25.797	0.010
		SA							
Form	2	1.139	0.566	2	2.729*	0.256	2	1.771	0.413
		MB							
Form	3	80.522	<0.001	3	25.939	<0.001	3	36.482	<0.001

Table 5
Generalized linear model analyses of a) the offspring fitness obtained after pollination treatments, i.e., legitimate and illegitimate pollinations, of *Oxalis pes-caprae* plants from the invaded area, b) the effect of form, i.e., 4x L-, 4x M- and 5x S-morph, on the offspring fitness of *O. pes-caprae* for each pollination treatment separately, and c) the effect of pollination treatment, i.e., legitimate and illegitimate pollinations, on the offspring fitness of *O. pes-caprae* of each form considered separately. d.f., degrees of freedom. Statistically significant differences are highlighted in bold. “-” not tested.

Factors	d.f.	Germination		Survival		Flowering	
		Wald χ^2	P	Wald χ^2	P	Wald χ^2	P
a) Offspring fitness							
Pollination treatment	1	6.022	0.014	3.101	0.078	0.789	0.163
Form	2	3.888	0.143	0.343	0.842	1.946	0.874
Pollination treatment × Form	2	3.385	0.184	1.280	0.527	0.787	0.675
b) Testing the effect of “form”							
Legitimate pollination	2	15.071	<0.001	1.341	0.511	4.351	0.114
Illegitimate pollination	2	3.888	0.143	0.343	0.842	0.270	0.874
c) Testing the effect of “pollination treatment”							
4x L-morph	1	6.022	0.014	3.101	0.078	1.946	0.163
4x M-morph	1	3.496	0.062	0.027	0.870	0.007	0.933
5x S-morph	1	8.233	0.004	1E ⁻⁴	0.993	-	-

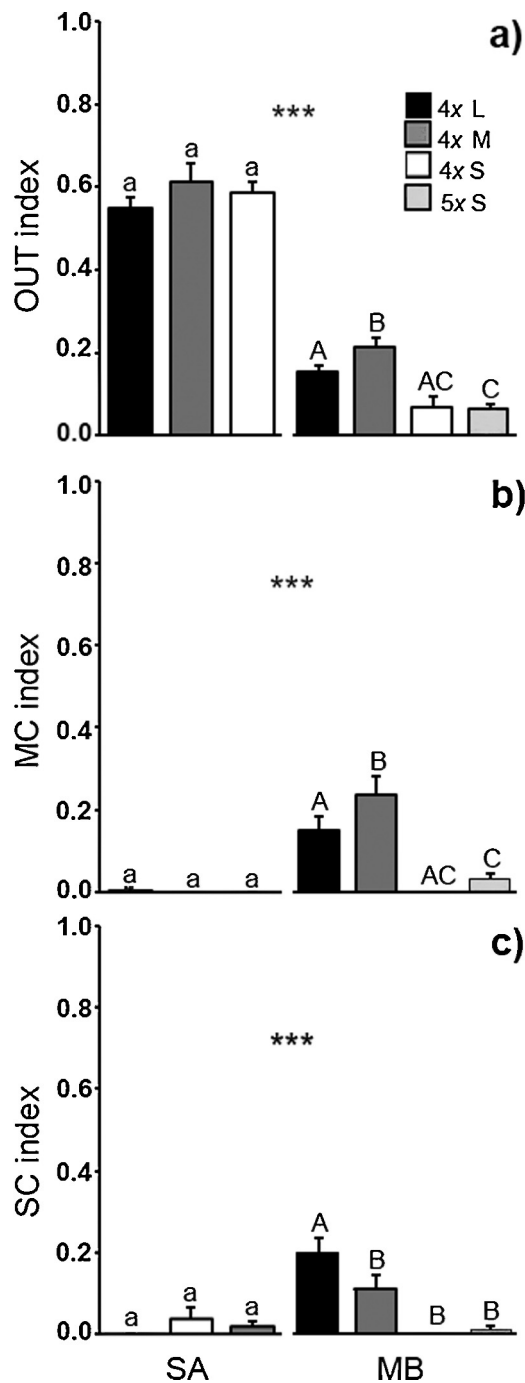


Fig. 3. Compatibility indices of *Oxalis pes-caprae* plants from South Africa (SA) and the Mediterranean basin (MB): (a) outcross (OUT) measure; (b) morph-compatibility (MC) index; (c) self-compatibility (SC) index. Results are mean and standard error. L, M, and S represent the L-morph, M-morph and S-morph, respectively. Different upper and lower case reveal statistically significant differences among forms within native and invasive populations, respectively, at $P < 0.05$. Asterisks refer to statistically significant differences between areas at $P < 0.001$.

reproductive indices (Table 4; Fig. 3), which were mostly driven by the reduced reproductive success of the 5x S- and 4x S-morph when compared with the other forms (Figs. 2 and 3).

3.4. Individual variation in the expression of incompatibility

By plotting the frequency of the individuals with different levels of MC and SC indices, we could observe the variation in morph- and

self-incompatibility within native and invasive populations (Fig. 4; Appendix B in Supplementary material).

Within native populations, most individuals were self- and morph-incompatible with a few individuals presenting low levels of morph-compatibility ($MC < 0.2$) being occasionally detected. Surprisingly, two individuals exhibited considerably high values of self-compatibility ($SC > 0.7$) when compared with the population's SC averages, in all cases not exceeding 0.15.

A higher variability in the expression of TSI was observed among invasive plants in comparison with native ones. Despite most of the invasive individuals were self- and morph-incompatible, with population averages of MC and SC indices being always below 0.3, individuals spanning the full range of possible values were detected (Fig. 4; Appendix B in Supplementary material). Plants from the Mediterranean basin ranged from complete incompatible (MC and $SC = 0$) to full compatible (MC and $SC = 1$).

3.5. Fitness of illegitimate offspring

The seeds obtained after legitimate pollinations had a significantly higher germination rate than the seeds obtained after illegitimate pollinations (Table 5; Fig. 5a). While form had no significant effect on the germination of seeds obtained after illegitimate pollinations (4x L-, $28.57 \pm 4.93\%$; 4x M-, $17.46 \pm 4.78\%$; 5x S-, $11.11 \pm 7.41\%$; Fig. 5a), differences among forms were detected after legitimate pollinations (Table 5), with the 4x M-morph having the lowest germination rate (4x L-, $45.14 \pm 4.15\%$; 4x M-, $30.33 \pm 4.16\%$; 5x S-, $52.76 \pm 3.59\%$; Fig. 5a). When comparing pollination treatments within form, seeds illegitimately sired by the 4x L- and 5x S-morph had a significantly lower germination rate than the ones legitimately sired, while no differences between treatments were observed for the 4x M-morph (Table 5; Fig. 5a).

Plant survival was always above 80% (Fig. 5b), and no differences were detected between pollination treatments and among forms, nor the interaction of the two (Table 5). Also, pollination treatment had no significant effect on the survival of plants sired by each of the forms considered (Table 5).

Except for the offspring illegitimately sired by the 5x S-morph that did not flower in the first year, between 40% and 70% of the plants that survived were able to produce inflorescences already in the first year (Fig. 5c), while the remaining stayed vegetative. No effect of pollination treatment, form and the interaction of the two were found for plant flowering (Table 5). Also, pollination treatment had no significant effect on the flowering of plants sired by each of the forms considered (Table 5).

4. Discussion

Our large scale comparison of the TSI between native and invasive populations of *O. pes-caprae* revealed several main findings: (1) native and invasive populations significantly differed in the strength of the TSI: plants from SA maintain a strong TSI, while invasives have a significantly higher reproductive success following illegitimate crosses than natives; (2) total reproductive success after intermorph pollinations was significantly higher for 4x natives than for 4x invasive plants; (3) morph-specific differences in the weakening of TSI were detected among invasive plants, with the L- and M-morphs being more self- and morph-compatible than the S-morph; (4) we detected variation in the expression of incompatibility among invasive plants, thus suggesting high standing genetic variation that might provide opportunities for natural selection on the strength of TSI; and finally, (5) the seeds produced in illegitimate crosses were able to germinate and survive to adult stage, with some plants remaining vegetative, while most of them were able to flower in the first year. Despite of the differences

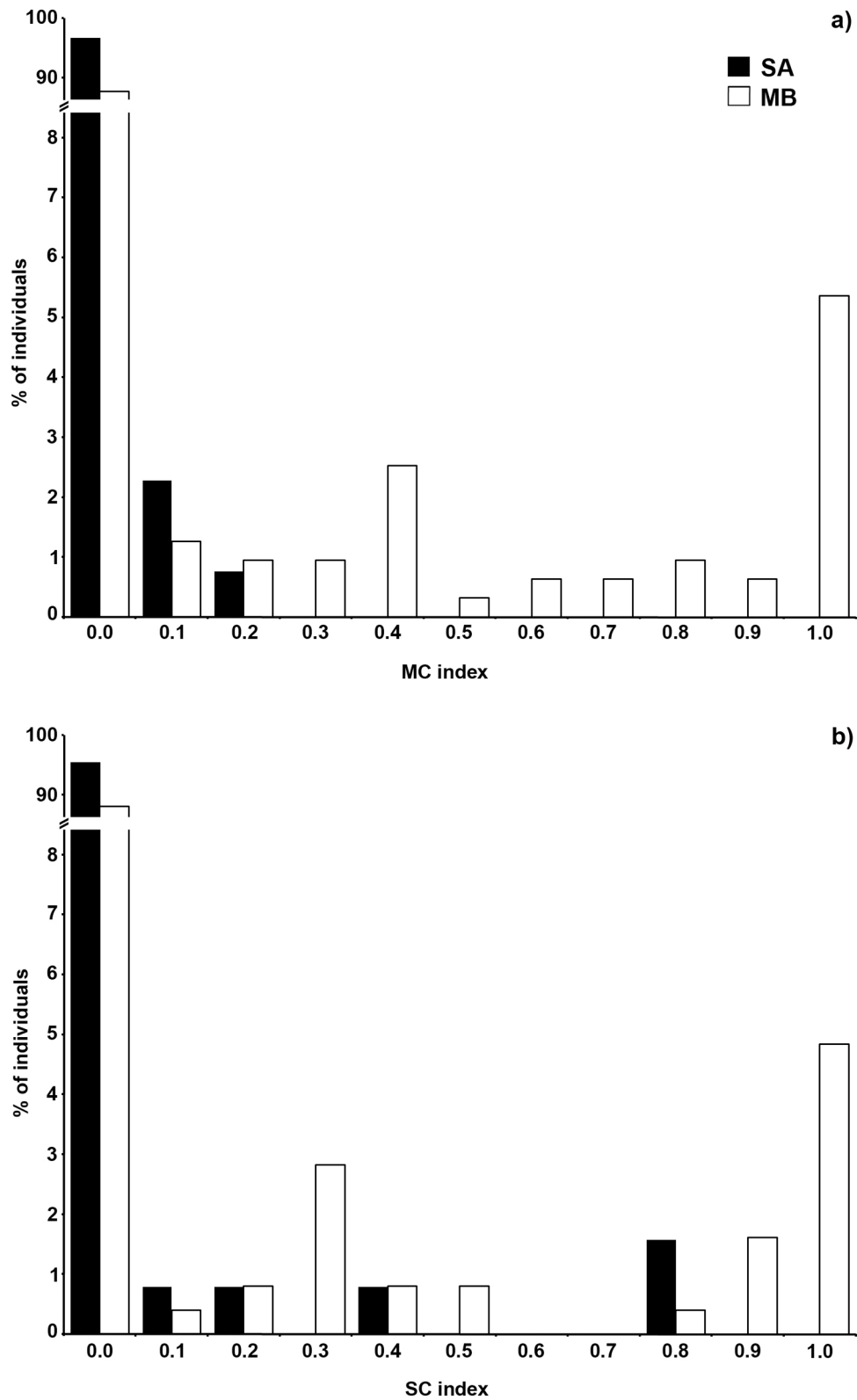


Fig. 4. Frequency of individuals of *Oxalis pes-caprae* given as percentage (%) from South Africa (SA) and the Mediterranean basin (MB) with variable levels of trimorphic incompatibility: (a) morph-compatibility index; (b) self-compatibility index.

detected in the TSI between ranges and the increase in compatibility in the invaded area, reproductive assurance is most probably guaranteed through asexual means. Notwithstanding, we detected some morph- and self-compatibility that might become selectively

advantageous in the invaded area. Below, we discuss our results in detail and focus their relevance for understanding the evolution of complex mating systems, such as heterostyly, during invasion.

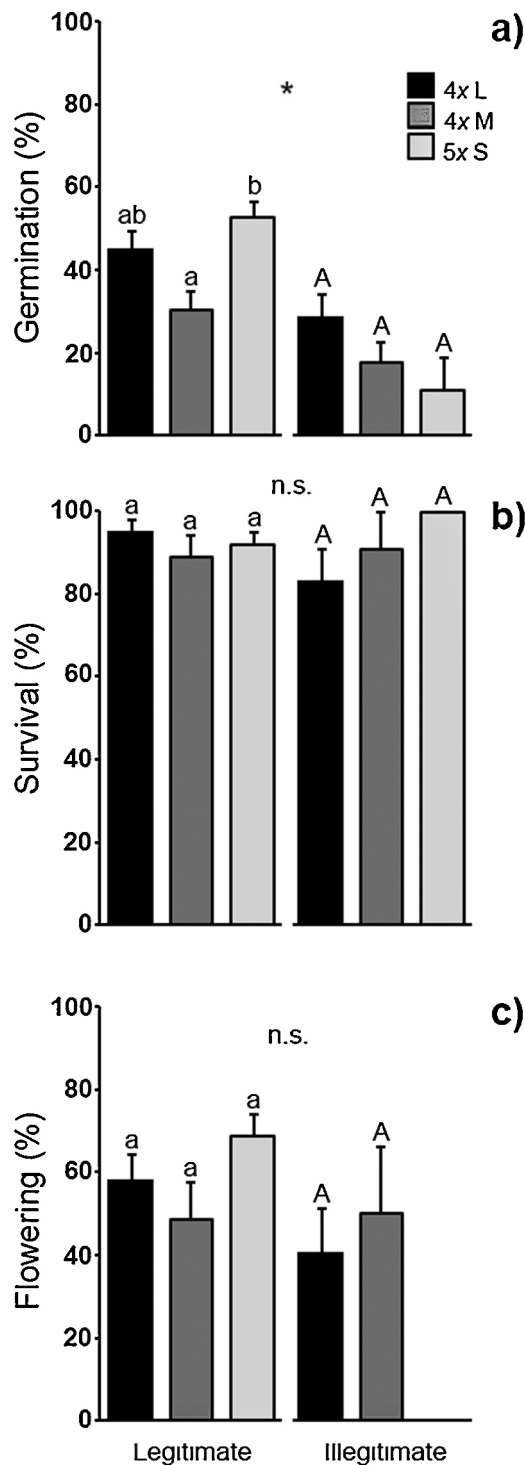


Fig. 5. Fitness of the offspring of invasive plants of *Oxalis pes-caprae* given as mean and standard error (%) after legitimate and illegitimate pollinations. (a) seed germination; (b) plant survival; (c) plant flowering. Different uppercase and lowercase letters reveal statistically significant differences among forms within pollination treatments, respectively, at $P < 0.05$. Asterisks refer to statistically significant differences between pollination treatments at $P < 0.05$. "n.s."—no statistically significant differences were found.

4.1. Trimorphic incompatibility system in the native area

Our results from controlled pollinations of South African plants confirmed that *O. pes-caprae* is strongly morph- and self-incompatible, which is in accord with earlier investigations (Ornduff, 1987). The maintenance of TSI in native populations is

expected for several reasons. First, negative frequency-dependent selection operating in these populations maintains the three style morphs, usually in isoplethy (Ornduff, 1987; Turketti, 2010; Ferrero et al., 2015). Second, these are homogeneously tetraploid and thus, compatible cytotypes co-occur (Krejčíková et al., 2013; Ferrero et al., 2015). Nonetheless, illegitimate crosses yielded small amounts of seeds, similar to what was also previously obtained by Ornduff (1987). Natural variation in the strength of the incompatibility system within and/or among populations of self-incompatible taxa (e.g., O'Neil, 1992; Barrett and Cruzan, 1994; López-García and Maillet, 2005; Mable et al., 2005; Arroyo et al., 2012) has already been reported. Variability in the incompatibility reactions in sporophytic incompatibility systems has been attributed to modifiers of the *S* gene activity that would allow the production of variable amounts of fruits and seeds after illegitimate pollinations of obligate outcrossers (Levin, 1996; Brennan et al., 2011). Such mechanism might explain the sporadic production of seeds by native plants after illegitimate pollinations, and in particular the high values of seed set obtained after selfing of two individuals from native range included in this study.

4.2. Increased compatibility during colonization

Native 4x plants performed better after legitimate pollinations than 4x individuals from the MB that yielded approximately half the number of seeds produced by natives. These differences were expected based on different historical processes in the two ranges. A recent comparison of patterns of genetic diversity between the two areas found that native populations of *O. pes-caprae* are genotypically more diverse than invasives, but the latter are not genetically uniform (Ferrero et al., 2015). Genetic drift during introduction was suggested to decrease genetic diversity, thus having strong negative impacts in reproductive success. However, some recombination by sexual means might explain why invasive populations are not genetically uniform. An additional factor might, however, contribute to the low genetic diversity reported: the presence in invasive populations of the 5x form, which has an even lower reproductive success. Plants with odd ploidy levels, e.g., 3x and 5x, show meiotic irregularities, resulting in a high production of aneuploids, and less frequently 1x, 2x, 3x, 4x and/or 5x gametes (Vignoli, 1937; Ramsey and Schemske, 1998; Risso-Pascotto et al., 2003). Thus, reproductive success of 5x invasives is expected to be strongly affected by ploidy and to be significantly lower than the 4x morphs as it was observed. Still, it is worth noticing that these plants are not entirely sterile and are able to produce some viable gametes and offspring (e.g., Brandham, 1982; Burton and Husband, 2000). Indeed, the results obtained here as well as from previous investigations in *O. pes-caprae*, show that the 5x S-morph is able to produce small amounts of fruits and seeds after illegitimate (Castro et al., 2013; Costa et al., 2014) and legitimate pollinations, and seeds are able to germinate and reach the adult stage.

Our large-scale comparison of the TSI between native and invaded areas gives further support to the preliminary findings of Castro et al. (2013) and Costa et al. (2014), who reported a weakening in the incompatibility reactions of *O. pes-caprae* based on experiments carried with a few populations from the MB only. Here, we detected differences in the mating system between native and invaded regions with increased self- and morph-compatibility in the later. A similar pattern was found for a few invasive species, as *Echium plantagineum* (Petanidou et al., 2012) and *Gomphocarpus physocarpus* (Ward et al., 2012). Theoretical models indicate that selection for self-compatibility depends on several factors, including the extent of the reproductive assurance provided by selfed progeny (Lloyd, 1992). This is particularly important after long-distance dispersal, because it allows the establishment and spread of a species in a new range under low compatible mates' den-

sity (Baker's law; Baker, 1955, 1967; Stebbins, 1957; Barrett, 2011; Pannell et al., 2015). For species with heteromorphic incompatibility systems (i.e., self- and morph-incompatibility), this situation should not only include single colonizers, but also small colonies formed exclusively by the same morph. Invasive individuals of *Oxalis pes-caprae* are under strong mate limitation, especially the 4x L- and 4x M-morphs, and although asexual reproduction is the main mechanism of spread (Castro et al., 2016), the ability to produce some self- and intramorph offspring might be selectively advantageous. Despite the plants raised from seed are able to germinate, survive and flower in the first year under glasshouse conditions, it is not clear how frequent the recruitment from seed occurs in natural populations. Thus, studies addressing these questions in natural conditions are desirable before further conclusions on selection of reproductive traits are reached.

4.3. Different reproductive strategies among style morphs during colonization

Different compatibility levels were observed among invasive forms. These patterns were surprising, but not unexpected. Populations of *O. pes-caprae* in the MB are predominantly monomorphic of the 5x S-morph with low sexual reproduction (Castro et al., 2007, 2013), being genetically depauperate (Ferrero et al., 2015). Thus, the opportunities for selection of compatibility in these individuals are expected to be limited. On the contrary, for the 4x L- and 4x M-morphs, still growing under strong mate limitation in populations dominated by the 5x S-morph, recombination opens the possibility for increased variability in the TSI. Indeed, our results revealed that the 4x L- and 4x M-morphs are more self- and morph-compatible, respectively, and this is in accordance with previous findings (Castro et al., 2013).

Strong mate limitation in the MB regardless of this species abundance might be driving the changes in the incompatibility reactions in the 4x L- and 4x M-morphs. First, the low availability of viable legitimate gametes in mixed populations caused by the occurrence of 5x S-morph plants and a deficiency/lack of 4x M-morph plants (Castro et al., 2007, 2013) might cause a selective environment driving the observed changes in the incompatibility system. Second, 5x S-morph plants may act as strong competitors via clonal propagation, investing more resources in producing large bulbs (Castro et al., 2016) overcoming the inability to produce the maximum/optimum number of viable gametes. Strong mate limitation also results from the rarity of the 4x S-morph in the MB (Castro et al., 2007, 2013) and its apparent inability to reproduce sexually in the absence of compatible mates (results herein). Altogether, these observations suggest that founder events after introduction or some selection against the S allele may be occurring and could be responsible for the rarity of the 4x S-morph in the invaded area.

Despite a few exceptions (Lewis and Jones, 1992), the S-morph (Ss-) is dominant over the M- (ssM-) and the L-morphs (ssmm), as it carries the S allele, which is dominant over the M allele (Heuch and Lie, 1985; Lewis and Jones, 1992). Theoretical simulations have shown that alleles governing tristyls differ in their frequency in populations at equilibrium ($S = 0.085$, $M = 0.151$, $m = 0.849$; Heuch, 1980). Consequently, style morphs differ in the probability of loss through drift and founder events, with the S-morph being more susceptible to loss than the other morphs (e.g., Eckert and Barrett, 1992). Additionally, some selection against the S allele has already been reported for other heterostylous species (Weller, 1992), and might be responsible for the low frequency of the 4x S-morph observed in our study. In *O. pes-caprae*, the 4x S-morph is thus expected to be more susceptible to loss, while populations with the 4x L- and 4x M-morphs are expected to be maintained. Under both scenarios, the 5x S-morph seems in disadvantage, depending mostly on its asexual reproduction capacity, which also revealed

to be significantly lower in comparison with the 4x floral morphs (Castro et al., 2016). Based on the available information, the dominance of the 5x S-morph in the western Mediterranean basin can be explained by the introduction of the three style morphs at different timings. Thus, it is highly probable that the introduction of the currently widespread 5x S-morph preceded a more recent set of introductions of the other two style morphs (4x L- and 4x M-morphs), for which the available data suggests higher sexual and asexual fitness than for the 5x S-morph [results herein for sexual reproduction under mate limitation; Castro et al. (2016) for sexual and asexual reproductive traits].

4.4. Changes to the mating system in the Mediterranean basin

Several features contribute for the undoubted success of *O. pes-caprae* as an invader of Mediterranean climate regions. Notably, this species has a profuse ability to reproduce vegetatively (Pütz, 1994), showing higher clonal reproduction in invasive than in native populations (Castro et al., 2016); it has successfully integrated the novel pollination networks in the MB (Ferrero et al., 2013); it inhabits disturbed and anthropogenized places (Gimeno et al., 2006); and finally, to our knowledge, no natural enemies have been reported so far in the MB. Additionally, our study demonstrated increased ability for reproduction under a strong mate limitation scenario via incompatibility weakening in invasive populations. We detected variability in the expression of TSI among invasive plants; individuals ranged from morph- and self-incompatible to full morph- and self-compatible with the self- and intramorph offspring being able to achieve sexual maturity in the first year after germination under glasshouse conditions. These findings might suggest a basis for natural selection acting on the mating system during invasion. Altogether, these characteristics make *O. pes-caprae* a successful invader difficult to eradicate. Moreover, if the ongoing changes in the incompatibility system allow the production of viable seeds after illegitimate pollination that are able to germinate, survive and flower in natural conditions, this might contribute to increase the genetic diversity in the populations (Ferrero et al., 2015), thus favoring the establishment and spread of more competitive and fitter genotypes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ppees.2016.12.002>.

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