Sexual reproduction of the pentaploid, short-styled Oxalis pes-caprae allows the production of viable offspring

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INTRODUCTION

One key factor for the successful establishment and spread of introduced species after overcoming long-distance dispersal is reproduction and, among other strategies, vegetative propagation has been largely correlated with invasion success (e.g. Godfrey et al. 2004; Lloret et al. 2005; Pyšek & Richardson 2007). Because clonality affects the spatial distribution of genets and their flowers determine opportunities for cross-fertilisation, clonal species are expected to have increased rates of self-pollination because of the higher probability of pollen dispersal between individuals of the same clone (Handel 1985; Charpentier 2002). In self-incompatible plants, an increase of self-pollination has important reproductive consequences negatively affecting both male (e.g. Harder & Barrett 1996) and female fitness (e.g. Vallejo-Marín & Uyenoyama 2004; Porcher & Lande 2005; Wang et al. 2005).

Conflicts between sexual and asexual reproduction can be even more intricate when the invader has a complex breeding system, such as heterostyly. In heterostylos populations, the plants present two or three floral morphs that differ reciprocally in the position of their sexual organs (Barrett 1992). Heterostyly plants are usually self-incompatible and, in addition, present an incompatibility system that only allows crosses among reciprocal anthers and stigmas of compatible morphs (intra-morph incompatibility). In these cases, when just one of the floral morphs is introduced in a new area, the sexual contribution to the fitness of the newly established plant/population is expected to be null (e.g. Oxalis per-caprae, Castro et al. 2007; O. debilis, Luo et al. 2006; O. corymbosa, Tsai et al. 2010).

Reproduction by vegetative means has several ecological advantages for an invader, enabling, for example, growth and persistence in the new range when conditions are unfavourable for sexual reproduction due to the absence of pollinators (Richardson et al. 2000) or to the loss of compatible mating partners (e.g. Barrett 1979; Castro et al. 2007). However, asexual reproduction also has strong negative consequences. Populations of obligate clonal plants are expected to have lower levels of genetic variability, being less able to respond adaptively to changing environments (Holsinger 2000). This is clearly a disadvantage for an invader in a new and unpredictable habitat. Under this scenario, selection may favour breakdown of the self-incompatibility, as individuals with some level of compatibility would have an advantage under low-density conditions and would be able to establish new populations after dispersal (Baker’s law; Baker 1955, 1967; Stebbins 1957). In heterostylos systems, such a phenomenon has been described in several taxa and is usually associated with a re-arrangement of sexual organ position (i.e. secondary homostyly), as a mechanism of reproductive assurance (e.g. distylos taxa: Amsinckia spp., Schoen et al. 1997; Primula spp., Mast et al. 2006; Turnera ulmifolia, Barrett & Shore 1987; Psychotria spp., Sakai & Wright 2008; tristylos taxa: Eichhornia spp., Barrett 1985, 2011; Oxalis corymbosa, Tsai et al. 2010).

Oxalis per-caprae L. is a polyploid (with reported diploids, 2x; tetraploids, 4x; and pentaploids, 5x) tristylos species (Fig. 1) native to South Africa, with a typical heteromorphic
incompatibility system responsible for self- and morph-incompatibility (Ornduff 1987). This geophyte has been introduced into Mediterranean climate regions throughout the world, where it has become a widespread invasive weed (Symon 1960; Baker 1965; Ornduff 1987). In most invaded regions, a shift to predominant asexuality was observed as a result of founder events after the introduction of the pentaploid short-styled morph alone (Baker 1965; Ornduff 1987). However, in the last years, new floral morphs (mid- and long-styled), cytotypes (4x) (Castro et al. 2007, 2013) and punctual events of sexual reproduction (Vignoli 1937; Ornduff 1987; Castro et al. 2007) have been described in the invaded range of the western Mediterranean Basin where the pentaploid short-styled morph (5x S-morph) is the most frequent floral morph (Castro et al. 2007, 2013).

The classical genetic studies in tristylos plants indicate that the floral morph is controlled by two loci, Ss and Mm, with the long-styled type being homozygous recessive for both of them (ssmm), the mid-styled dominant over the long one but recessive for the other (ssMx) and the short-styled dominant over both (Sxxx) (Lewis & Jones 1992). This system has also been demonstrated in some Oxalis species (Weller 1976). In a parallel study, Castro et al. (2013) suggest that the occurrence of new morphs in the invaded area could have resulted from a breakdown of the incompatibility system that enabled the occurrence of sexual reproduction events in the short-styled morph and/or from multiple introduction events.

The objective of the present study was to test the ability of the pentaploid short-styled (5x S-morph) O. pes-caprae from the invaded region of the western Mediterranean Basin to produce viable offspring in the absence of compatible mates. It is hypothesised that one of the pathways involved in the emergence of new morphs in this invaded range is by (occasional) sexual reproduction through a breakdown of the incompatibility system in the 5x S-morph. If this is the case, legitimate (between morph) crosses will yield offspring, proving the ability of the 5x S-morph to produce viable gametes, and illegitimate (within morph) crosses will yield viable offspring, proving the ability of the 5x S-morph to reproduce sexually in the absence of compatible mates. Under natural conditions, this will actively contribute to an increase in the genetic diversity of the invasive populations and, ultimately, may have serious consequences for the invasiveness of this species.

**MATERIAL AND METHODS**

**Plant material and study area**

*Oxalis pes-caprae* L. (Oxalidaceae) is a perennial bulbous plant with a profuse production of bulbs that, associated with the contractile properties of its roots, confers a high ability to reproduce asexually (Pütz 1994). *O. pes-caprae* is a tristylos species (short-, mid- and long-styled floral morph, S-morph, M-morph and L-morph, respectively; Fig. 1), with actinomorphic yellow flowers arranged in terminal umbellate cymes (Coutinho 1939; Ornduff 1987; Sánchez-Pedraja 2008). In the invaded region of the Mediterranean Basin, flowering occurs from early January to late March.

This study was carried out during 2009 and 2010 with plants from Almôçageme (Estremadura province, Portugal), a population previously known to be trimorphic (Castro, personal observation). The use of a trimorphic population allowed us to test the ability of the 5x S-morph to produce viable offspring and to assess if it could be involved in the emergence of other floral morphs. The percentage of floral morphs within the population in February 2009 was: 56.0% of the S-morph, 11.9% of the M-morph and 32.1% of the L-morph. Floral morph composition was assessed by evaluating the morph of 100 individuals every 5 m along two longitudinal transects across the population. Plants were collected in the field during December 2009 before flowering. Thirty-five plants per floral morph (S-morph, M-morph and L-morph) were directly transplanted to pots, identified with an ID number and maintained at ambient outdoor conditions in the experimental garden of the Botanical Garden of the University of Coimbra. The selected plants were collected at least 5 m apart to avoid re-sampling of the same genets.

**Ploidy level analysis**

Because there are two cytotypes reported to occur in the invaded range of the Mediterranean region (4x and 5x; Castro et al. 2009) and 5x cytotypes have been described...
et al. 2007, 2013), ploidy level of all plants collected was analysed using flow cytometry (FCM). Samples were prepared following the procedure of Galbraith et al. (1993) and the two-step nuclear isolation method with Otto’s buffers (Otto 1992; Doležel & Göhde 1995). Briefly, nuclei from fresh leaves of O. pes-caprae and Bellis perennis (internal reference standard with $2C = 3.38$ pg; Schönswetter et al. 2007) were released after chopping the leaves in 0.5 ml Otto I solution (100 mM citric acid, 0.5% (v/v) Tween 20); the solution was filtered after centrifugation to a cytometer sample tube using a 50-μm nylon filter and 1 ml Otto II solution (400 mM Na2PO4.12H2O) added; finally, 50 μg ml$^{-1}$ propidium iodide was added to stain the nuclei and 50 μg ml$^{-1}$ RNAsé for digestion of the double stranded RNA (Doležel et al. 2007). At least 3000 nuclei per sample were analysed in a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany). The flow cytometer was equipped with a green solid state laser (Cobolt Samba 532 nm, 100 mW; Cobolt, Stockholm, Sweden) for PI excitation. Only histograms with a coefficient of variation (CV) below 5% for both sample and standard G0/G1 peaks were accepted as a quality standard. The DNA index was calculated for all the samples by dividing the O. pes-caprae G0/G1 peak mean fluorescence by that of B. perennis and plants were identified as 4x or 5x for genome size values of $1.37 \pm 0.056$ (n = 39) and $1.66 \pm 0.030$ (n = 248; mean ± SD, followed by sample size in parenthesis), respectively (Castro et al. 2007).

Hand-pollination experiments
To assess the ability of the 5x S-morph to produce offspring, both illegitimate and legitimate pollinations were performed (Fig. 1). Illegitimate pollinations were carried out to assess the self- and morph-incompatibility of the 5x S-morph and the following treatments were performed: self-pollinations with pollen from the mid- and long-anther levels (selfing 5x Sm and selfing 5x Ss, respectively) and intra-morph pollinations with pollen from the mid- and long-anther levels (5x S × 5x Sm and 5x S × 5x Ss, respectively; Fig. 1). Legitimate pollinations were carried out to assess the ability of the 5x S-morph to produce viable offspring through its ovules and pollen grains and, thus, the following treatments were performed: inter-morph legitimate pollinations with 5x S-morph as pollen recipient (5x S × 4x M, and 5x S × 4x Ls) and as pollen donor (4x M × 5x Sn and 4x L × 5x S0 Fig. 1). Plants were covered with a nylon mesh before flowering to prevent natural pollinations and maintained bagged until fruiting. Recipient flowers were emasculated to prevent self-pollination. Up to 33 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.

When the ovaries started to swell, most stigmas and styles were cut and harvested in ethanol 70% to assess pollen germination and pollen tube development in the style. Stigmas and styles were softened with 8 N sodium hydroxide for 3 h, washed in distilled water and placed overnight in 0.05% (w/v) aniline blue prepared in 0.1 N potassium phosphate (Dafni et al. 2005). Then they were placed on a microscope slide with a drop of glycerine 50%, squashed beneath a coverslip and observed using a Nikon Eclipse 80i epifluorescence microscope (Nikon Instruments, Kanagawa, Japan) with the UV-2A filter cube. Pollen germination and pollen tube development along the style were assessed by counting the number of germinated grains from 50 randomly selected grains deposited in the stigmatic papillae and by counting the number of pollen tubes in the lower part of the style, respectively. The mean number of ovules of each floral morph was also assessed in more than 15 flowers from distinct individuals under fluorescence microscopy following the procedure of Dafni et al. (2005), as described above.

The fruit and seed production were recorded when mature fruits and seeds were characterised as morphologically viable or aborted. Fruit set was calculated for each pollination treatment as the percentage of treated flowers that developed into fruits.

Seed germination
The seeds obtained from the hand-pollination experiments were placed to germinate in 6 × 6 cm pots filled with common garden substrate and maintained at ambient outdoor conditions protected from the rain in the experimental garden of the Botanical Garden (University of Coimbra) in September 2010. Pots were monitored weekly over 3 months to count the number of seedlings. Ploidy level of the germinated offspring was assessed following the procedure described in the section ‘Ploidy level analysis’.

Statistical analysis
Descriptive statistics (mean and SE of the mean) were calculated for pollen germination, number of pollen tubes developed in the lower part of the style, fruit set, number of morphologically viable and aborted seeds per fruit, and seed germination.

Differences among pollination treatments in pollen germination, number of pollen tubes along the style and aborted seeds were analysed using a generalized linear model (GLZ) with a gamma distribution and a power(-1) link function. A similar approach was used for fruit set with a binomial distribution and logit link function. Least square means were used to analyse differences between treatments. The number of viable seeds and seed germination were analysed using a one-way ANOVA, followed by Tukey test for multiple comparisons. The results for viable seeds, aborted seeds and seed germination from the 5x S × 5x S0 cross were not included in the analysis because there was only one sample. All the analyses were carried in SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS
Results from hand-pollination experiments are given in Fig. 2 and Appendix S1. Pollen grains from the 5x S-morph, 4x M-morph and 4x L-morph were able to germinate on the recipient stigma but statistically significant differences were observed in germination rates ($\chi^2 = 14.57, P = 0.0419$); higher germination rates were observed in legitimate crosses (although no significant differences were found for 5x S × 4x Ls and 4x L × 5x S0 crosses) and in self- and intra-morph pollinations when pollen from the mid-anthers of the 5x S-morph was used (Fig. 2A). Pollen tube development was observed in all illegitimate and legitimate crosses, despite the significant differences observed between pollination treatments ($\chi^2 = 9.14, P < 0.0001$), with legitimate pollinations having significantly more pollen tubes than illegitimate ones (Fig. 2B).

The mean number of ovules produced by each floral morph was not significantly different (mean ± SE: 39.6 ± 1.0;
Fruit production and number of viable and aborted seeds per fruit were significantly different between pollination treatments ($\chi^2_2 = 65.65$, $P < 0.0001$; $F_4 = 3.50$, $P = 0.0126$; $\chi^2_4 = 30.74$, $P < 0.0001$, respectively; Fig. 2C–E). Selfing crosses did not yield any fruit and significantly higher fruit set was found in legitimate crosses when the $5xS$-morph was used as pollen recipient (Fig. 2C). Legitimate crosses tended to produce more viable seeds per fruit than illegitimate crosses, but no significant differences were found between them and the $5xS \times 5xS_1$ (Fig. 2D). Concerning the number of aborted seeds, in legitimate crosses two statistically different groups could be distinguished, with higher seed abortion in pollinations where the $5xS$-morph was used as pollen donor (Fig. 2E).

Seed germination revealed no statistically significant differences among pollination treatments ($F_2 = 1.69$; $P = 0.1667$) and ranged between 11.1% and 34.7%, it being possible to obtain seedlings from both illegitimate and legitimate crosses (Fig. 2F). Flow cytometric analysis of the germinated offspring revealed that both $4x$ and $5x$ were produced in illegitimate and legitimate crosses (Appendix S1). The low number of seedlings obtained from illegitimate crosses made it difficult to disentangle the cytotype patterns in the offspring. In legitimate crosses, $5x$ offspring was only obtained when the $5xS$-morph was used as pollen donor; nevertheless, $4x$ was the most frequent cytotype in the offspring; when the $5xS$-morph was used as pollen donor, the offspring was composed only of $4x$ (Appendix S1).

**DISCUSSION**

After long-distance dispersal, reproductive strategies are of major importance for the successful colonisation of invasive
species (e.g. Pyšek & Richardson 2007; Barrett 2011). In heterostyloous plants, the introduction of only one floral morph leads to the loss of compatible mates, forcing, in many cases, the emergence of novel reproductive adaptations to the new conditions (e.g. Barrett 1979). Under low-density of mating partners and pollen limitation, the transition from incompatibility to compatibility is expected to be advantageous because selection will favour self- and/or morph-compatible individuals (Allee et al. 1949; Baker 1966; Charlesworth 1979; Barrett et al. 1987). Self-incompatibility breakdown has already been documented in several heterostylos species (Ornduff 1972; Barrett 1989, 1992; Weller 1992), including some invasive ones (Barrett & Shore 2008; Colautti et al. 2010). Although fruit and seed production were not completely ruled out in the invaded range of O. pes-caprae where the 5x S-morph dominated (Vignoli 1937; Ornduff 1987; Ater 2005; Castro et al. 2007), this is the first study quantifying its potential production of viable offspring as a result of a partial breakdown in the morph-incompatibility system.

In the native range, O. pes-caprae is known to present a sporophytic, heteromorphic incompatibility system (Ornduff 1987); however at which level the incompatibility occurs is still unknown. Incompatibility responses in heterostyloous plants include lack of adhesion, hydration and germination of pollen, inability of pollen tubes to penetrate the stigmatic zone, and cessation of pollen tube growth in the style and ovary (Dulberger 1992; Barrett & Cruzan 1994). The present study shows that in the invaded area of the western Mediterranean region, the self-incompatibility system is still operating, as no fruit and seed production were observed after self-pollinations. However, as pollen tube development along the style was observed, the incompatibility system seems to be operating at several levels of the style and ovary, which suggests a possible late-acting self-incompatibility system in O. pes-caprae. This system has been described in several other species such as Cyrtanthus breviflorus (Vaughton et al. 2010), Narcissus spp. (Dulberger 1964; Sage et al. 1999; Navarro et al. 2012), Anchusa officinalis (Schou & Philipp 1983), Asclepias exaltata (Lipow & Wyatt 2000) and Spathodea campanulata (Bittencourt et al. 2003), however further work must be done in order to confirm this in O. pes-caprae.

Contrary to self-pollinations, intra-morph crosses resulted in the production of fruits, seeds and seedlings, showing a partial breakdown in the morph-incompatibility system of O. pes-caprae in this invaded area. Nevertheless, pollen tube development and fruit and seed production were lower than in legitimate crosses, indicating that the breakdown was not complete and that morph-incompatibility still reduces the reproductive success of within-morph pollinations at several levels of the style and ovary. Low levels of sexual reproduction may have been occurring for some time (Vignoli 1937; Ornduff 1987; Ater 2005), and Castro et al. (2007) reported sporadic pollen tube development (although no fruit production was observed) after within-morph pollinations in other populations of O. pes-caprae from the same geographic range. Indeed, a recent large-scale reassessment of natural reproductive success across this range reported a remarkable diversity in floral morph and genotype composition, with variable sexual reproductive outcomes across the surveyed area (Castro et al. 2013). The acquisition of morph-compatibility increases the number of mating partners within the population and has major implications for the population dynamics and, potentially, for genetic structure (Ray & Chisaki 1957; Ganders 1979; O’Brien & Calder 1989). In addition, a partial breakdown in the morph-incompatibility system may be one of the factors involved in the occurrence of additional floral morphs reported recently in this invaded range, although multiple introduction events could also be involved in the process. The partial breakdown in the morph-incompatibility (results herein) and higher sexual reproductive success of M- and L-morphs (Castro et al. 2013) may also be involved with the recently observed spread patterns of these morphs.

Legitimate pollinations were performed to assess the ability of the 5x S-morph to produce viable offspring through its ovules and pollen grains. Plants with odd ploidy levels, such as triploids and pentaploids, are reported to have meiotic abnormalities and to produce a high number of aneuploids, as well as 2x, 3x, 4x and/or 5x gametes in lower numbers (Ramsey & Schemske 1998; Risso-Pascotto et al. 2003), consequently they are expected to be mostly sterile (Ramsey & Schemske 1998). Meiotic abnormalities producing microspores with a variable number of chromosomes have been described in O. pes-caprae (Vignoli 1937). Although no differences were observed in pollen tube development along the style, the 5x S-morph individuals were more successful as pollen recipient than as pollen donor. Still, our results showed that 5x S-morph individuals were able to produce some viable pollen grains and ovules that, after legitimate pollinations, yielded viable offspring. The prevalence of 4x in the offspring also seems to indicate that 2x gametes were favourably recruited for seed production. Bi-nucleate microspores and 2n microspores resulting from nucleus restitution were already reported in the pentaploid Brachiaria brizantha (Risso-Pascotto et al. 2003). Moreover, exploring the ploidy of the offspring produced by triploids of Aloinaeae, Brandham (1982) showed that plants with odd ploidy levels (3x) still have some fertility, contributing either 1x or 2x gametes when crossed with 2x or 4x plants, respectively. The bias in the frequency of progeny ploidy levels resulted from seed abortion when the ratio of maternal to paternal genomes in the endosperm tissue deviated from 2:1 (Brandham 1982; Grossniklaus et al. 2001). A similar mechanism could be actually guiding the prevalence of 4x seedlings in the 4x × 5x and 5x × 4x crosses with O. pes-caprae plants.

In conclusion, a partial breakdown in the morph-incompatibility system, combined with the ability of the 5x S-morph to produce some viable gametes, opened the possibility of sexual reproduction and may be one of the mechanisms involved in the emergence of new floral morphs and cytotypes of O. pes-caprae in this invaded region. These results are in accordance with our hypothesis; however, in order to fully understand the patterns of the incompatibility breakdown and their contribution to the reproductive success and morph proportions of O. pes-caprae in this invaded region, large-scale pollination experiments are currently being performed throughout the invasive range of the western Mediterranean region.

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SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article:
Appendix S1. Results from controlled-holl-pollination experiments

Incompatibility system breakdown in the invasive Oxalis pes-caprae