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Sexual reproduction of the invasive pentaploid short-styled *Oxalis pes-caprae* L.

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"The hermaphrodite class contains two interesting sub-groups, namely, heterostyled and cleistogamic plants; but there are several other less important subdivisions, presently to be given, in which flowers differing in various ways from one another are produced by the same species."

Charles Darwin

(*In:* Darwin, C. (1877). *The different forms of flowers on plants of the same species*. London.)

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i. Abbreviations

2C – two copies of the nuclear DNA content

$2x$ – diploid

$2n$ – diploid number of chromossomes

$3x$ – triploid

$4x$ – tetraploid

$5x$ – pentaploid

Aus – Australia

Ca – California

Ch – Chile

CV – coefficient of variation

e.g. – (L. *exempli gratia*) for example

et al. – (L. *et alia*) and other

FCM – flow citometry

GLM/ GLZ – general linear model/ generalized linear model

ID – identification

i.e. – (L. *id est*) that is

L-morph – long-styled floral morph

LSmeans – least square means

MB – Mediterranean basin

M-morph – mid-styled floral morph

n – number of

$Na_2PO_4.12H_2O$ – sodium phosphate dodecahydrate

pg – picograms

PI – propidium iodide

SA – South Africa

SD – standard deviation

SE – standard error

S-morph – short-styled floral morph

sp. – (L. *species*) species

spp. - (L. *species*) species in plural

St – sterile multipetal form

x – monoploid number of chromosomes

Note: all the units used follow the SI (Système International d'Unités)

ii. Resumo

A reprodução é um factor chave no estabelecimento e dispersão de uma espécie exótica, determinando as oportunidades para a adaptação local. *Oxalis pes-caprae* é uma espécie tristílica dotada de um sistema de auto- e morfo-incompatibilidade. Na área invadida da bacia do Mediterrâneo ocidental, esta planta foi forçada à assexualidade como resultado da introdução de um único morfotipo floral. No entanto, novas formas florais e citotipos, assim como eventos de reprodução sexual foram recentemente detectados em algumas populações. Os objectivos desta tese de Mestrado foram 1) estudar o sistema de incompatibilidade heteromórfica de *O. pes-caprae* nesta região invadida e 2) determinar o sucesso reproductivo em populações naturais da área invadida com diferentes composições de morfotipos florais. Para tal, o sistema de auto- e morfo-incompatibilidade, assim como a capacidade do morfotipo curto 5*x* produzir gâmetas viáveis foram testados através de polinizações controladas. Para responder ao segundo objectivo, foram seleccionadas três populações com diferentes composições de morfotipos florais (populações mono-, di- e trimórficas), nas quais se monitorizou o comportamento dos polinizadores e se quantificaram os sucessos reproductivos masculino e feminino. Os resultados revelaram uma quebra no sistema de morfo-incompatibilidade, assim como a produção de gâmetas viáveis, permitindo dessa forma a reprodução sexual na área de estudo. *O. pes-caprae* revelou-se uma planta generalista em termos de polinizadores, tendo já estabelecido novas interacções mutualísticas na área invadida que permitiram o fluxo de pólen e, consequentemente, a produção de frutos e sementes. As relações mutualísticas estabelecidas com polinizadores nativos assim como a capacidade do morfotipo curto 5*x* se reproduzir sexuadamente podem ter importantes consequências na dinâmica das populações invasoras de *O. pes-caprae*, sendo este um dos possíveis factores envolvidos na ocorrência de populações com diferentes composições de formas florais nesta região invadida.

Palavras-chave: espécie invasora; heterostilia; pentaplóide; polinizadores; sistema de incompatibilidade.

*Este resumo não foi escrito segundo o novo acordo ortográfico em vigor.

iii. Abstract

Reproduction is a key factor for the successful establishment and spread of exotic species determining the opportunities for local adaptation. *Oxalis pes-caprae* is a tristylous species with a self- and morph-incompatibility system that, in the invaded range of the Mediterranean basin, was forced to asexuality due to the introduction of only one floral morph. Recently, in Portugal, new floral morphs and cytotypes and the occurrence of sexual reproduction in some populations were detected. The main objectives of this MSc thesis were: 1) to test the heteromorphic incompatibility system of *O. pes-caprae* in the invaded range and 2) to assess its sexual reproductive success in natural populations from the invaded range differing in morph's composition. To achieve the first objective, the ability of the 5*x* S-morph to produce viable offspring was evaluated through controlled hand-pollinations to assess self- and morph-incompatibility and the production of viable gametes by the 5*x* S-morph. Regarding the second objective, mono-, di- and trimorphic populations were selected, pollinator's assemblage and behavior were monitored and male and female reproductive success were quantified. Results revealed that the self-incompatibility system is still operating, but a breakdown in the morph-incompatibility system combined with the production of viable gametes was observed, allowing its sexual reproduction in the study area. Sexual reproductive success of *O. pes-caprae* depended of generalist pollinators, with new mutualistic interactions having already been established in the invaded range. This allowed pollen movement within the populations and, consequently, fruit and seed production was observed in both di- and trimorphic populations. The mutualistic interactions already established and the ability of the 5*x* S-morph to reproduce sexually may have major consequences on the dynamics of the invasive populations of *O. pes-caprae* and could be one of the factors involved in the occurrence of populations with new floral morph's composition in this invaded area of the Mediterranean basin.

Key words: heterostyly; incompatibility system; invader; pollinators; sexual reproduction.

INTRODUCTION

0.1. Floral biology: brief historical considerations

In Nature, flower traits such as colour, size and shape are found to fluctuate under a continuous of variation. Because of plant immobility, this variability is extremely important for mating success of flowering plants depending on their pollen transport vectors (*e.g.*, Lloyd and Barrett 1996; Barrett 2010). Sexual characters are so important that Linnaeus used them as the basis for the plant classification presented in *Systema Naturae* in 1735. Still, the study of floral biology was only born in the 18th and 19th centuries and aimed to understand the functioning of flowers and the role of floral design in pollinator's attraction (*e.g.*, reviewed in Ferrero 2009; Barrett 2010). The first experimental studies on pollination biology were undertaken by manipulating floral rewards, *e.g.*, nectar, or by altering pollinator's senses through antennae removal. These manipulative studies were important because they provided insights on plant-pollinators coevolution (Kearns and Inouye 1993). The sexual systems of flowering plants are highly diverse and have long intrigued biologists. In fact, the ancestral condition of the flower sexual system, *i.e.*, hermaphroditism, has always attracted biologist's attention. This can be confirmed by the work developed by many authors since the 19th century. For example, the important contributions given by Müller (1983), Kerner von Marilaun (1902) or Percival (1965) regarding floral biology in a descriptive way or the significant contributions of Darwin (1862, 1876, 1877) and Stebbins (1950) with numerous studies of floral biology as a mechanism to understand evolution.

0.2. Sexual polymorphisms and heterostyly

Hermaphroditic plants are an interesting study group because they experienced a huge physiological and morphological variability to enable cross-fertilization, while preventing selfing (Barrett 2010). To promote cross-pollination, some hermaphroditic plants developed different sexual polymorphisms that are characterized by the presence, in the same population, of distinct morphological mating groups of the same species, differing in their sexual characters (Barrett 2002).

Heterostyly is a stylar polymorphism that comprises populations of a given species bearing two (distyly, Fig. 1A) or three floral morphs (tristyly, Fig. 1B) (Barrett *et al*. 2000; Barrett 2002; Ferrero 2009). These morphs differ in the reciprocal arrangement of anthers and stigmas within the flowers (Fig. 1; Barrett and Shore 2008). In distylous populations, long-styled flowers (L-morph) have the stigma at the highest level and the anthers below, while the short-styled flowers (S-morph) are characterized by a whorl of anthers at the highest position and the stigma below (Fig. 1A). Similarly, tristylous populations have L-morph and S-morph flowers, but also mid-styled flowers (M-morph) with the stigma located between the two sets of anthers (Fig. 1B). Additionally to the reciprocal arrangement of anthers and stigmas, known as reciprocal herkogamy, heterostylous species present a diallelic sporophytic heteromorphic incompatibility system apparently controlled by two loci, *Ss* and *Mm* (Lewis and Jones 1992). This incompatibility system is responsible for self- and morph-incompatibility, with legitimate pollinations occurring only between reciprocal anthers and stigma of flowers from different individuals (Barrett and Shore 2008; Ferrero 2009). Finally, ancillary characters such as differences between morphs in pollen size and production, papillae size and shape or corolla size can also occur in heterostylous species (Barrett *et al*. 2000; Ferrero 2009).

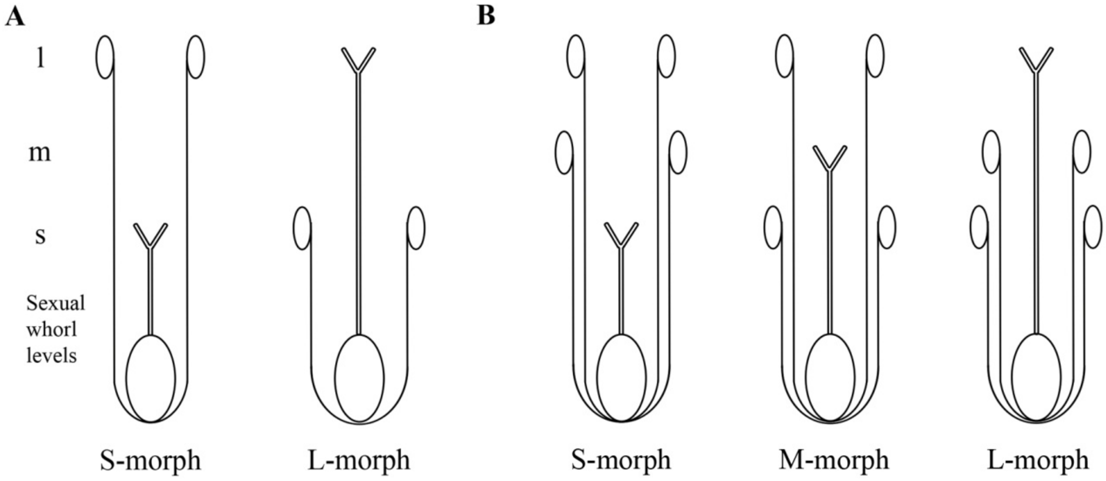

Figure 1. Schematic representation of heterostylous flowers: **A.** Distyly; **B.** Tristyly. Floral morphs: S-morph, short-styled; M-morph, mid-styled; L-morph, long-styled. The whorls of anthers are also illustrated: l, m and s for long, mid and short anther levels, respectively.

Other sexual polymorphisms have been described with the common feature of a variable position of the stigma in relation to the anthers (Barrett *et al*. 2000). As examples: stylar dimorphism, in which only the stigma length varies in relation to the anthers (Barrett *et al*. 1996, 2000); enantiostyly, involving flowers comprising mirror images (Barrett *et al*. 2000; Jesson *et al*. 2003); flexistyly, involving stigma movement out of the way when anthers are dehiscent (Li *et al*. 2001); and inversostyly, a polymorphism in which the floral morphs display reciprocal vertical positioning of sexual organs (Pauw 2005).

0.3. Functional significance of heterostyly

Sexual polymorphisms have been described in approximately 28 botanical families (Barrett *et al*. 2000; Barrett and Shore 2008). Darwin postulated that reciprocal herkogamy was of major importance in promoting efficient cross-pollinations between reciprocal floral morphs (*disassortative mating*; Darwin 1877; Barrett 1992). This hypothesis has been successfully tested by several authors through controlled pollination experiments in heterostylous species (reviewed in Lloyd and Webb 1992). Currently, it is well recognized that heterostyly enhances both female and male sexual fitness (Barrett 2002). On one hand, the reciprocal arrangement of anthers and stigma between floral morphs has been described as a mechanism to (1) minimize sexual interference between male and female functions and to (2) increase the precision of pollen transfer between reciprocal floral morphs, promoting cross-pollination (Fig.2; Barrett 2002). This is achieved by a precise deposition of pollen along the pollinator's body corresponding to the reciprocal level of stigma, thus favoring male function requirements (Barrett 2002). On the other hand, the sporophytic heteromorphic incompatibility system prevents self-fertilization, as well as, intra-morph pollinations (*assortative mating*), reducing inbreeding depression and contributing to the maintenance of genetic variability of the species, thus enhancing female function (Barrett 2002).

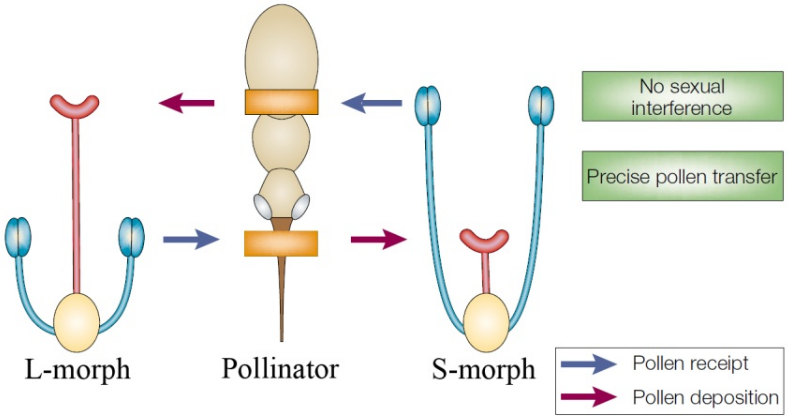

Figure 2. Illustration of the pollen deposition along the pollinator's body and transference between reciprocal floral morphs in a distylous species (adapted from Barrett 2002).

Due to the incompatibility system described above, heterostylous species are pollinator's dependent in order to spread its pollen and reach a reciprocal stigma. After long-distance dispersal and facing a new and unpredictable environment, the absence of compatible mates (Baker 1955, 1965) and the loss of pollinator mutualisms may negatively affect sexual reproduction (Traveset and Richardson 2006; Roig 2008). Thus, the replacement of the native mutualisms for new ones is a key factor for the successful establishment and subsequent colonization success of species with peculiar reproductive systems, like the heterostylous ones (Mitchell *et al*. 2006).

0.4. Establishment of new mutualisms and reproduction during invasion

Biological invasions are a serious threat to biodiversity, leading to significant ecological and evolutionary consequences, both for the invaded communities and for the invasive species themselves (*e.g.*, Mack *et al*. 2000; Marchante *et al*. 2011). After introduction, one main barrier must be overpassed in order to a species become invasive: reproduction. When an alien is introduced in a new range, the replacement of the native mutualisms by others is the first step for successful sexual reproduction; this hypothesis is commonly known as the *mutualism facilitation hypothesis* (Mitchell *et al*. 2006). The establishment of new mutualistic interactions is particularly important in self-incompatible species due to the need of pollination vectors for successful seed production (Traveset and Richardson 2006; Roig 2008). However, this issue is frequently overpassed because most invasive species are pollinator generalists and easily establish new pollination interactions (Traveset and Richardson 2006). Another problem that invasive species may face to reproduce is the absence of compatible mates (Baker 1955, 1967). This question is particularly important in species with special reproductive systems, such as sexual polymorphisms (*e.g.*, heterostyly; Barrett 1979; Luo *et al*. 2006; Castro *et al*. 2007). It is known that founder events during invasion processes frequently lead to the loss of floral morphs in heterostylous populations and this effect is often preserved for long periods, limiting the sexual reproduction of the species (Barrett and Shore 2008). In this case, invasive heterostylous species may become strictly clonal (*e.g.*, *Oxalis debilis*, Luo *et al*. 2006; *O. corymbosa*, Tsai *et al*. 2010) or they may experience a breakdown in the self- and/or morph-incompatibility, which allows their sexual reproduction (*e.g.*, *Eichhornia paniculata*, Barrett 1979; *Lythrum salicaria*, Colautti *et al*. 2010).

0.5. Study system: *Oxalis pes-caprae*

Oxalidaceae family is composed by approximately 880 species distributed in five genera of herbaceous annuals and perennials plants: *Averrhoa* L., *Biophytum* DC., *Dapania* Korth., *Oxalis* L. and *Sarcotheca* Blume. This family is distributed all over the world, especially in tropical and subtropical regions, with few species also occurring in temperate climate areas (Sánchez-Pedraja 2008). Heterostylous flowers, namely tristylous ones are frequently found in several species of this family (Weller 1992).

The genus *Oxalis* consists of about 800 species (Hussey *et al*. 1997) and is found, mostly, in South America and Africa (Luo *et al*. 2006), with some invasive species occurring in other parts of the world, such as Mediterranean climate regions (Ornduff 1987; Castro *et al*. 2007) and Asia (Luo *et al*. 2006; Tsai *et al*. 2010).

Figure 3. *Oxalis pes-caprae*: **A. – C.** S-, M- and L-morph, respectively; **D.** Multipetal sterile form; **E.** Invaded field; **F.** Bulbs produced by this invasive species.

Oxalis pes-caprae L. is a south-African geophyte that was introduced as an ornamental plant in several areas of the world and has become a widespread invasive weed in regions with Mediterranean climate (Fig. 3E; Ornduff 1987; Vilà *et al*. 2006; Castro *et al*. 2007). In its native range, this species displays tristylous flowers (Fig. 1B; Fig. 3A-C) and presents three cytotypes (2*x*, 4*x* and 5*x*) (Fig. 4, Ornduff 1987). In the invaded area of the Mediterranean basin, a shift to obligate asexuality through clonal propagation was observed as a result of founder events, as a consequence of the introduction of only one floral morph, the S-morph (Ornduff 1987). Successful clonal propagation is guaranteed in *O. pes-caprae* not only by the high production of bulbils (Fig. 3F; Ornduff 1987; Pütz 1994), but also by the contractive capacities of its roots (Galil 1968; Pütz 1994). However, in the last years, new floral morphs and cytotypes (Castro *et al*. 2007; Castro *et al*. 2009; Ferrero *et al* 2011) and the sporadic observation of fruits have been described in the invaded range of the Mediterranean basin (Costa *et al*. 2010; Ferrero *et al.* 2011). Facing these observations, *O. pes-caprae* revealed to be an excellent study system to address questions concerning its reproductive strategy during the invasion process and providing new insights on the function and evolution of heterostyly.

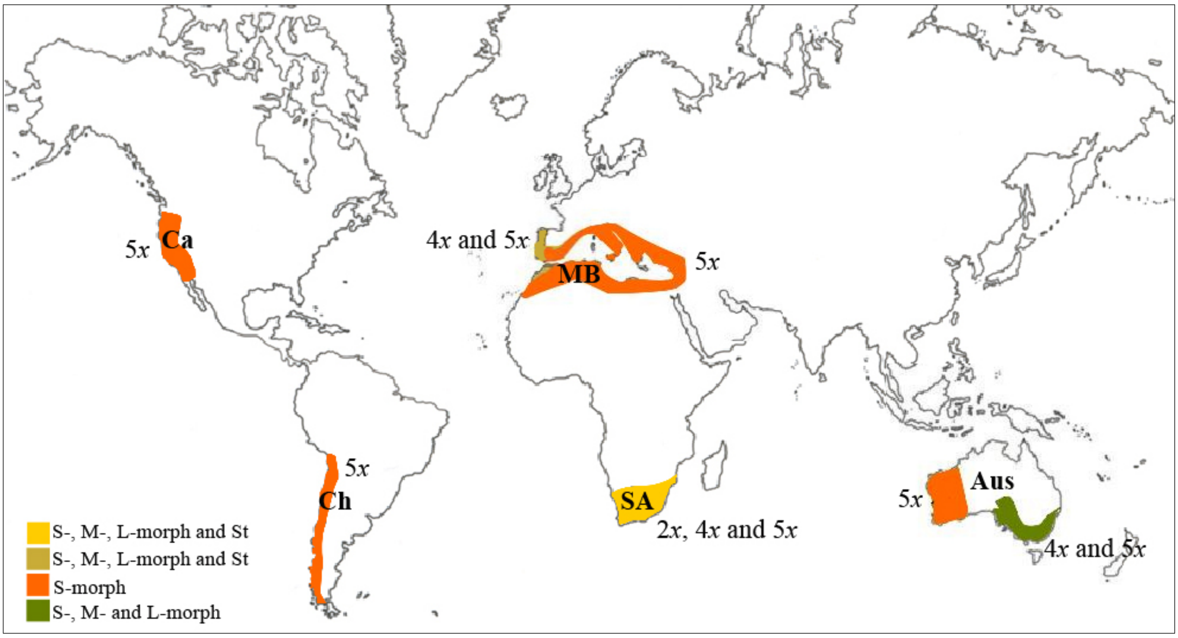

Figure 4. Distribution patterns of *Oxalis pes-caprae* from its native and invasive ranges, South Africa and Mediterranean climate regions of the world, respectively. The floral morphs and cytotypes reported for each area are also provided (South Africa, California, Chile, Ornduff 1987; Mediterranean basin, Castro *et al* 2007, Castro *et al* 2009; and Australia, Symon 1961, Michael 1964). Additionally, a multipetal sterile form (St) is reported for South Africa and for the Mediterranean region (see also Fig. 3D).

0.6. Objectives and structure of the thesis

This Master Thesis was integrated within a broader FCT project on the evolutionary changes of reproductive systems during the invasion process of the polyploid *O. pes-caprae* and had two main objectives centred in the invaded range of the western Mediterranean region: 1) to assess the reproductive system of *O. pes-caprae* in the invaded range by investigating the ability of the 5*x* S-morph to produce viable offspring; and 2) to determine the new mutualistic interactions at the pollination level established in the invaded area and their role in the successful sexual reproduction of the 5*x* S-morph.

The first objective addresses part of a broader question aiming to assess the origin of the new floral morphs and cytotypes recently detected in the invaded range of the Mediterranean basin, where the following two hypothesis (not mutually exclusive) were proposed: 1) the new forms have originated in this region through incompatibility breakdown (tested in this Thesis) and/or 2) the new forms have originated after multiple introductions (in progress). The second objective addresses the new mutualistic interactions established in the invaded range and their role in successful sexual reproduction and invasion, and is also integrated in a broader question aiming to assess sexual reproductive success in invaded (studied in this thesis) *versus* native ranges (in progress). The answers to these questions will contribute for a better comprehension on the processes involved in the reacquisition of sexuality, and consequent production of viable offspring, which may have several important implications for the continuous spread of this invasive species. The present study combines an experimental approach integrating both greenhouse experiments with controlled hand-pollinations and field observations on pollinators' behaviour.

In accordance with the proposed objectives, this Master Thesis was organized in two main chapters as follows:

Chapter I: *Reacquisition of sexual reproduction in the invasive short-styled Oxalis pes-caprae.* In this chapter, the morph- and self-incompatibility system of this invasive species were tested through controlled hand-pollination experiments in order to assess if its breakdown could be one of the factors involved with the emergence of new forms.

Chapter II: *Reproductive success of Oxalis pes-caprae in populations with different morph proportions*. In this chapter, through floral visitor's observation in natural populations, pollinator's assemblage and foraging behaviour were determined to assess the role of the new established mutualisms in the successful sexual reproduction of this species in the invaded area.

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Chapter I

Reacquisition of sexual reproduction in the invasive short-styled *Oxalis pes-caprae*

1.1. Introduction

One key factor for the successful establishment and spread of introduced species, at least 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 and Richardson 2007). Because clonality affects the spatial distribution of genets and its flowers determining the opportunities for cross-fertilization, 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 affecting negatively both male (*e.g.*, Harder and Barrett 1996) and female fitness (*e.g.*, Vallejo-Marín and Uyenoyama 2004; Porcher and 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 heterostylous populations, the plants present two or three floral morphs that differ reciprocally in the position of their sexual organs (Barrett 1992). Heterostylous plants are usually self-incompatible and, in addition, present an incompatibility system that only allows crosses among reciprocal stamens 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 pes-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, the growth and persistence in the new range when the 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 bears 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 the breakdown of the self-incompatibility, as individuals with some levels of compatibility would have advantage under low density conditions and would be able to establish new populations after dispersal (*Baker's law*, Baker 1955; Stebbins 1957; Baker 1967). In heterostylous systems, such phenomena 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.*, distylous *taxa*: *Amsinckia* spp., Schoen *et al*. 1997; *Primula* spp., Mast *et al*. 2006; *Turnera ulmifolia*, Barrett and Shore 1987; and *Psychotria* spp, Sakai and Wright 2008; tristylous *taxa*: *Eichhornia* spp., Barrett 1985; Barrett 2011; and *Oxalis corymbosa*, Tsai *et al*. 2010).

Oxalis pes-caprae L. is a tristylous species (Fig. 1) native from South Africa with a typical heteromorphic incompatibility system responsible for self- and morph-incompatibility (Ornduff 1987). This geophyte was introduced in Mediterranean climate regions all over the world where it became a widespread invasive weed (Symon 1960; Baker 1965; Ornduff 1987). In most invaded regions a shift to obligate asexuality was observed as a result of founder events after the introduction of the short-styled morph, only (Baker 1965; Ornduff 1987). However, in the last years, new floral morphs (mid- and long-styled) and the occurrence of sexual reproduction have been described in the invaded range of the western Mediterranean basin where the pentaploid short-styled morph (5*x* S-morph) is the most frequent floral morph (Castro *et al*. 2007, Ferrero *et al*. 2011).

The classical genetic studies in tristylous plants indicates that the floral morph is controlled by two loci, *Ss* and *Mm*, with the long-styled 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 and Jones 1992). This system has also been demonstrated in some *Oxalis* species (Weller 1976). In a parallel study, Ferrero *et al*. (2011) 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 assess the incompatibility system of *O. pes-caprae* in the invaded region of the western Mediterranean basin by investigating the ability of the short-styled morph to produce viable offspring. For this, controlled hand pollinations involving legitimate (between morphs) and illegitimate (within morph) crosses were performed and pollen germination, pollen tube development along the style, fruit and seed production and seed germination were assessed. The ploidy level of the offspring was also estimated.

1.2. Material and Methods

1.2.1. Plant material and study area

Oxalis pes-caprae L. (Oxalidaceae) is a perennial bulbous plant with a profuse production of bulbils that, associated with the contractile properties of its roots, confers a high ability to reproduce asexually (Pütz 1994; see also Fig. 3E-F from *Introduction*). *O. pes-caprae* is a tristylous species (short-, mid- and long-styled floral morphs, 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 its native range, this species is composed by three cytotypes (diploids, 2*x*; tetraploids, 4*x*; and pentaploids, 5*x*) (Ornduff 1987), while in the invaded region of the Mediterranean basin a shift to obligate asexuality was observed as a result of founder events with the introduction of the 5*x* S-morph, only (Ornduff 1987). Recently, new floral morphs (M-morph, L-morph and a sterile form) and cytotypes (4*x*) have been described in this invaded area (Castro *et al*. 2007; Ferrero *et al*. 2011; see also Fig. 4 from *Introduction*). The flowering occurs from early January to late April.

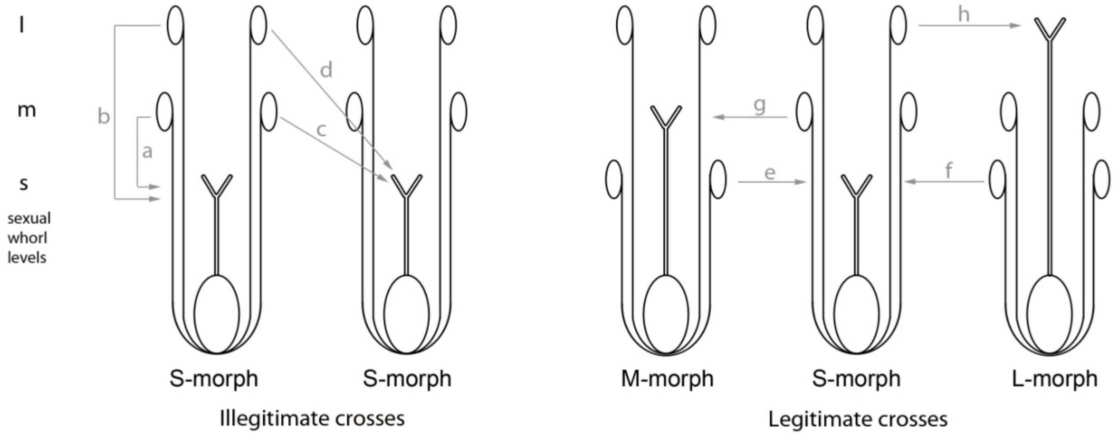

Figure 1. *Oxalis pes-caprae* floral morphs and crosses performed in hand pollination experiments: self-pollinations with pollen from the mid- (a) and long-anther levels (b) and intra-morph pollinations with pollen from the mid- (c) and long-anther levels (d) and inter-morph legitimate pollinations with 5*x* S-morph as pollen recipient (e, f) and as pollen donor (g, h). S-morph, M-morph and L-morph for short-, mid- and long-styled floral morphs. The anther levels are represented by l, m and s for long-, mid- and short-whorl, respectively.

This study was carried out during 2009 and 2010 with plants from Colares (Estremadura province, Portugal). Plants were collected in the field during winter of 2009 before flowering. Thirty-five plants per floral morph (S-morph, M-morph and L-morph) were directly collected to pots, identified with an ID number and maintained in the nurseries of the Botanical Garden of the University of Coimbra under natural conditions. The plants collected were separated at least 5 m apart to guarantee the sampling of different individuals.

1.2.2. Ploidy level analysis

Because there are two cytotypes reported to occur in the invaded range of the Mediterranean region (4*x* and 5*x*; Castro *et al.* 2007; Ferrero *et al.* 2011), ploidy level of all plants collected was analysed using flow cytometry (FCM). Samples were prepared following Galbraith *et al.* (1993) procedure and the two-step nuclear isolation method with Otto's buffers (Otto 1992; Doležel and 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 of Otto I solution (100 mM citric acid, 0.5 % (v/v) Tween 20); the solution was filtered into a cytometer sample tube using a 50 μm nylon filter and 1 ml of Otto II solution (400 mM $Na_2PO_4.12H_2O$) was added; finally, 50 μg mL^{-1} propidium iodide was added to stain the nuclei and 50 μg mL^{-1} of RNAse 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'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 fluorescence by that of *B. perennis* and plants were identified as 4*x* or 5*x* for genome size values of 1.37 ± 0.056 (n = 39) and 1.66 ± 0.030 (n = 248)(mean ± SD, followed by sample size in parenthesis), respectively (Castro *et al.* 2007).

1.2.3. Hand pollination experiments

To assess the ability of the 5*x* 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 5*x* S-morph and the following treatments were performed: self-pollinations with pollen from the mid- and long-anther levels (selfing 5*x* S_m and selfing 5*x* S_l, respectively) and intra-morph pollinations with pollen from the mid and long anther levels (5*x* S × 5*x* S_m and 5*x* S × 5*x*

S_l, respectively) (Fig. 1). Legitimate pollinations were carried out to assess the ability of the 5*x* S-morph to produce viable offspring through its ovules and pollen grains and, thus, the following treatments were performed: inter-morph legitimate pollinations with 5*x* S-morph as pollen recipient (5*x* S × 4*x* M_s and 5*x* S × 4*x* L_s) and as pollen donor (4*x* M × 5*x* S_m and 4*x* L × 5*x* S_l) (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 3-5 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 3h, 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 in 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 upper 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 using the procedure described above.

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

1.2.4. Seed germination

The seeds obtained from the hand pollination experiments were placed to germinate in 6 × 6 cm pots filled with common garden substrate at the nurseries of the Botanical Garden (University of Coimbra) under natural conditions in September 2010. Pots were monitored weekly during 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*.

1.2.5. Statistical analysis

Descriptive statistics (mean and standard error of the mean) were calculated for pollen germination, number of pollen tubes developed along 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, number of viable seeds and seed germination were analysed using a 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. LSmeans were used to analyse differences between treatments. All the analyses were performed in STATISTICA 7.0 (Stat Soft. Inc., Tulsa, OK, USA), except LSmeans that were carried in SAS version 9.2 (SAS Institute Inc, Cary, North Carolina).

1.3. Results

Results from hand pollination experiments are given in Figure 2 and Appendix 1.1. Pollen grains from 5*x* S-morph, 4*x* M-morph and 4*x* L-morph were able to germinate on the recipient stigmas but statistically significant differences were observed in germination rates ($\chi_7^2 = 14.57$, $P = 0.0419$): higher germination rates were observed in legitimate crosses (although no significant differences were found for 5*x* S × 4*x* L_s and 4*x* L × 5*x* S_l crosses) and in self and intra-morph pollinations when pollen from the mid-anthers of 5*x* S-morph was used (Fig. 2A). Pollen tube development was observed in all illegitimate (Fig. 3A) and legitimate crosses despite the significant differences observed between pollination treatments ($\chi_7^2 = 9.14$, $P < 0.0001$), with legitimate pollinations having significantly higher pollen tubes than in illegitimate ones (Fig. 2B).

The mean number of ovules produced by each floral morph was not significantly different (mean ± SE: 39.6 ± 1.0; $F = 0.53$, $P = 0.59$). Fruit production and number of viable and aborted seeds per fruit were significantly different between pollination treatments ($\chi_7^2 = 65.65$, $P < 0.0001$; $\chi_5^2 = 2.90$, $P = 0.0005$; $\chi_5^2 = 33.26$, $P < 0.0001$ respectively; Fig. 2C-E). Selfing crosses did not yield any fruits and significantly greater fruit set was found in legitimate crosses when 5*x* S-morph was used as pollen donor (Fig. 2C). Legitimate crosses tend to produced greater numbers of viable seeds per fruit (Fig. 3B) than illegitimate crosses but no significant differences were found between them and the 5*x* S × 5*x* S_l (Fig. 2D). Concerning the number of aborted seed in

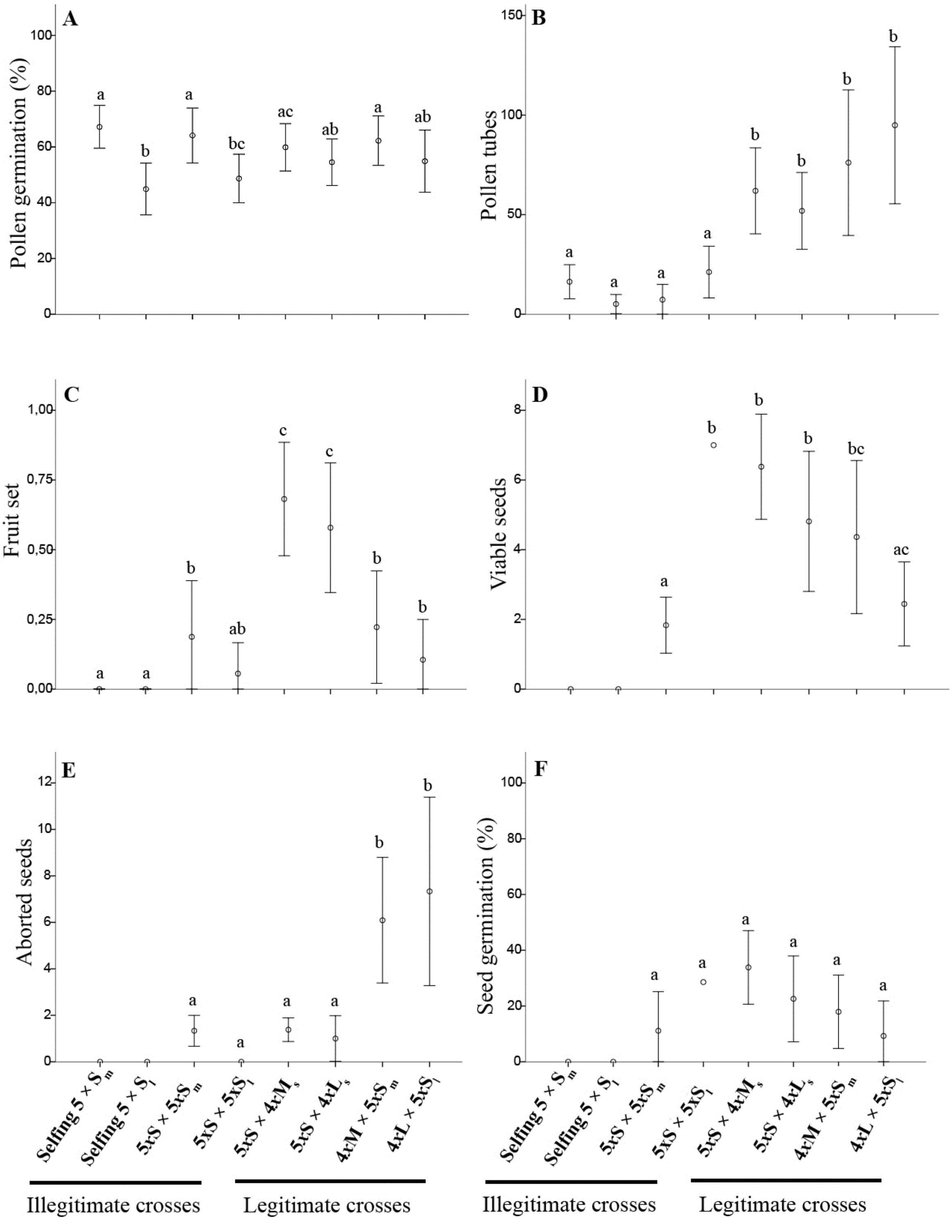

Figure 2. *Oxalis pes-caprae* sexual reproduction in the invaded range of the Mediterranean basin: **A.** percentage of pollen germination; **B.** mean number of pollen tube development along the style; **C.** fruit set; **D.** mean number of viable seeds; **E.** mean number of aborted seeds and **F.** percentage of seed germination. In pollination treatments, the first individual represents the pollen receptor and the second the pollen donor; for pollen donors anther level is also provided: s, m and l for short, mid and long whorls of anthers, respectively.

legitimate crosses two statistically different groups could be distinguished with greater seed abortion in pollinations where 5*x* S-morph was used as pollen donor (Fig. 2E).

Seed germination revealed no statistically significant differences among pollination treatments ($\chi_5^2 = 1.38$, $P = 0.8891$) and ranged between 11.1 and 34.7%, being possible to obtain seedlings from both illegitimate and legitimate crosses (Fig. 2F and 3C). Flow cytometric analysis of the germinated offspring revealed that both 4*x* and 5*x* were produced in illegitimate and legitimate crosses (Appendix 1). The low number of seedlings obtained from illegitimate crosses made it difficult to entangle the cytotype patterns in the offspring. In legitimate crosses, 5*x* offspring was only obtained when 5*x* S-morph was used as pollen recipient; still, the 4*x* was the most frequent cytotype in the offspring; when 5*x* S-morph was used as pollen donor, the offspring was composed by 4*x*, only (Appendix 1).

Figure 3. *Oxalis pes-caprae* sexual reproduction in the invaded range of the Mediterranean basin. **A.** pollen germination and pollen tube development in the style after illegitimate pollinations (5*x* S × 5*x* S_m); **B.** fruit with morphologically viable seeds after legitimate pollinations (5*x* S × 4*x* M_s; bar = 1 mm); **C.** seedlings obtained after illegitimate pollinations.

1.4. Discussion

After long-distance dispersal, reproductive strategies are of major importance for the successful colonization of invasive species (*e.g.*, Pyšek and Richardson 2007; Barrett 2011). In heterostylous 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 been already documented in several heterostylous species (Ornduff 1972; Barrett 1989; Barrett 1992; Weller 1992), including some invasive ones (Barrett and Shore 2008; Colautti *et al.* 2010). Despite fruit and seed production had not been completely ruled out in the invaded range of *O. pes-caprae* where the 5*x* 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 breakdown in its 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 heterostylous 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 and 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 suggest a possible late-acting self-incompatibility system in *O. pes-caprae*. This system has been described in several others species such as *Cyrtanthus breviflorus* (Vaughton *et al.* 2010), *Narcissus* spp. (Dulberger 1964, Sage *et al.* 1999), *Anchusa officinalis* (Schou and Philipp 1983), *Asclepias exaltata* (Lipow and Wyatt 2000) and *Spathodea campanulata* (Bittencourt *et al.* 2003), however further work must be done in order to confirm this in *O. pes-caprae*.

Contrarily to self-pollinations, intra-morph crosses resulted in the production of fruits, seeds and seedlings, showing a breakdown in the morph-incompatibility system of *O. pes-caprae* in this invaded area. Still, pollen tube development and fruit and seed production were slightly 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. Despite no fruit production was observed, Castro *et al.* (2007) had already reported sporadic pollen tube development 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 cytotype composition with variable sexual reproductive outcomes across the surveyed area (Ferrero *et al*. 2011). 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 its genetic structure (Ray and Chisaki 1957; Ganders 1979; O'Brien and Calder 1989). In addition, the 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, despite multiple introduction events could be also involved in the process.

Legitimate pollinations were performed to assess the ability of the 5*x* 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 1*x*, 2*x*, 3*x*, 4*x* and/or 5*x* gametes in lower numbers (Ramsey and Schemske 1998; Risso-Pascotto *et al*. 2003). Consequently, they are expected to be mostly sterile (Ramsey and Schemske 1998). Meiotic abnormalities producing microspores with variable number of chromosomes have been described in *O. pes-caprae* (Vignoli 1937). Despite no differences were observed in pollen tube development along the style, 5*x* S-morph individuals were more successful as pollen recipient than as pollen donor. Still, our results showed that 5*x* S-morph individuals were able to produce some viable pollen grains and ovules that, after legitimate pollinations, yielded viable offspring. The prevalence of 4*x* in the offspring also seems to indicate that 2*x* gametes were favourably recruited for seed production. Bi-nucleate microspores and 2*n* 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 *Aloineae*, Brandham (1982) showed that plants with odd ploidy levels (3*x*) still bear some fertility contributing with either 1*x* or 2*x* gametes when crossed with 2*x* or 4*x* plants, respectively. The bias in the frequency of progeny ploidy levels resulted from seed abortion when the ratio of material 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 4*x* seedlings in the 4*x* × 5*x* and 5*x* × 4*x* crosses with *O. pes-caprae* plants.

In conclusion, the breakdown in the morph-incompatibility system combined with the ability of the 5*x* S-morph to produce some viable gametes opened the possibility for the sexual reproduction and may be one of the mechanisms involved in the emergence of new floral morphs and cytotypes 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 for reproductive success and morph proportions of *O. pes-caprae* in this invaded region, large-scale pollination experiments are currently being performed through the invasive range of the western Mediterranean region.

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Appendix 1.1. Results from controlled hand pollination experiments.

	Pollen				Seeds			Seed	Offspring
Pollination treatment	***n***	**Germination**	**Pollen tubes**	**Fruit set**	***n***	**Viable**	**Aborted**	**germination**	**ploidy**
Illegitimate crosses									
Selfing 5*x* S_m	20	67.0 ± 0.04 ^{a}	16.3 ± 4.3 ^{a}	0.0 (22) ^{a}	-	-	-	-	-
Selfing 5*x* S_l	17	45.0 ± 0.05 ^{b}	5.1 ± 2.4 ^{a}	0.0 (26) ^{a}	-	-	-	-	-
5*x* S × 5*x* S_m	16	63.2 ± 0.04 ^{a}	20.4 ± 6.5 ^{a}	21.4 (28) ^{b}	6	1.8 ± 0.4 ^{a}	1.3 ± 0.4 ^{a}	11.1 ± 7.0 (6) ^{a}	5*x* (1)
5*x* S × 5*x* S_l	18	49.0 ± 0.04 ^{bc}	21.2 ± 6.5 ^{a}	4.4 (23) ^{ab}	1	7.0 ^{b}	0.0 ^{a}	28.6 (1) ^{a}	4*x* (2)
Legitimate crosses									
5*x* S × 4*x* M_s	22	60.0 ± 0.04 ^{ac}	62.0 ± 10.9 ^{b}	70.0 (30) ^{c}	21	6.4 ± 0.8 ^{b}	1.4 ± 0.3 ^{a}	34.7 ± 6.9 (20) ^{a}	4*x* (15); 5*x* (5)
5*x* S × 4*x* L_s	19	55.0 ± 0.04 ^{ab}	51.9 ± 9.7 ^{b}	61.5 (26) ^{c}	16	4.8 ± 1.0 ^{b}	1.0 ± 0.5 ^{a}	25.8 ± 8.5 (14) ^{a}	4*x* (12)
4*x* M × 5*x* S_m	18	62.0 ± 0.04 ^{a}	76.1 ± 18.3 ^{b}	39.3 (28) ^{b}	11	4.4 ± 1.1^{bc}	6.1 ± 1.4 ^{b}	18.2 ± 7.6 (9) ^{a}	4*x* (9)
4*x* L × 5*x* S_l	19	55.0 ± 0.06 ^{ab}	94.9 ± 19.7 ^{b}	27.3 (33) ^{b}	9	2.4 ± 0.6 ^{ac}	7.3 ± 2.0 ^{b}	11.9 ± 8.4 (7) ^{a}	4*x* (1)
Statistical test		$\chi_7^2 = 14.57$, $P = 0.0419$	$\chi_7^2 = 40.61$, $P < 0.0001$	$\chi_5^2 = 32.25$, $P < 0.0001$		$\chi_5^2 = 22.19$, $P = 0.0005$	$\chi_5^2 = 35.46$, $P < 0.0001$	$\chi_5^2 = 1.70$, $P = 0.8891$	

Notes: Floral morphs: S, S-morph; M, M-morph; L, L-morph. Cytotypes: 4*x*, tetraploid; 5*x*, pentaploid. '-', absence of seed production. In pollination treatments, the first individual represents the pollen receptor and the second the pollen donor; for pollen donors anther level is also provided: s, m and l for short, mid and long whorls of anthers, respectively. Values are given as mean and standard error of the mean. Sample size is given as *n* for pollen and seed variables; sample size for the remaining variables is provided in parentheses. Germination provides the percentage of germinated pollen grains in the stigmatic papillae and pollen tubes the number of pollen tubes along the style. Fruit set and seed germination are also given in percentage. Statistical comparisons among pollination treatments are also provided in *Statistical test* for all the variables. Different letters reveal statistically significant differences. The ploidy of the offspring, followed by the number of seedlings analyzed in parentheses, is also provided.

Chapter II

Reproductive success of *Oxalis pes-caprae* in populations with different morph proportions

2.1. Introduction

Under a global changing World, biological invasions are among the most concerning threats to Biodiversity (Walker and Steffen 1997; Richardson and Pyšek 2008; Vilà *et al.* 2011). After long-distance dispersal, the reproductive strategies of alien plants are one of the critical steps for their establishment and spread (Lloret *et al.* 2005; Pyšek and Richardson 2007). According to *Baker's Law* (Baker 1955, 1967), pollinator's limitation and lack of compatible mates are the major barriers to invader's sexual reproduction in the new range, possibly forcing them to clonality or selfing. Vegetative reproduction has already been reported for several invasive species (*e.g., Oxalis pes-caprae*, Ornduff 1987; Castro *et al*. 2007; *Elodea canadiensis*, Bowmer *et al*. 1995; *Fallopia japonica*, Forman and Kesseli 2003), acting as an initial strong advantage that enables their persistence and growth in the new area (Richardson *et al*. 2000). Nevertheless, exclusive clonal populations are expected to present less genetic diversity, which may be disadvantageous in long-term when facing new and unpredictable scenarios (Holsinger 2000). Another strategy is selfing; individuals with some levels of compatibility will have advantage in the establishment of new populations under low density conditions in comparison with self-incompatible ones (Baker 1955; Stebbins 1957; Baker 1967).

Pollination mutualisms play an important role in plant's diversification, with most flowering plants depending on pollinators to reproduce (Bronstein *et al.* 2006). Thus, when an exotic plant is introduced in a new area, the scarcity or inexistence of pollinators may limit the reproductive success of the introduced plant and, consequently, restrict their expansion range (Baker 1955, 1967). The replacement of the lost plant-pollinator mutualisms from the native range by new ones from the novel area is fundamental for a successful invasion and is commonly recognized as the *mutualism facilitation hypothesis* (Richardson *et al.* 2000; Mitchell *et al.* 2006). However, because exotic plants are mostly pollinator's generalists, their integration into the new mutualistic networks is usually straightforward (*e.g.,* Crawley 1989; Richardson *et al.* 2000; Traveset and Richardson 2006; Lopezaraiza-Mikel *et al.* 2007). Indeed, it has been suggested that the absence of compatible mates, rather than the limitation in pollination services, is one of the main barriers for the establishment of alien species (van Kleunen and Johnson 2007). This is especially critical for obligate out-crosser plants, such as heterostylous or strong self-incompatible species (*e.g.,* Mal *et al.* 1992; Harrod and Taylor 1995). Compatible mates limitation has already been observed not only in large scale surveys (van Kleunen and Johnson 2007), but also in particular invasive species (*e.g., Centaurea* spp. and *Acroptilon repens*, Harrod and Taylor 1995; *Lytrhum salicaria*, reviewed in Mal *et al.* 1992).

Heterostylous species are characterized by the presence of two or three floral morphs (distyly and tristyly, respectively) differing reciprocally in the positioning of their sexual organs (anthers and stigmas; see Fig. 1 from *Introduction*; Barrett 1992). Most heterostylous species are self-incompatible and, additionally, only crosses between reciprocal stamens and stigmas of compatible morphs are allowed (intra-morph incompatibility; Barrett 1992). Through negative-frequency dependent selection, disassortative mating together with heteromorphic incompatibility leads natural populations of heterostylous species to isoplethy (*i.e.,* equal floral morph proportions). However, deviations from isoplethy may occur in clonal species, in newly established populations, and/or after population disturbance (Morgan and Barrett 1988; Barrett 1992). Founder events after the introduction of a single morph in a new range will also lead to anisoplethic populations with strong negative consequences on the plant's sexual reproductive success due to the lack of compatible mates (*e.g.*, *Oxalis pes-caprae*, *Castro et al*. 2007; *O. debilis*, Luo *et al.* 2006). Thus, studies assessing reproductive success in anisoplethic populations from the invaded range are of major importance to understand the contribution of reproduction to the successful spreading of heterostylous species.

Oxalis pes-caprae L. is a tristylous invasive species in regions with Mediterranean climate (Ornduff 1987; Castro *et al.* 2007) that was forced to asexuality as a result of founder events due to the introduction of only one floral morph (the S-morph; Michael 1964; Ornduff 1987). However, the occurrence of mixed populations composed by different floral morphs and cytotypes has been recently reported in the western Mediterranean basin (Castro *et al.* 2007; Ferrero *et al.* 2011). A weakening in the self-incompatibility and a breakdown in the morph-incompatibility system in this area was shown in Chapter I and was proposed as a possible explanation for the appearance of new forms. Thus, after observing the recent reacquisition of sexuality in this invasive species, the next step is now to assess the sexual reproductive success in the invasive populations under natural conditions. In addition, deviations from isoplethy are a relatively common feature in some species of *Oxalis* in the native range (*e.g.*, Marco and Arroyo 1998; Turketti 2010). Facing all these observations, the main objective of the present study was to assess the sexual reproductive success of *O. pes-*

caprae in invasive populations from the western Mediterranean basin presenting different floral morph compositions. It is expected that, (1) *O. pes-caprae* establishes new interactions with pollinators from the novel area independently of the population's morph composition, and, regardless of the breakdown in the incompatibly system (Chapter I), (2) an increasing morph number within the population leads to higher disassortative pollen flow and, consequently, higher female reproductive success. To achieve this objective, mono-, di- and trimorphic populations were selected, floral morphs were characterized morphologically, pollinator assemblage and behaviour were monitored and the male and female reproductive success were quantified.

2.2. Material and methods

2.2.1. Plant material and study area

Oxalis pes-caprae L. (Oxalidaceae) is a south-African bulbous plant that was introduced as ornamental in several areas of the world and has become a widespread invasive weed in regions with Mediterranean climate (Ornduff 1987; Vilà *et al.* 2006; Castro *et al.* 2007). A rosette of leaves emerges from the rhizome apex with green heart-shaped leaflets usually presenting purple spots. The flowers are actinomorphic yellow and are arranged in terminal umbellate cymes (Coutinho 1939; Sánchez-Pedraja 2008). This species is described as tristylous, being composed by three floral morphs (short-, mid- and long-styled floral morphs; S-, M- and L-morphs, respectively; see Figure 1 from Chapter I; Ornduff 1987). In the invaded range of the western Mediterranean basin, it flowers from January to April (Castro *et al.* 2007).

This study was carried out during the flowering season of 2012 in three natural populations from the invaded range differing in the floral morphs composition: Coimbra, monomorphic population of the S-morph (40°12'21''N, 8°25'26''W); Cortegaça, dimorphic population of the S- and L-morphs (40°56'25''N, 8°39'19''W); and Alto da Praia Grande, trimorphic population bearing the three floral morphs (38°47'52''N, 9°28'35''W).

2.2.2. Floral characterization

Two to three longitudinal transects across each population studied were performed to assess floral morph proportions. The floral morph of a minimum of 100 individuals separated 5 m apart was recorded. One flower per plant from 10 distinct individuals of each floral morph was collected and harvested in 70% ethanol for morphological measurements. In the laboratory, the corolla was removed, the sexual organs were photographed and the following parameters were measured using Image Tool v. 3.00 for Windows (Wilcox *et al.* UTHSCSA): (1) corolla length; (2) style length (from the corolla insertion up to the stigma); and (3) stamen height (from the corolla insertion up to the midpoint of the anther for each of the two anther whorls). Descriptive statistics (mean and standard error of the mean) were calculated for all the floral measurements. The reciprocity indices were calculated for dimorphic and trimorphic populations using the Excel macro RECIPRO (Sánchez *et al.* submitted). The reciprocity index enables to compare stigma and stamen height gaps among potential mates in the population, considering both distance and dispersion of this measure without influence of the morph frequency (Sánchez *et al.* 2008; Sánchez *et al.* submitted). This index enables comparisons between populations and species and varies between 0 (not reciprocal) and 1 (maximum reciprocity) (Sánchez *et al.* 2008; Sánchez *et al* submitted). Due to the presence of only one morph the reciprocity index in the monomorphic population is zero.

2.2.3. Floral visitor's assemblage

To assess the mutualistic interactions established between *O. pes-caprae* and the native insects in the invaded range, floral visitor's assemblage was determined by direct field observations. The observations were performed during the flowering peak of 2012 in the three populations studied. Six plots of approximately 2 m^2 were arbitrarily selected in each population. The observer was placed at approximately 1 m away from the plot being able to monitor all the flowers without disturbing the pollinator's behaviour. When more than one floral morph was present, stakes with flags with two or three different colours were used to identify them; this procedure enabled to record pollinator's movements between and within floral morphs. Observation sessions of 15 min per plot were conducted at different hours of the day (from 1130 to 1615h, GMT, the period of the day of corolla opening). The following variables were recorded: insect's visitor and number, morph and sequence of the flowers visited. A minimum of 18h of observation per population were performed. At the end, one specimen of each insect type/*taxon* was collected for further identification. After identification, the insects were assembled in functional groups concerning their taxonomical position, behaviour, morphology and type of collected rewards (pollen and/or nectar).

Within each population, the percentage of floral interactions to each morph was calculated for all the insect *taxa* by dividing the number of visits of the insect to a given morph by the total number of visits to that floral morph. The visitation rate to each morph within population was calculated for each functional group by dividing the number of flowers visited by the insects of each functional group by the total number of open flowers of the morph in the plot. Descriptive statistics (mean and standard error of the mean) were calculated for the number of legitimate (between morphs) and illegitimate (within morphs) pollinator visits per population and for visitation rates per population along the day. The probability to receive a visit in a 15 min period was calculated for each population by dividing the total number of flowers visited by the total number of flowers monitored (global visitation rate for each population studied); a similar approach was used to calculate the probability of a given morph to receive a visit in a 15 min period (floral morph visitation rates within a population).

2.2.4. Male reproductive success

Male efficiency was assessed using fluorescent powder dyes as pollen analogues (Waser and Price 1982; Campbell and Waser 1989). Despite of some differences in the properties of fluorescent powder dyes and pollen grains (Thomson *et al.* 1986), it has been found that powder dye closely resembles pollen, being a useful and realistic tool to asses pollen flow in the field (*e.g.*, Waser and Price 1982; Adler and Irwin 2006; Van Rossum *et al.* 2011). Thus, in each plot selected (see *Floral visitor's assemblage*), five flowers per morph were randomly chosen and fluorescent powder dye was applied to its anthers, with each floral morph having its own colour. After three days, up to 150 inflorescences per floral morph were collected across the population and the open flowers were observed in a stereo binocular microscope with UV light. The presence/absence, colour and place of dye deposition were recorded for all the flowers observed.

To assess the natural pollen flow within populations with different morph compositions, the percentage of flowers with fluorescent dye from the total number of observed flowers was calculated. To assess where the pollen was deposited (in the stigma or in other structures), the percentage of flowers with dye in the stigma from the total number of flowers that have received dye (*e.g.*, in the style or anthers), independently of its colour, was calculated. To assess the provenience of the pollen, the percentage of disassortative versus assortative dye transfer (considering transfer to the stigmas, only) was calculated; disassortative dye transfer was the percentage of stigmas with dye from a reciprocal morph and assortative dye transfer was the percentage of stigmas with dye from the same floral morph.

2.2.5. Female reproductive success

Sexual reproductive success was assessed in natural conditions by recording fruit and seed production in the populations studied. During fruiting, a minimum of 17 infructescences per morph were randomly collected for paper bags. Afterwards, the number of flowers and fruits per inflorescence and the number of morphologically viable seeds per fruit were counted using a stereo binocular microscope.

Descriptive statistics (mean and standard error of the mean) were calculated for fruit set (percentage of flowers that developed into fruit) and mean number of seeds per fruit. The overall reproductive success of each population was calculated by summing the fruit set of each floral morph present in the population weighted by its proportion in the corresponding population.

2.2.6. Statistical analysis

Differences in visitation rates among populations were assessed using Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's method for pairwise multiple comparisons. Differences in visitation rates among morphs and in legitimate *versus* illegitimate visits were analyzed independently for both di- and trimorphic populations, using GLZ with gamma and Poisson distributions, respectively, and a log link function. Additionally, differences in the legitimate *versus* illegitimate visits among populations were assessed using a comparison of more than two proportions (Zar 1984). To assess if the functional groups had a preference for a specific morph, differences in visitation rates among floral morphs and populations (using only di- and trimorphic populations) were tested for each functional group using a GLM approach. A GLZ with gamma distribution and a log function was used when normality assumptions were not fulfilled. Functional groups that only visited one morph within population were not considered in the analysis.

Differences among populations and floral morphs for natural dye flow, dye loss and disassortative *versus* assortative dye transfer were analysed using GLZ models (binomial distribution for the first and the last variable and multinomial for dye loss; and logit link function for all). Differences in fruit set and seed production among populations and morphs were assessed using Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's method for pairwise multiple comparisons. All the analyses were performed in STATISTICA 7.0 (Stat Soft. Inc., Tulsa, OK, USA).

2.3. Results

Floral morph composition and floral measurements of the populations studied are provided in Table 1 and Figure 1 (raw data is also given in Appendix 2.1). The S-morph had bigger flowers, followed by the M-morph with intermediate ones and the L-morph with the smallest flowers (Appendix 2.1). This was already visible in the field where corolla lengths enabled easy recognition of floral morphs. When analyzing sexual organ's disposition within morph, it was possible to observe that the stigmas of the S-morph tend to approach the first levels of the anthers, while the L-morph tend to have smaller anthers whorls and thus a bigger separation between stigmas and anthers (Figure 1). As a consequence, in di- and trimorphic populations, the level of anthers reciprocal to the stigma of the S-morph was the mid-level of the L-morph (Figure 1B-C). The low anther levels are located approximately at the same height in M- and L-morphs, not being reciprocal to the stigmas of the S-morph (Figure 1B-C). The monomorphic population had a reciprocity index of zero, while di- and trimorphic populations had high values of reciprocity (Table 1).

O. pes-caprae floral visitor's assemblage is presented in Appendix 2 and the most common visitors are illustrated in Figure 2. Significant differences were observed in visitation rates between populations ($H_2 = 105.28$, $P < 0.001$), with the monomorphic population having the highest global visitation rates and the trimorphic having the lowest ($P < 0.05$; Table 2). Concerning the visitation rates among floral morphs in di- and trimorphic populations, significant differences between morphs were only obtained in the trimorphic population ($\chi_2^2 = 3.66$; $P = 0.06$ and $\chi_2^2 = 6.76$; $P = 0.03$, respectively). In any case, the S-morph always had the highest visitation rates (Table 2). Within population (considering di- and trimorphic, only), illegitimate visitation rates were always significantly higher than legitimate ones ($\chi_1^2 = 681.11$ and $\chi_1^2 = 68.97$, $P < 0.001$, for di- and trimorphic populations, respectively). As expected, the illegitimate visitation rates were significantly higher in the monomorphic population ($\chi_1^2 = 246.87$; $P < 0.05$), but not significantly different between the di- and trimorphic populations ($P < 0.05$).

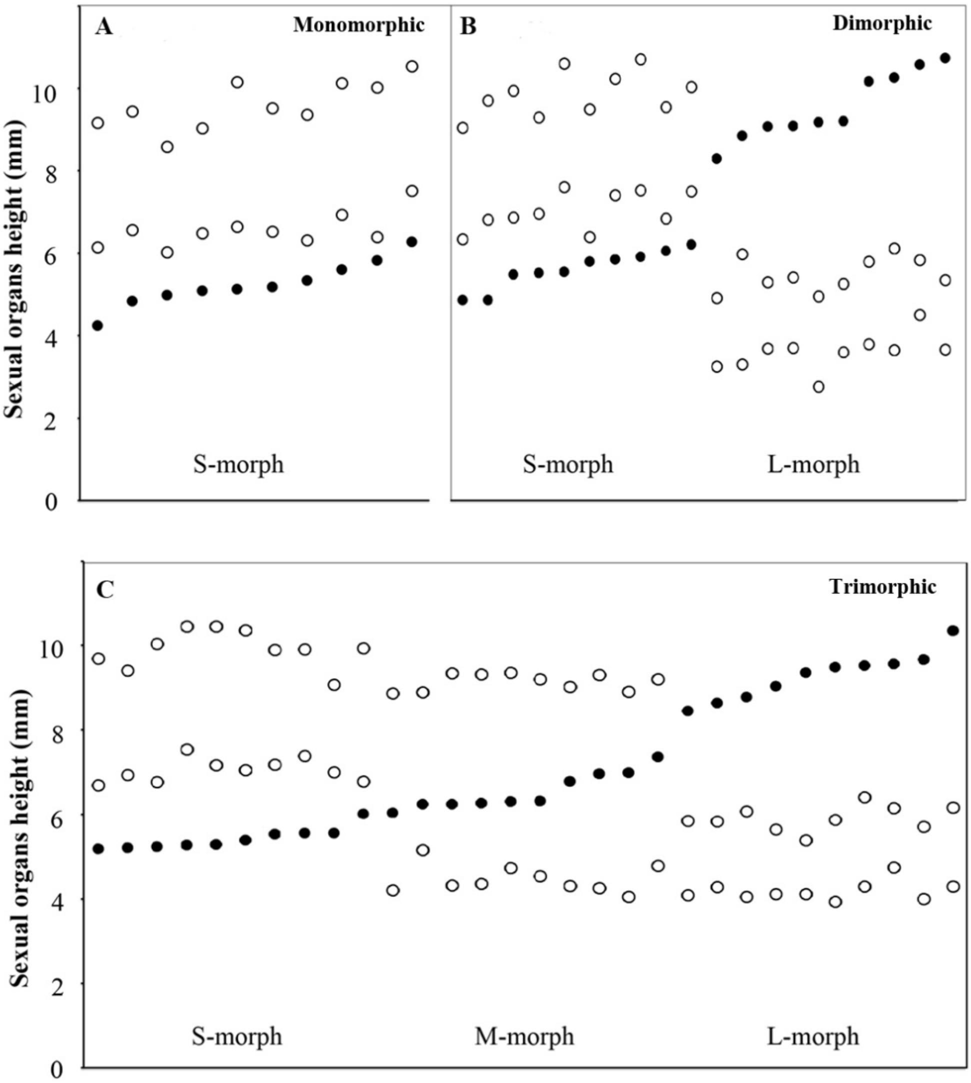

Figure 1. Sexual whorls morphometric measurements for the populations studied: **A.** Coimbra, monomorphic population; **B.** Cortegaça, dimorphic population; **C.** Alto da Praia Grande, trimorphic population. Stigma: closed circles, anthers levels: open circles. Floral morphs: S-, M- and L- for short-, mid- and long-styled floral morphs, respectively.

Despite of some common functional groups, pollinator assemblage differed among populations (Figure 3; Appendix 2.2). In general, floral visitors did not have a preference for a specific morph, except for *Psithyrus* sp. and Lepidoptera that mostly foraged S-morph flowers in the dimorphic population, and Syrphidae that visited preferentially the M-morph in the trimorphic population (Figure 3 and Appendices 2.2 and 2.3).

Results obtained for male efficiency measured as fluorescent powder dye flow are illustrated in Figure 4. Natural dye flow varied significantly between morphs (χ_2^2=

7.27; $P = 0.03$) but, surprisingly, not among populations with different morph compositions ($\chi_2^2 = 2.15$; $P = 0.34$), ranging from 13% to 21% (Figure 4 A-C).

Statistically significant differences were observed for the pollen lost in other floral structures rather than in the stigma among populations ($\chi_2^2 = 71.44$; $P < 0.05$). Despite similar dye flow (Figure 4 A-C), the monomorphic population had significantly higher pollen deposition in other structures (Figure 4 D-F; $P < 0.05$). The dimorphic population had the lowest dye loss, independently of the floral morph. Despite not significant, in the trimorphic population the L-morph had the highest values of dye deposition in the stigma (see Appendix 2.3).

Table 1. Location, floral morph composition (%) and reciprocity index for each population studied.

Population	**Geographical coordinates**		**Population type**	**Floral morphs (%)**			**Reciprocity index**
				S-	**M-**	**L-**	
1. Coimbra	40º12'21''N	8º25'26''W	Monomorphic	100.0	-	-	0.00
2. Cortegaça	40º56'25''N	8º39'19''W	Dimorphic	57.4	-	42.6	0.74
3. Alto da Praia Grande	38º47'52''N	9º28'35''W	Trimorphic	21.2	27.2	51.6	0.70

Notes: Floral morphs: S-, M- and L- for short-, mid- and long-styled floral morphs, respectively; "-" indicates absence of a given floral morph in the population. Reciprocity index was calculated using RECIPRO and varies between 0 (not reciprocal) and 1 (maximum reciprocity; Sánchez *et al* submitted).

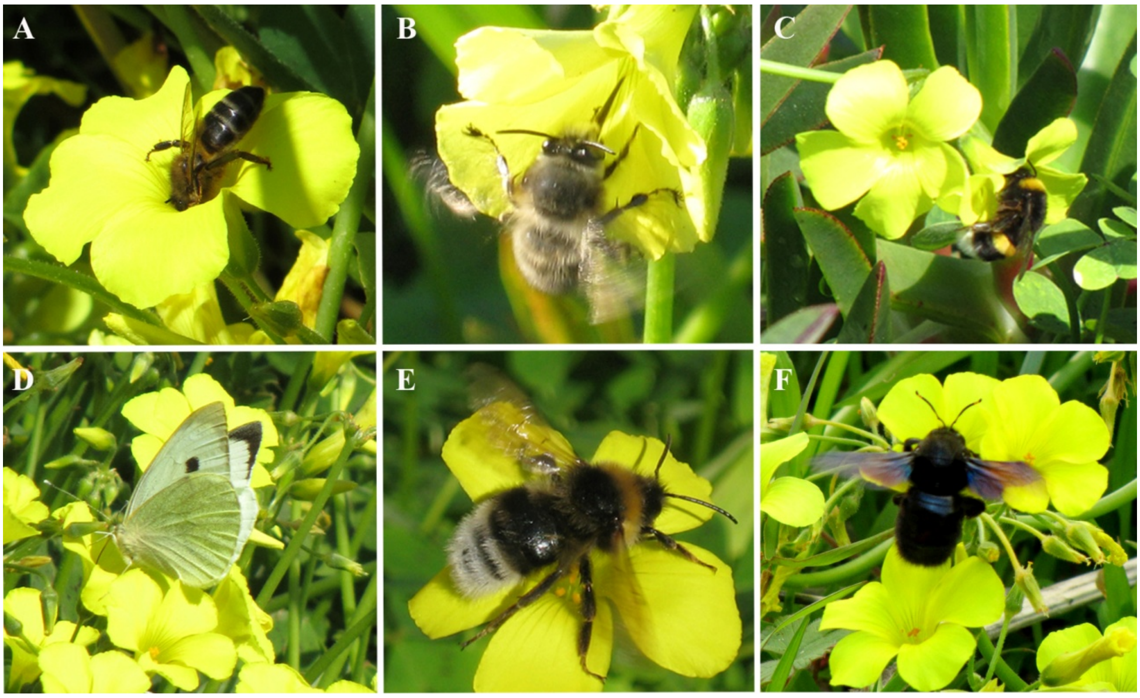

Figure 2. *Oxalis pes-caprae* pollinators. **A.** *Apis mellifera*; **B.** *Anthophora* sp.; **C.** *Bombus terrestris;* **D.** *Pieris brassicae*; **E.** *Psithyrus* sp.; **F.** *Xylocopa violaceae.*

Disassortative and assortative dye deposition differed significantly among populations ($\chi_2^{\,2}$ = 43.32; $P < 0.05$) and floral morphs ($\chi_2^{\,2}$ = 13.24; $P < 0.05$; Figure 4 G-I). As a result of the monomorphic condition of the Coimbra population, all the dye deposition was assortative. Overall, there was an increase in disassortative dye deposition from di- to trimorphic populations. Considering the exposed stigmas, it was surprising that the L-morph had the lowest disassortative dye deposition in comparison with the other morphs in both di- and trimorphic populations; the S- and M-morphs had higher values especially in the trimorphic population (Figure 4 G-I).

The results of female efficiency are provided in Figure 5 and Appendix 2.4. Statistically significant differences among populations were obtained for fruit production (H_2 = 90.05; $P < 0.001$; H_2 = 118.29; $P < 0.001$, respectively), with the monomorphic population having no sexual output and the others having similar fruit production ($P < 0.05$; Figure 3). Statistically significant differences in fruit production were also observed among floral morphs (H_2 = 118.29; $P < 0.001$), with the S-morph having lower fruit production than the others morphs ($P < 0.05$).

With the exception of the monomorphic population where no fruit was observed, all the floral morphs yielded morphologically viable seeds (Appendix 2.4). When considering the di- and trimorphic populations, no statistically significant differences were obtained for seed set among populations and morphs (Appendix 2.3 and 2.4).

Table 2. Floral morph visitation rates and global visitation rates for each population studied.

Population	***n***	**Floral morphs visitation rate**			**Global visitation rate**
		S-	**M-**	**L-**	
1. Coimbra	82	0.30 ± 0.994	-	-	0.30 ± 0.994
2. Cortegaça	80	0.22 ± 0.028	-	0.09 ± 0.017	0.16 ± 0.017
3. Alto da Praia Grande	76	0.04 ± 0.018	0.01 ± 0.004	0.01 ± 0.002	0.02 ± 0.006

Notes: Floral morphs: S-, M- and L- for short-, mid- and long-styled floral morphs, respectively; “-“ indicates absence of a given floral morph in the population. The number of census performed is also provided (*n*). Values are given as mean and standard error of the mean.

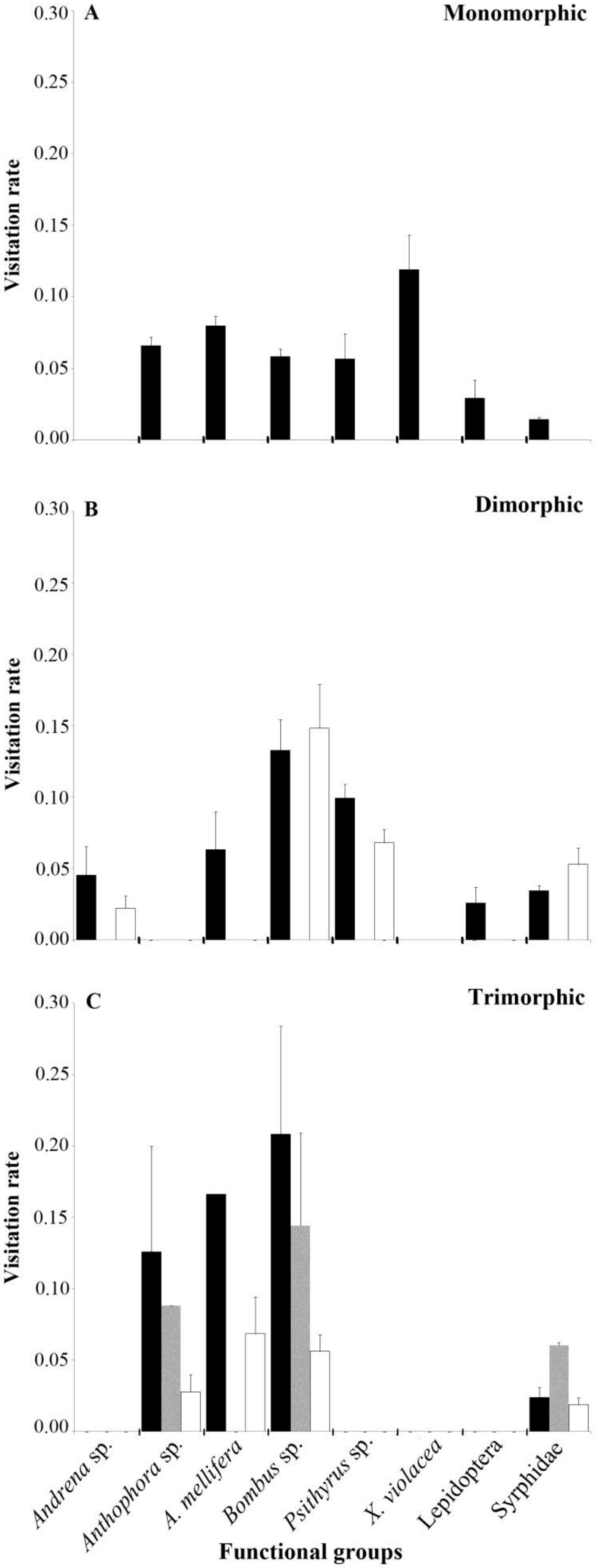

Figure 3. Visitation rates of each functional group per morph for the three populations studied: **A.** Coimbra, monomorphic population; **B.** Cortegaça, dimorphic population; **C.** Alto da Praia Grande, trimorphic population. Values are given as mean and standard error of the mean. Black, grey and white bars for for short-, mid- and long-styled floral morphs, respectively.

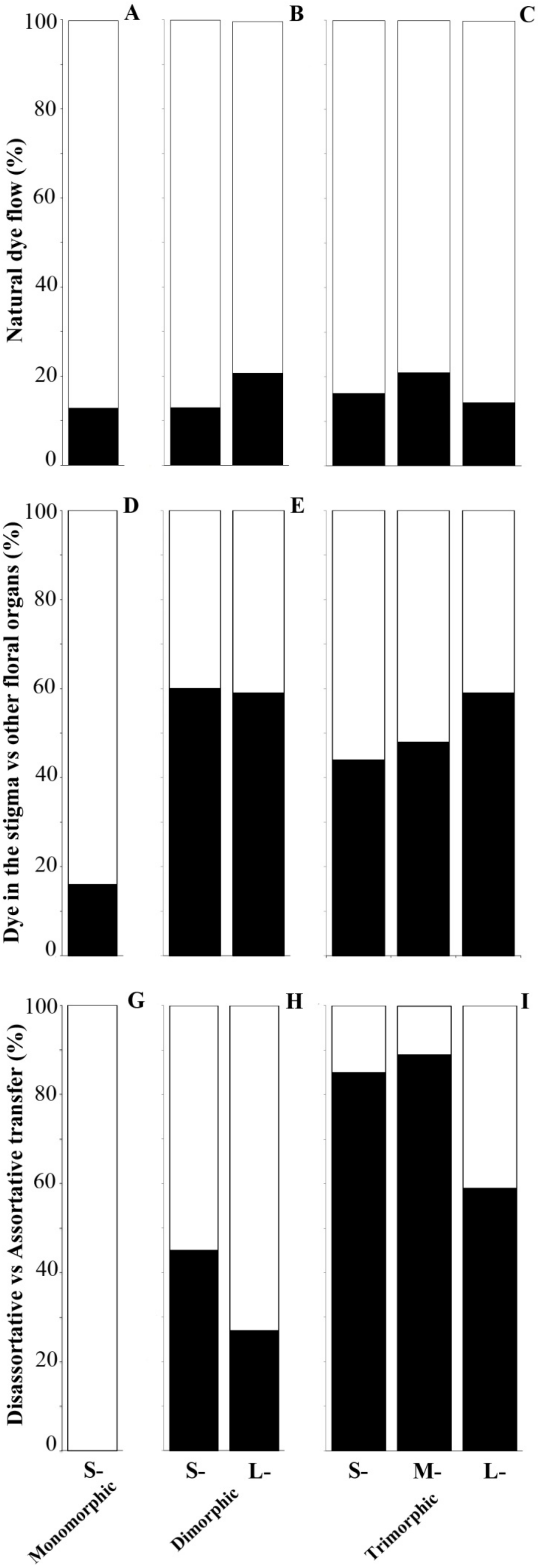

Figure 4. Male reproductive success within the populations studied given as dye flow per floral morph: **A-C.** Natural dye flow (percentage of flowers with and without dye are represented by black and white bars, respectively); **D-F.** Percentage of flowers with dye deposited in the stigma (black bars) *versus* lost in other organs (white bars); **G-I.** Percentage of disassortative (black bars) *versus* assortative (white bars) dye deposition in the stigma.

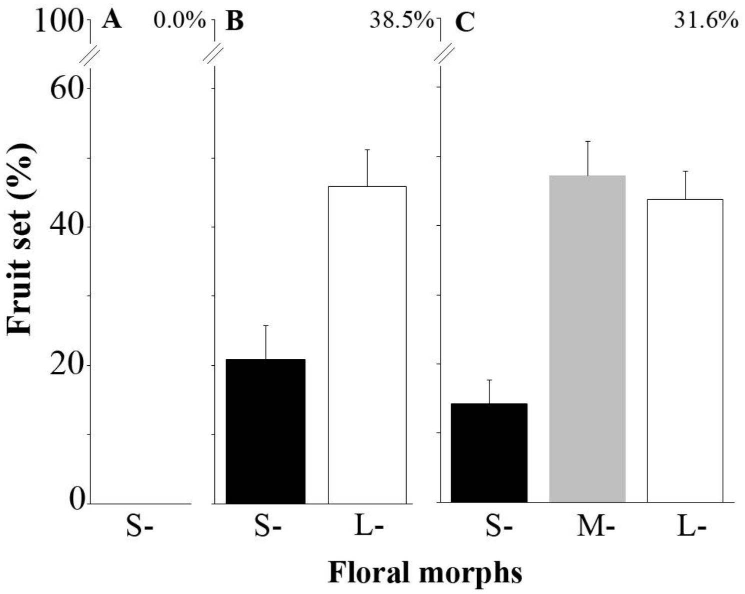

Figure 5. Female reproductive success within the populations studied given as fruit set: **A.** Coimbra, monomorphic population; **B.** Cortegaça, dimorphic population; **C.** Alto da Praia Grande, trimorphic population. The overall fruit set (%) for each population is given in the left side of the graphs. Values are given in percentage, as mean and standard error of the mean. Floral morphs: S-, M- and L- for short-, mid- and long-styled floral morphs, respectively.

2.4. Discussion

Reproduction is a key factor for the successful establishment of an exotic species after introduction (García-Ramos and Rodríguez 2002; Kinlan and Hastings 2005). Lack of suitable pollinators and compatible mate’s loss are known to negatively affect sexual reproduction of heterostylous species during the invasion processes (reviewed in Barrett and Shore 2008; Barrett *et al.* 2008). In the present study it was observed that the invasive *O. pes-caprae* established new mutualistic interactions at the pollination level with different insect’s functional groups from the invaded range as expected for a generalist species; and that, regardless of a breakdown in the incompatibly system (Chapter I), an increase in morph diversity (*i.e.*, compatible mates diversity) increased disassortative pollen flow and, consequently, the female reproductive success. Up to date, to our knowledge, this is the first study assessing both male and female contributions to the reproductive success of an invasive heterostylous species in populations with different morph compositions in its invaded area.

The morphometric analysis of *O. pes-caprae* flowers revealed a close positioning of the stigma and the first level of anthers in the S-morph. Also, the two levels of anthers in the L-morph were closer together than with the stigma in a way that the second level of anthers was more reciprocal with the stigmas of the S-morph, rather than the lower level. These findings indicate that the system is dynamic and might be changing towards semi-homostyly, *i.e.*, flowers composed by a long whorl of anthers and a short level that coincides with the stigma in height (Lewis 1954; Barrett 1989). This evolution from tristyly towards semi-homostyly (Lewis 1954) may result from recombination phenomena and from the accumulation of modifier genes (Ganders 1979) in a medium-long term and has already been described for several heterostylous species (reviewed in Turketti 2010). For example, in some *Oxalis* species, the semi-homostylous flowers observed seemed to point out for a modification of the S- and M-morphs (Ganders 1979; Ornduff 1972). Also, in *Eichhornia* genus, semi-homostylous flowers resulted from the breakdown of tristyly, which was associated with a change in the breeding system from out-crossing to selfing. The relaxation and subsequent loss of self-incompatibility preceded modifications in floral structures, and both had major impacts in population dynamics, floral morph composition and flower architecture (Barrett 1988, 1989; reviewed in Weller 1992). Despite the genetic basis of semi-homostyly in tristylous species is rather complex, *O. pes-caprae* might be following a similar pathway: incompatibility system is collapsing (Chapter I) and, regardless of the few observed flowers, some individuals were semi-homostylous (results herein). Large scale morphometric analyses in the subsequent years should be performed to assess how floral morphology is evolving.

O. pes-caprae flowers were visited by a wide array of insects from different functional groups and the majority of them were moving pollen and, consequently, pollinating the flowers. Considering the open corolla with rewards accessible to most floral visitors, *O. pes-caprae* profits from a substantial variety of different pollinators, and thus can be viewed as a pollinator's generalist. Indeed, few invaders are pollinator's specialists and, consequently, the integration of an exotic plant species into the existent plant-pollinator's networks is quite common (Richardson *et al.* 2000; Traveset and Richardson 2006). Indeed, this has already been confirmed for several invasive species (*e.g.*, reviewed in Richardson *et al.* 2000; *Cytisus scoparius*, Morales and Aizen 2002;

Impatia glandulifera, Lopezaraiza-Mikel *et al.* 2007; *Opuntia maxima*, Padrón *et al.* 2009), and pollinators are not usually considered among the barriers that a plant must overpass to become a successful invader (Rambuda and Johnson 2004).

Different floral visitor's assemblage and visitation rates were observed between *O. pes-caprae* populations, with the monomorphic population having the highest visitation rate, and the trimorphic one the lowest. Several factors are known to influence floral visitors assemblage and abundance, namely environmental conditions (reviewed in Burkle and Alarcón 2011) and food resources, such as the presence of co-flowering species that may be offering better and/or more floral rewards (*e.g.*, Horvitz and Schemske 1988; Stone *et al.* 2003). In the mono- and dimorphic populations, *O. pes-caprae* was the only resource available for insects and thus, it was continuously foraged for nectar and pollen. Contrarily, in the trimorphic population, *O. pes-caprae* was flowering simultaneously with *Acacia longifolia*. Species from the genus *Acacia* are known to produce high amounts of floral rewards (Stone *et al.* 2003). Thus, *A. longifolia* was probably actively competing with *O. pes-caprae* for pollinators, significantly reducing its visitation rates. In addition, the environmental conditions of this population were more adverse the pollinators; this population is located near the coast, having strong winds and low temperatures during the census, overall contributing to lower pollinator's abundances than in the other two populations.

When analyzing visitation rates to each floral morph within population, the S-morph flowers had higher visitation rates than the flowers of the other morphs in both di- and trimorphic populations, despite its low representation in the later. It has been demonstrated that larger corollas attract more insects (*e.g.*, Ganders 1979; Brown *et al.* 2002), and the higher visitation rates to S-morph flowers could be due exactly to this. Higher visitation rates of the S-morph flowers have been obtained for some other heterostylous species, but not always the corolla's size was the attracting factor (*e.g.*, *Pontederia cordata*, Wolfe and Barrett 1987; *Eichhornia paniculata*, Husband and Barrett 1992). Insects from distinct functional groups with different behaviours were observed visiting *O. pes-caprae* flowers, including mostly Hymenoptera, but also, Syrphidae and Lepidoptera. The Syrphidae have suctorial or sponging mouthparts and were mainly feeding on pollen. They stayed for long periods in the same flower in the longest sexual whorl and as a result of their feeding preferences they had a clear preference for the S-morph. In some study systems, they do not play any role on plant's reproduction (*e.g.*, *Geonoma irena*, Borchsenius 1997; *Lonicera japonica*, Larson *et al.*

2002); however, in other plant species, depending on their body and tongue sizes (Gilbert *et al.* 1985; Stone *et al.* 2003), they revealed to be constant and efficient pollinators (*e.g.*, Gilbert 1980; Sugiura 1996; Goulson and Wright 1997). In *O. pes-caprae*, given their behaviour during field observations, this does not seem to be the case, but future studies are necessary to assess the efficiency of these visitors as pollinators. Within Hymenoptera, *O. pes-caprae* was visited by several bees with different body and proboscis sizes and different foraging strategies when exploiting the flowers for nectar and pollen. However, in most cases they performed quick visits moving rapidly across the population and visiting many flowers. Most of them inserted the tongue and/or proboscis in the corolla and, depending on their size, touched the anthers depositing pollen at different heights along their bodies, potentially allowing the segregation of the pollen from different anther levels and subsequent disassortative pollen transfer (Barrett 2002). It is however important to notice that many were clumsy and clearly had pollen all over the body. Lepidoptera were also sporadically observed collecting nectar in *O. pes-caprae* flowers and, according with previous studies, are considered to be more accurate in pollen transfer (Ferrero *et al.* 2011b).

Pollen flow in all the populations surveyed was registered and, in the monomorphic population, surprisingly, fluorescence dye deposition in the stigmas of the S-morph was observed. The flower morphology discussed above combined with each insect's particular pattern of visiting the flowers (Lloyd and Webb 1992) and with a latter redistribution of pollen along their bodies during the flight (Wolfe and Barrett 1989), may have led to some pollen transfer to the stigmas of the S-morph in this population. Given the presence of only one floral morph, the considerably high dye loss observed was expected.

Disassortative pollen analogue deposition was higher when the three floral morphs were growing together, despite the low visitation rates registered. Considering the high level of reciprocity of both di- and trimorphic populations, the efficient pollen deposition along the pollinator's body (Lau and Bosque 2003; Sánchez *et al.* 2008) that contributed to the inter-morph dye transfer was expectable. The low level of disassortative dye transfer in dimorphic population was related with its most common floral visitor, *Psithyrus* sp. Its hairless thorax prevents pollen removal from the anthers (Thorp 2000) and together with its bumbling behaviour (flight near the ground, frequently rising and falling from flower to flower) contributes to its negligible role on pollination. The S-morph from di- and trimorphic populations and the M-morph received the highest levels of disassortative dye transfer in comparison with the L-morph. This pattern is similar to the one that was found for the tristylous *Pontederia cordata* (Wolfe and Barrett 1989) and can be attributed to higher exposition of the stigma in the L-morph. In fact, higher percentages of random pollinations are expected to occur as a result of the exposed stigma location in L- morph, consequently decreasing the opportunity for disassortative pollen transfers in this floral morph (reviewed in Dulberger 1992).

An increase in disassortative dye flow from di- to trimorphic populations was observed, thus allowing fruit production. Considering the monomorphic population, no fruit set was recorded. This was most probably due to a combination of factors; first, to low pollen transfer to the stigmas between flowers of the same morph (results herein); second, the incompatibility system may still be operating (completely or in some degree) in this population (Chapter I; Castro *et al.* 2007; Ferrero *et al.* 2011a) and finally, the pentaploid level of the individuals limits the development of viable gametes (Chapter I; Castro *et al.*, 2007). For a more detailed discussion on this subject see *Discussion* from Chapter I. Despite the proportional increase in disassortative dye transfer with the diversity of floral morphs (*i.e.*, compatible mates) the trimorphic population registered significantly lower fruit production. The factors affecting pollinator's assemblage and abundance discussed above (*i.e.*, environmental conditions and co-flowering species) are clearly involved in the reduced fruit set observed. However, no differences in the seed set between both populations were found. Thus, pollinators from the trimorphic population can be considered more efficient than the ones from dimorphic population.

O. pes-caprae revealed to be a generalist plant concerning pollinators, having already established new mutualistic interactions in the invaded range of the western Mediterranean basin. This condition allowed pollen flow in populations differing in morph composition. High levels of disassortative fluorescence dye transfer revealed inter-morph pollinations, allowing fruit and seed production, regardless of the biased floral morph ratios in di- and trimorphic populations. Factors affecting pollen transfer (*i.e.*, pollinator's assemblage, abundance and behaviour; environmental conditions and co-flowering species) played a crucial role in pollen transfer efficiency and, consequently, in fruit and seed set. The absence of fruits in the monomorphic population reveals that the reported breakdown in the morph-incompatibility (Chapter I) may not be a generalized event in the entire invaded range. A positive correlation between floral morph's diversity and efficient pollen transfer was also confirmed. Future work encompassing more populations characterized at the vegetation and co-flowering species level and contemplating pollen grain's analyzes from the different whorls of anthers and subsequent capture of pollinator's to analyse pollen segregation along their bodies, will allow a better comprehension on how this invasive species is successfully reproducing by sexual means in the invaded area of the Mediterranean basin.

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Appendix 2.1. Floral morphometric measurements.

Population	**Floral morph**	***n***	**Corolla length (mm)**	**Stigma height (mm)**	**Anther level (mm)**		
					s	**m**	**l**
1. Coimbra	S-morph	10	24.6 ± 0.6	5.2 ± 0.2	-	6.6 ± 0.1	9.6 ± 0.2
2. Cortegaça	S-morph	10	22.6 ± 0.4	4.7 ± 0.1	-	5.8 ± 0.1	8.3 ± 0.1
	L-morph	10	13.8 ± 0.3	8.0 ± 0.2	3.0 ± 0.1	4.6 ± 0.1	-
3. Alto da Praia Grande	S-morph	10	22.9 ± 0.5	5.4 ± 0.1	-	7.1 ± 0.1	9.9 ± 0.1
	M-morph	10	20.5 ± 0.7	6.6 ± 0.1	4.5 ± 0.1	-	9.1 ± 0.1
	L-morph	10	18.2 ± 0.6	9.3 ± 0.2	4.2 ± 0.1	5.9 ± 0.1	-

Notes: Values are given as mean and standard error of the mean. Floral morph: S-, M- and L-morph for short-, mid- and long-styled floral morph. Anther level is given as follows: s, m and l for short-, mid- and long-whorls of anthers, respectively. Sample size is also provided (*n*).

Appendix 2.2. Number of interactions of *Oxalis pes-caprae* floral visitors in the three populations studied.

Floral visitors	**Floral reward**	**Coimbra**	**Cortegaça**		**Alto da Praia Grande**		
		S-morph	**S-morph**	**L-morph**	**S-morph**	**M-morph**	**L-morph**
Order Coleoptera	P		5 (0.4)				
Order Diptera							
Episyrphus balteatus (Syrphidae)	P	10 (0.3)	15(1.2)	1 (0.2)			
Eristalis tenax (Syrphidae)	P	2 (0.1)					
Scaeva sp. (Syrphidae)	P		4 (0.3)				
Eupeodes sp. (Syrphidae)	P		71 (5.6)	2 (0.4)		2 (13.3)	
Unknown (Syrphidae)	P	66 (2.3)	11 (0.9)	4 (0.8)	10 (7.5)	1 (6.7)	5 (10.4)
Order Hymenoptera							
Anthophora sp. (Anthophoridae)	N	852 (29.7)			9 (6.7)	3 (20.0)	4 (8.3)
Andrena sp. (Andrenidae)	N/P		59 (4.6)	4 (0.8)			
Apis mellifera (Apidae)	N/P	934 (32.6)	27 (2.1)		1 (0.7)		16 (33.3)
Bombus sp. (Apidae)	N/P		34 (2.7)	1 (0.2)			
Bombus pascuorum (Apidae)	N/P		10 (0.8)	1 (0.2)	15 (11.2)	4 (26.7)	6 (12.5)
Bombus terrestris (Apidae)	N/P	538 (18.8)	258 (20.3)	139 (26.7)	99 (73.9)	5 (33.3)	17 (35.4)
Psithyrus sp. (Apidae)	N	32 (1.1)	757 (59.6)	368 (70.8)			
Xylocopa violacea (Xylocopidae)	N	414 (14.5)					
Order Lepidoptera							
Macroglossum stellatarum (Sphingidae)	N	5 (0.2)					
Pieris brassicae (Pieridae)	N		16 (1.3)				
Pieris rapae (Pieridae)	N	12 (0.4)	3 (0.2)				
Total no. of visits		**2865**	**1270**	**520**	**134**	**15**	**48**

Notes: Floral rewards: P, pollen and N, nectar. Floral morphs: S-, M- and L- for short-, mid- and long-styled floral morphs, respectively. Values are given as total number of flowers visited followed by the percentage of the total floral interactions per morph in parentheses (%).

Appendix 2.3. Results of the statistical analyses for differences among populations and floral morphs in pollinator's assemblage and male and female reproductive success variables.

Variables	**Factors**	
	Population	**Morph**
Pollinator's assemblage		
Global visitation rates	**$H_2 = 105.28; p < 0.001$**	-
Andrena sp.	-	$\chi^2_1 = 1.52; p = 0.29$
Anthophora sp.$^{\#}$	-	$F_2 = 1.52; p = 0.35$
A. mellifera $^{\#}$	$F_1 = 4.30; p = 0.13$	$F_1 = 3.45; p = 0.16$
Bombus sp.	$\chi^2_1 = 0.34; p = 0.56$	$\chi^2_2 = 0.38; p = 0.83$
Lepidoptera	$\chi^2_1 = 0.06; p = 0.81$	-
Psithyrus sp.	-	**$\chi^2_1 = 6.73; p < 0.05$**
Xylocopa violaceae	-	-
Syrphidae	$\chi^2_1 = 1.65; p = 0.20$	**$\chi^2_2 = 6.08; p = 0.04$**
Legitimate *vs* illegitimate visits	**$\chi^2_2 = 246.87; p < 0.05$**	-
Male reproductive success		
Natural dye flow	$\chi^2_1 = 2.15; p = 0.34$	**$\chi^2_2 = 7.27; p = 0.03$**
Total dye lost	**$\chi^2_4 = 71.44; p < 0.05$**	$\chi^2_2 = 5.70; p = 0.22$
Disassortative *vs* assortative	**$\chi^2_2 = 43.32; p < 0.05$**	**$\chi^2_2 = 13.24; p < 0.05$**
Female reproductive success		
Fruit set	**$H_2 = 90.05; p < 0.05$**	**$H_2 = 118.29; p < 0.05$**
Seed set	$H_1 = 0.005; p = 0.94$	$H_2 = 4.19; p = 0.12$

Notes: "#" indicates the functional groups tested with GLM; in bold are highlighted the statistically significant results; "-" indicates that no statistical test was performed.

Appendix 2.4. Female reproductive success in natural populations given as fruit set and mean number of seeds per fruit.

Population	Population type	Fruit set (%)			Overall fruit set (%)	Mean no. of seeds per fruit		
		S-morph	M-morph	L-morph		S-morph	M-morph	L-morph
1. Coimbra	Monomorphic	0.0 ± 0.0 (92)	-	-	0.0	-	-	-
2. Cortegaça	Dimorphic	20.9 ± 4.9 (43)	-	45.9 ± 5.3 (55)	38.5	2.5 ± 0.5 (17)	-	1.1 ± 0.3 (39)
3. Alto da Praia Grande	Trimorphic	14.3 ± 3.4 (64)	47.3 ± 4.9 (50)	43.8 ± 4.1 (85)	31.6	2.0 ± 0.4 (41)	2.1 ± 0.6 (21)	2.3 ± 0.6 (60)

Notes: Floral morphs: S-, M- and L- for short-, mid- and long-styled floral morphs. Fruit set was calculated as the percentage of flowers developing into fruit and overall fruit set was calculated by summing the fruit set of each floral morph present in the population weighted by its proportion in the corresponding population. Values are given as mean and standard error of the mean. Sample size is given in parenthesis.

CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions

The results obtained in this MSc thesis allowed obtaining further insights regarding the reproductive system of *Oxalis pes-caprae* in the invaded area (Chapter 1) and the sexual reproduction success in natural populations from the invaded range differing in morph composition (Chapter 2). The obtained results proved that a breakdown in the morph-incompatibility system of *O. pes-caprae* occurred in the study area. Additionally, we detected the ability of the 5*x* S-morph to produce some viable gametes, which opened the possibility for the sexual reproduction to occur in the invaded area of the Mediterranean basin. The ability to reproduce sexually may, thus, be one of the mechanisms involved in the emergence of new floral morphs and cytotypes in this range of the invaded area. Regarding sexual reproduction in natural populations, it was confirmed that *O. pes-caprae* is a pollinators' generalist plant that has already integrated the existent pollination networks in the invaded range of the western Mediterranean basin. These interactions allowed pollen flow within the populations and, ultimately, fruit and seed production. The biased floral morph ratios resulted in different rates of seed set among populations.

The work developed opens new insights in the knowledge of the invasion process of a primarily obligate out-crosser in a new environment. The shift from strict clonality for sexual reproduction confirms the importance of studies like this to understand the dynamics associated with the invasion of species with a peculiar sexual system such as heterostyly.

Future Perspectives

The results obtained shed light in some important questions concerning the reproductive system during the invasion process of *O. pes-caprae.* However, the answers obtained lead to new and pertinent questions for future work.

In order to reach a full understanding of the patterns associated with the incompatibility breakdown and their contribution for the reproductive success and morph biased populations of *O. pes-caprae* in this study region, large-scale pollination experiments through the invasive range of the western Mediterranean basin are necessary. Additionally, improvements of the FCM methodology using pollen grains are needed to confirm the ploidy level of the gametes produced by the 5*x* S-morph.

Also, a more exhaustive field work encompassing a higher number of populations, differing in morph composition, characterized at vegetation and co-flowering species level are needed to fully understand the patterns of fruit production obtained. A deeper nectar characterization would also be interesting to completely rule out the importance of the floral rewards provided by *O. pes-caprae.* Palinological studies of the pollen grains from the different whorls of anthers together with pollinator's capture and observation of pollen segregation along their bodies are also necessary for a better evaluation of the sexual reproduction success of this invasive species in the Mediterranean basin.