

INVASION GENETICS: THE BAKER AND STEBBINS LEGACY

Invasion genetics of the Bermuda buttercup (*Oxalis pes-caprae*): complex intercontinental patterns of genetic diversity, polyploidy and heterostyly characterize both native and introduced populations

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Abstract

Genetic diversity in populations of invasive species is influenced by a variety of factors including reproductive systems, ploidy level, stochastic forces associated with colonization and multiple introductions followed by admixture. Here, we compare genetic variation in native and introduced populations of the clonal plant *Oxalis pes-caprae* to investigate the influence of reproductive mode and ploidy on levels of diversity. This species is a tristylous geophyte native to South Africa. Invasive populations throughout much of the introduced range are composed of a sterile clonal pentaploid short-styled form. We examined morph ratios, ploidy level, reproductive mode and genetic diversity at nuclear microsatellite loci in 10 and 12 populations from South Africa and the Western Mediterranean region, respectively. Flow cytometry confirmed earlier reports of diploids and tetraploids in the native range, with a single population containing pentaploid individuals. Introduced populations were composed mainly of pentaploids, but sexual tetraploids were also found. There was clear genetic differentiation between ploidy levels, but sexual populations from both regions were not significantly different in levels of diversity. Invasive populations of the pentaploid exhibited dramatically reduced levels of diversity but were not genetically uniform. The occurrence of mixed ploidy levels and stylar polymorphism in the introduced range is consistent with multiple introductions to the Western Mediterranean. This inference was supported by variation patterns at microsatellite loci. Our study indicates that some invasive populations of *Oxalis pes-caprae* are not entirely clonal, as often assumed, and multiple introductions and recombination have the potential to increase genetic variation in the introduced range.

Keywords: biological invasions, clonal reproduction, genetic variation, heterostyly, polyploidy

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Introduction

Biological invasions provide outstanding opportunities for investigating microevolutionary process over

contemporary timescales. A key factor in studies of the genetics of colonizing species concerns the kinds and amounts of genetic variation that occurs within invasive populations (Baker & Stebbins 1965; Barrett 1992; Sakai *et al.* 2001; Lee 2002; Petit *et al.* 2004). Reproductive systems play an important role in determining patterns of genetic variation in invasive populations and are of

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special importance in plants because of their wide diversity of sexual and asexual strategies and variable mating systems (Barrett 2002). In particular, the degree of asexuality in populations and whether a species is predominantly outcrossing or selfing are of considerable importance (Novak & Mack 2005; Barrett 2011). Clonal invasions in which sexual reproduction is limited or absent can commonly involve striking genetic bottlenecks (Kliber & Eckert 2005; Zhang *et al.* 2010). Moreover, in selfing populations, restricted recombination as a result of inbreeding often preserves multilocus associations established through founder events (Brown 1983; Husband & Barrett 1991). Therefore, the degree of sex and recombination will play a major role determining levels of genetic diversity in invasive populations.

Invasion processes frequently cause transitions to uniparental reproduction (e.g. clonality and selfing) in the introduced range of plants (reviewed in Barrett 2011). Vegetative reproduction in particular has several ecological advantages in novel environments, such as persistence in habitats where sexual reproduction is not possible, the ability of clones to forage for resources in heterogeneous environments, and the capacity for physiological integration and division of labour among clonal modules (Silander 1985; Caraco & Kelly 1991; Stuefer *et al.* 1996; Roiloa *et al.* 2010). However, extensive clonality combined with restrictions on sexual recruitment reduces levels of genetic diversity and may even be associated with a complete loss of sexual reproduction in some clonal populations (Vallejo-Marín *et al.* 2010). Loss of sex can occur for a variety of reasons, but in invasive species asexuality may often arise because long-distance dispersal can result in the stochastic loss of sexual morphs thus disabling sexual systems and preventing outbreeding and seed production (Barrett *et al.* 1993; Hollingsworth & Bailey 2000; Eckert 2002; Wang *et al.* 2005). In such cases, clonal propagation is required for persistence and well-developed phenotypic plasticity may be necessary for populations to cope with environmental heterogeneity.

Other processes such as genome duplication (polyploidization) may also play an important role in facilitating plant invasions (te Beest *et al.* 2012). Polyploidization is expected to increase genetic variability because of the increase in genome size; however, in cases where polyploidy is associated with hybridization and the origin of sterile polyploids (e.g. $3x$, $5x$ cytotypes), it may also cause a dramatic decrease in the genetic diversity of populations. Clonal propagation may often become the only means of numerical increase and spread in asexual populations (García-Verdugo *et al.* 2013). In polyploid clonal invasive species, this can lead to extensive areas of genetic uniformity in the introduced range.

The Bermuda buttercup (*Oxalis pes-caprae* L., Oxalidaceae) is a polyploid, highly clonal geophyte, native to the Greater Cape Floristic Region of South Africa (Goldblatt & Manning 2000; Born *et al.* 2007), with a centre of distribution in the Western and Northern Cape Provinces (Salter 1944). However, today *O. pes-caprae* is also considered a widespread and noxious invasive weed occurring in disturbed sites in all Mediterranean climatic regions of the world (Rappa 1911; Baker 1965; Ornduff 1986, 1987; Lambdon 2006; Ferrero *et al.* 2013). The species was introduced into the Mediterranean Basin in 1796, reaching the Iberian Peninsula by 1825 (D'Austria 1884; Gimeno *et al.* 2006). In its native range, both diploid ($2n = 2x = 14$ chromosomes) and tetraploids cytotypes ($2n = 4x = 28$ chromosomes) are reported. Tetraploidy is the most common ploidy level (te Beest *et al.* 2012), and in a few restricted areas, diploids and tetraploids occur in parapatry (Krejčíková *et al.* 2013). The sterile pentaploid cytotype ($2n = 5x = 35$ chromosomes) is the most widespread form throughout the introduced range (Baker 1965; Ornduff 1987; Rottenberg & Parker 2004; Castro *et al.* 2007, 2013; V. Ferrero & S. C. H. Barrett, unpublished observations), although a small number of populations containing tetraploids have been reported from the Mediterranean Basin (Castro *et al.* 2007, 2013) and Australia (Symon 1961; V. Ferrero, unpublished results). Surprisingly, there is only one record of pentaploid plants from the native range (Franklin in Michael 1964), and a recent large-scale survey of *O. pes-caprae* populations (Krejčíková *et al.* 2013) failed to locate pentaploid plants in the Cape region.

Oxalis pes-caprae is tristylous with populations in the native range usually containing the three floral morphs (long-styled, mid-styled, short-styled hereafter, L-, M- and S-morphs) that characterize this floral polymorphism (reviewed in Barrett 1993). Tristylous species commonly possess a trimorphic incompatibility system that prevents self- and cross-fertilization among plants of the same style morph promoting outcrossing and maintaining genetic diversity in populations. In the introduced range, populations most commonly contain a single sterile pentaploid form and thus rely entirely on clonal reproduction through underground bulbils. Recently, $4x$ L-, M- and S-morphs and a sterile double-flowered form have been reported from a few locations in the Western Mediterranean region (Castro *et al.* 2007, 2013). The presence of populations polymorphic for style length in the Western Mediterranean enables sexual reproduction (Castro *et al.* 2013; Costa *et al.* 2014), but at this stage, it is unclear how prevalent recruitment from seed is in these populations.

Here, we address three specific questions by comparing patterns of genetic diversity in a sample of native *O. pes-caprae* populations from South Africa with invasive

populations from the Western Mediterranean: (i) Do populations in which there is more than one style morph contain significantly higher levels of diversity than those composed of the sterile pentaploid form? If sexual reproduction is occurring in populations polymorphic for style morph, we predict significantly higher levels of genetic diversity than in predominantly clonal 5x populations. (ii) What is the most likely origin of the 4x L- M- and S-morphs recently discovered in the Western Mediterranean (Castro *et al.* 2007, 2013)? We evaluated two hypotheses. First, the new forms could have arisen from separate introductions from South Africa, in which case we would expect them to be genetically differentiated from the sterile 5x S-morph. Second, it is possible, as suggested by Ornduff (1987), that the 4x style morphs have arisen from residual fertility in the 5x race (Costa *et al.* 2014) and a breakdown in its incompatibility system, and thus, we would expect them to be genetically closer to the sterile 5x S-morph. (iii) What is the origin(s) of the 5x S-morph cytotype that predominates throughout the introduced range? We expect that the 5x cytotype originated in South Africa and was then introduced to Mediterranean climatic regions of the world. However, given previous surveys of South Africa showing its overall rarity, we discuss other possible scenarios.

Materials and methods

Population sampling

We sampled 10 populations of *Oxalis pes-caprae* in South Africa and 12 populations from the Western Mediterranean region in 2010–2012. In the Western Mediterranean region, we included in our sampling a subset of populations that were likely to be sexual because of the presence of more than one style morph. We adopted this strategy because we were particularly interested in comparing populations showing all possible combinations of ploidy level and reproductive system observed in each area. All populations in South Africa contained the three style morphs. In each population, we determined style morph frequencies and the occurrence of the double-flowered sterile mutant (for a discussion of this form see Castro *et al.* 2007) by sampling 100 plants randomly distributed throughout the population. Our sampling followed methods outlined in Castro *et al.* (2007, 2013) and involved setting out transects in each population and sampling clones at 5 m intervals to reduce the likelihood of the repeated inclusion of the same clone. We measured fruit production by sampling infructescences from 10 plants from each morph detected in populations. We collected leaves and bulbils from 3 to 10 randomly chosen plants of each morph in

a population, and the leaves were dried in silica gel for subsequent DNA extraction. Once again, a special effort was made to reduce repeated sampling of the same clone by collecting material from individuals that were widely separated. Bulbils were grown in a common garden at the Centre for Functional Ecology (University of Coimbra, Portugal), and plants were used for ploidy level analyses using flow cytometric analyses (see details below) and for DNA extractions, when dried leaf material resulted in poor quality DNA samples. Our study included a total of 380 individuals, 207 from South Africa and 173 from the introduced range. Detailed information about the geographical location of populations, number of individuals sampled per population, style morph proportions, inferred reproductive mode and fruit set in each population is summarized in Table 1 and Fig. 1. Permits to collect and undertake scientific research were obtained to sample in South Africa; no permit was necessary to sample in the introduced range.

Ploidy level analysis

We determined the ploidy level of all individuals using flow cytometry on fresh leaves that emerged from bulbils. Nuclei were isolated following the procedure of Galbraith *et al.* (1983) by chopping simultaneously with a razor blade, 1 cm² of leaf tissue of *O. pes-caprae* and 1 cm² of leaf tissue of *Solanum lycopersicum* 'Stupické' (internal reference standard with 2C = 1.96 pg, Doležel *et al.* 1992) in 1 mL of WPB buffer (0.2 M Tris-HCl, 4 mM MgCl₂·6H₂O, 1% Triton X-100, 2 mM EDTA Na₂·2H₂O, 86 mM NaCl, 10 mM metabisulphite, 1% PVP-10, pH adjusted to 7.5 and stored at 4 °C; Loureiro *et al.* 2007). After filtration with a 50 µm nylon filter, we added 50 µg/mL of propidium iodide and 50 µg/mL of RNase to the nuclear suspension to stain the DNA and remove double-stranded RNA, respectively. We analysed samples in a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) equipped with a green solid state laser (Cobolt Samba 532 nm, operating at 30 mW; Cobolt, Stockholm, Sweden). More than 3000 nuclei were analysed per sample. We used a pooled sampling strategy, in which leaflets from five individuals were analysed simultaneously (Castro *et al.* 2013); when several peaks were obtained, individual samples were prepared and ploidy levels assigned to each plant. Results were obtained using PARTEC FLOMAX software (v. 2.5). As a quality standard, only histograms with a coefficient of variation (CV) lower than 5% for both samples and standard G₁ peaks were accepted. We determined ploidy level by estimating the DNA index, that is the ratio between the mean fluorescence of *O. pes-caprae* G₀/G₁ peak with that of *S. lycopersicum*. Each plant was

Table 1 Features of *Oxalis pes-caprae* populations investigated in this study. Location (including range, population name and geographical coordinates), sample size used for the molecular analysis, ploidy level (with the percentage of 5x individuals in mixed populations in parenthesis), expected mode of reproduction, fruit set and morph frequencies are provided for each population. Populations were classified as sexual whenever there was more than one fertile sexual morph present. Asexual populations included those monomorphic for the 5x cytotype and mixed populations composed of 5x and 4x sterile double-flowered individuals

Range	Population	Coordinates		Sample size	Ploidy (N)	Reproductive mode	Fruit set	Morph frequency			
								L	M	S	St
Native: South Africa	Barrydale	33°47.247'S	21°08.652'W	21	4x	Sex	0.60	0.37	0.23	0.40	0.00
	Cape Point	34°09.413'S	18°26.100'W	20	4x + 5x (15%)	Sex	0.54	0.15	0.76	0.08	0.00
	Gouritsmond	34°17.703'S	21°49.356'W	20	4x	Sex	0.73	0.30	0.38	0.32	0.00
	L'Agulhas	34°41.391'S	20°01.198'W	21	4x	Sex	0.61	0.24	0.24	0.52	0.00
	Lamberts Bay	32°11.517'S	18°19.924'W	20	4x	Sex	0.84	0.28	0.26	0.46	0.00
	Nuwerus	31°10.006'S	18°21.005'W	21	2x	Sex	0.69	0.36	0.28	0.36	0.00
	Oudtshoorn	33°32.827'S	21°50.612'W	21	4x	Sex	0.84	0.39	0.56	0.05	0.00
	Springbok	30°13.242'S	17°54.176'W	21	2x	Sex	0.75	0.42	0.26	0.32	0.00
	Yzerfontein	33°20.979'S	18°09.302'W	21	4x	Sex	0.85	0.32	0.22	0.46	0.00
Worcester	33°33.671'S	19°54.072'W	21	4x	Sex	0.83	0.40	0.37	0.22	0.00	
Introduced: Western Mediterranean Basin	Almogrove	37°38.885'N	8°47.320'E	19	4x + 5x (47%)	Asex	0.10	0.00	0.00	0.91	0.09
	Armação de Pêra	37°04.856'N	8°17.201'E	6	4x + 5x (67%)	Asex	0.04	0.00	0.00	0.81	0.19
	Coimbra	40°12.363'N	8°25.431'E	10	5x	Asex	0.00	0.00	0.00	1.00	0.00
	Colares I	38°48.015'N	9°28.061'E	21	4x + 5x (29%)	Sex	0.48	0.50	0.22	0.27	0.00
	Colares III	38°48.752'N	9°28.394'E	21	4x + 5x (29%)	Sex	0.47	0.39	0.13	0.48	0.00
	Lavras	41°14.436'N	8°42.871'E	10	5x	Asex	0.00	0.00	0.00	1.00	0.00
	Marinha Grande	39° 44.403'N	8°56.063'E	20	4x + 5x (50%)	Sex	0.16	0.09	0.00	0.91	0.00
	Melides	38°07.843'N	8°46.961'E	15	4x + 5x (53%)	Asex	0.07	0.00	0.00	0.69	0.31
	Moulay- Bousselham	34°52.542'N	6°17.831'E	6	4x + 5x (33%)	Sex	0.00	0.31	0.00	0.69	0.00
	São P. da Maceda	40°55.264'N	8°39.625'E	20	4x + 5x (50%)	Sex	0.10	0.22	0.00	0.78	0.00
	Troia	38°29.495'N	8°54.386'E	15	4x + 5x (53%)	Sex	0.09	0.05	0.00	0.95	0.00
Vieirinhos	40°00.163'N	8°48.341'E	10	5x	Asex	0.00	0.00	0.00	1.00	0.00	

Sex, sexual population; Asex, asexual population; L, L-morph; M, M-morph; S, S-morph; St, sterile form.

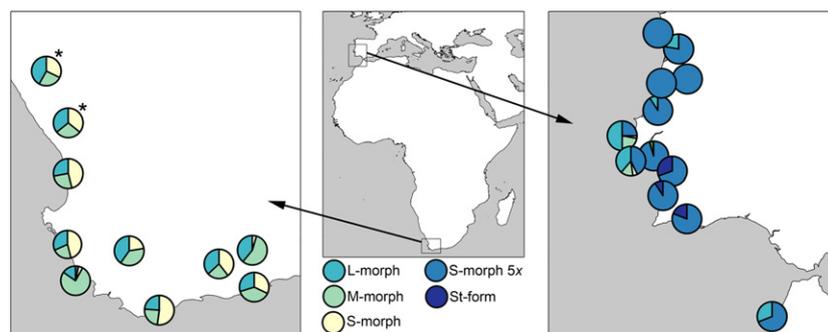


Fig. 1 Location of populations of *Oxalis pes-caprae* that were investigated in this study: native range, South Africa (left) and introduced Western Mediterranean range (right). The Pie diagrams represent the percentage of each style morph in populations. The 5x short-styled form and the sterile double-flowered form (St-form 4x) are shown in different colors. Populations with 2x individuals are marked with an asterisk.

identified as either a diploid, for DNA indices of 0.35 ± 0.01 (mean \pm SD), a tetraploid for DNA indices of 0.70 ± 0.03 and a pentaploid for DNA indices of 0.86 ± 0.02 .

DNA extraction and nSSR amplification

We extracted total genomic DNA from 100 mg of fresh leaves or 20 mg of dried leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. For microsatellite development, we made an effort to maximize variation by selecting samples from different locations from throughout the geographical range we studied. The set of samples involved seven plants, each from a different population, with five from the native range and two from the introduced range. We initially tested 12 putative polymorphic nuclear microsatellite (nSSR) loci developed for *O. pes-caprae* by Genoscreen (Lille, France). Seven of the 12 primer pairs showed polymorphism and thus were used in our study (Table 2).

We prepared multiplex reactions using primers that amplify fragments of different size. We performed polymerase chain reactions (PCR) in a total volume of 20 μ L, containing 20 ng of DNA template, 2x QIAGEN Multiplex PCR Master Mix (Type-it Microsatellite PCR Kit) and 1 μ M of each forward (F) and reverse (R) primers and RNase-free water. All PCRs were run in a Gene Amp PCR System 9700 (Applied Biosystems) using the following program: initial denaturation step for 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C annealing temperature and 60 s at 72 °C and a final extension step for 10 min at 72 °C and cooling down to 4 °C. PCR products were checked on 2% TBE agarose gels stained with Gel Red Nucleic Acid Stain (Biotium). PCRs that did not produce bands or that had

different sizes were repeated. We combined the amplification products with formamide and a size standard (GeneScan-500 LIZ, Applied Biosystems), and these were separated on a 3730 ABI automated sequencer. We scored sample profiles manually using GENEMARKER v2.4 (Applied Biosystems).

Data analysis

To test whether the morph ratio of the total sample showed a deviation from the expected isoplethic equilibrium (1:1:1), we used the G-test for goodness-of-fit (Sokal & Rohlf 2011). To assess how changes in ploidy level and reproductive mode may affect genetic variability during invasion, we conducted several different analyses. The quantification of genetic variability in polyploids is difficult because they bear more than two alleles per individual at a given locus and can carry multiple copies of a particular allele (Obbard *et al.* 2006; Sampson & Byrne 2012; García-Verdugo *et al.* 2013; Teixeira *et al.* 2014). Thus, indices such as expected heterozygosity (*He*) cannot be used to study genetic diversity in polyploid complexes. In addition, most software packages designed for population genetic analyses do not cope with polyploid data, particularly sets of mixed ploidies (see recent review by Dufresne *et al.* 2014). To overcome these problems, we used GenoDive (Meirmans & Var Tienderen 2004) to calculate the number of alleles per locus, the effective number of alleles, heterozygosity within each population and the number of private alleles. We grouped populations according to the origin, ploidy level and 'expected mode of reproduction'. For the expected mode of reproduction, we considered sexual populations those with more than one fertile style morph, whereas asexual populations were composed of the

Table 2 Characterization of 12 microsatellite loci in *Oxalis pes-caprae*; the first seven primers were polymorphic and used for genotyping in this study. Size indicates the range of observed alleles

Primer name	Forward primer sequence	Reverse primer sequence	Motif	Size (bp)
OX_02	GCTAATCGCCATCTTCATCG	GAATCCATGGTGACTCCTGC	CTT ₅	236–260
OX_07	GATACAATTCGATCACCGTGC	CTCCTGACGAGCAAAAAGGTC	AG ₈	119–157
OX_15	GGACAGACTCGTCTTGACACA	TGGAATCTAGGGTATCGGAAA	TC ₉	135–171
OX_29	TCCTCAGTCTGTGCCAGTAAAG	CGCAAAGAGGGAAGCTGTAT	AC ₁₀	226–242
OX_31	CGAACAACATCGAAGCTAAACA	GTTTCTCGATAAAGGCGGC	GA ₁₀	109–125
OX_32	ACAGAAATTAACAACCCACATAGC	CGCTTACAACCTTCCATCACC	AG ₁₀	232–250
OX_42	AAACCGATCAAAGCATCTTCA	CCTAGCCAAAACCTAAGGCA	AG ₁₆	94–110
OX_26	TCTCCCTTCCAGTATCACAACA	GCTGTGGAGTTTAGTATTCTTTGTCTT	TG ₁₀	140
OX_34	ACACGGATTATGTTTCTCCCT	GAAGCTCTAACCTCATGGCG	CTT ₁₁	130
OX_35	TGAGATTAATTTCTGGAATAGATTTGC	TTCTCACTTAACCTTCTAATGACG	AG ₁₁	105
OX_37	TTGAACTGGGAAGCAAAAACA	TCTCCTTTAAAGTCTCAAAACACTG	GA ₁₂	101
OX_44	TTCTTTCCACACTTTTAATCCTAGC	AGCAGGGAAGAAAATGAAGTT	CTT ₁₇	125

bp, base pairs.

sterile pentaploid form or a mixture of this form and the double-flowered sterile mutant. We used ANOVA followed by Tukey's multiple comparisons of means to compare the number of alleles (A_n), number of effective alleles (AE_{eff}) and heterozygosity (H_t) between groups. All analyses were carried out in R v3.1.1 using libraries 'stats', 'gridExtra' and 'ggplot2' (R Development Core Team 2014).

To assess the most probable origin of populations in the Western Mediterranean region and to determine the relationships between native and introduced populations, we investigated the patterns of differentiation between regions and among ploidy levels. We used the original genotypic data to calculate a pairwise distance matrix between all samples using the Bruvo distance, as implemented in POLYSAT (Clark & Jasieniuk 2011). This method takes into account the ambiguity of allele copy number in polyploids and works effectively with populations of mixed ploidy (Bruvo *et al.* 2004; Clark & Jasieniuk 2011). The distance matrix obtained was used to perform a Principal Coordinate Analysis (PCoA) – a method that explores and visualizes similarities and dissimilarities in data – within POLYSAT and a Neighbour-joining tree using the package 'ape' (Paradis *et al.* 2004). We performed all analyses in R, and trees were further edited using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Results

Ploidy level and style morph representation in populations

In native South African populations, three ploidy levels (2x, 4x, 5x) and three style morphs (L-, M-, S-morph) were represented in the region sampled (Table 1; Fig. 1). All populations were trimorphic, with the average morph ratio (\pm SE) among the 10 populations sampled not significantly different from isoplethy: L-morph = 0.32 ± 0.08 ; M-morph = 0.36 ± 0.18 ; S-morph = 0.32 ± 0.16 ; $G_{pooled} = 2.82$; d.f. = 2; $P = 0.244$; $n = 672$ plants. Significantly, given its widespread distribution in the introduced range, the 5x cytotype was exceedingly rare and only three individuals were detected in a single 4x population (Cape Point); the remaining populations were either 2x ($n = 2$) or 4x ($n = 7$). In the introduced region, populations varied in the presence and proportion of style morphs. Among the 12 populations that we sampled three contained only the sterile 5x short-styled form, three contained mostly this form but also the sterile 4x double-flowered form, and of the remaining populations, four were dimorphic and three were trimorphic composed of mixtures of 4x and 5x forms, including the 4x L-morph, the 4x M-morph and the 4x S-morph (Table 1).

Genetic variation

Analyses of genetic variation involved all sampled populations from South Africa and the Western Mediterranean region. Based on their origin, ploidy level and expected mode of reproduction they were grouped as follows: 2x native sexual populations; 4x native sexual populations (the three 5x individuals found in one population were excluded from this analysis); and from the introduced range, asexual 5x populations, sexual mixed ploidy (4x + 5x) populations and asexual mixed ploidy (4x-sterile form + 5x) populations (see Table 1 for details).

We found significant differences between groups for the number of alleles ($F_{4,17} = 23.420$, $P < 0.001$), the number of effective alleles ($F_{4,17} = 6.052$, $P = 0.003$) and heterozygosity ($F_{4,17} = 8.325$, $P < 0.001$). The 2x native populations tended to have lower genetic diversity than 4x native populations and similar or slightly higher genetic diversity than populations from the introduced range. Overall, genetic diversity was significantly higher in 4x populations from South Africa than in all populations from the Western Mediterranean region (Fig. 2). Within the introduced range, mixed populations tended to have higher values for the number of alleles and heterozygosity than pentaploid monomorphic populations, although the differences were not significant. A total of seven private alleles were found in the native range whereas none were identified in the introduced range (Table 3). No statistical analysis for the number of private alleles could be conducted due to their low number.

PCoA and Neighbour-joining tree

Results from the PCoA indicated significant differentiation between individuals of different ploidy level, particularly between diploids and polyploids but less obviously between tetraploids and pentaploids (Fig. 3). No clear distinction between ranges was detected, as there was some overlap between samples from native and introduced areas (Fig. 3). The Neighbour-joining tree corroborated the relatedness of individuals according to their ploidy level (Fig. 4). Diploid individuals were considered as the basal group due to their genetic differentiation, as illustrated by the PCoA (Fig. 3), and also because they are expected to be the most probable parental source of polyploids. In the tree, tetraploids formed distinct clusters with some differentiation according to their range, although some groups included tetraploids from both areas. All pentaploids constitute a differentiated terminal group, including the three individuals detected from the native area (Fig. 4). The double-flowered sterile form appeared multiple times in the tree within the 4x cluster (Fig. 4).

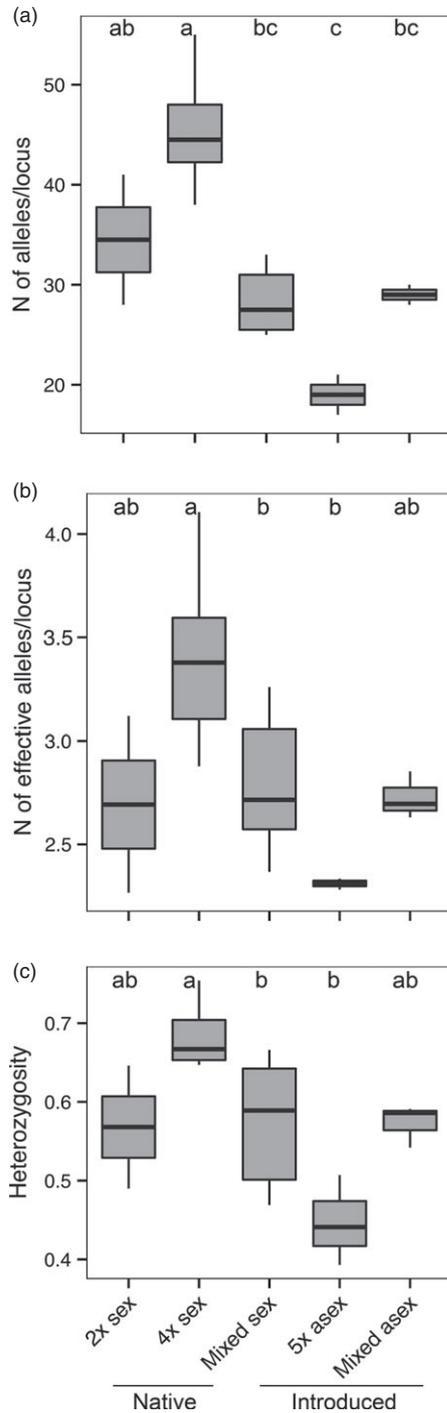


Fig. 2 Box plots of indices of genetic diversity in native and introduced populations of *Oxalis pes-caprae*: a) number of alleles per locus; b) effective number of alleles per locus; c) total heterozygosity. Comparisons were carried among groups of populations considering origin, ploidy level and reproductive mode of individuals (2x sexual native; 4x sexual native; 4x + 5x sexual mixed populations introduced; 5x asexual introduced; 4x + 5x asexual mixed populations introduced). Dissimilar superscripts indicate differences following Tukey's post hoc tests ($P < 0.05$).

Discussion

Oxalis pes-caprae illustrates the complexity of biological invasions for species with polyploidy and dual sexual/asexual reproductive systems. Our comparison of genetic diversity in native and introduced populations of *O. pes-caprae* revealed several major findings: (i) Populations polymorphic for style morph contained significantly higher levels of genetic diversity than predominantly clonal populations of the 5x cytotype. However, an important finding was that populations of the 5x cytotype were not genetically uniform, as often assumed, and were composed of numerous genotypes. (ii) The recently discovered 4x L-, M- and S-morphs in the Western Mediterranean most likely resulted from multiple introductions from the native range rather than originating *in situ* from the 5x cytotype. (iii) The 5x cytotype was restricted to just three individuals in a single population in South Africa. The rarity of this form in the native range therefore raises intriguing questions concerning how and where it originated. Below, we discuss our results in detail and consider their relevance to broader questions in invasion genetics.

Effect of shifts in reproductive mode and ploidy level on genetic variability

Our comparison of native vs. introduced populations of *O. pes-caprae* found no substantial difference in the overall levels of genetic diversity between regions. However, this finding is unlikely to reflect differences in the average amount of genetic diversity within populations between the regions because our sampling of populations in the introduced range was not random. Instead, we targeted populations that were likely to be sexual because of the presence of more than one style morph. A completely random sample of populations from the Western Mediterranean region would have significantly increased the proportion of sterile 5x populations in our sample, as these predominate throughout this region (e.g. see Fig. 2 in Castro *et al.* 2007, 2013). As expected, we found much reduced levels of genetic diversity in the three 5x populations that we sampled from the introduced range (Fig. 2), most probably as a result of bottlenecks and near exclusive clonal propagation in these populations.

Our comparison of levels of genetic diversity between native and introduced populations of *O. pes-caprae* revealed significant differences between groups defined by ploidy level and reproductive mode (Fig. 2). In the native range, 4x populations tended to have more genetic variability than 2x, as might be expected. In the introduced range, we found significantly lower variability in populations containing only the sterile 5x

Table 3 Indices of genetic diversity in populations of *Oxalis pes-caprae* from the native and the introduced ranges

Range	Population	N	A_n	AE_{ff}	H_t	PA
Native: South Africa	Barrydale	21	48	4.106	0.754	1
	Cape Point	20	44	2.878	0.65	1
	Gouritsmond	20	55	3.866	0.743	3
	L'Agulhas	21	40	3.387	0.691	0
	Lamberts Bay	20	38	3.169	0.647	0
	Nuwerus	21	41	3.119	0.646	0
	Oudtshoorn	21	43	3.504	0.674	1
	Springbok	21	28	2.266	0.49	0
	Worcester	21	48	3.368	0.654	0
	Yzerfontein	21	45	2.911	0.66	1
Introduced: Western Mediterranean Basin	Almogrove	19	30	2.853	0.591	0
	Armação de Pêra	6	29	2.631	0.586	0
	Coimbra	10	17	2.334	0.507	0
	Colares I	21	32	2.855	0.61	0
	Colares III	21	33	3.259	0.653	0
	Lavras	10	21	2.314	0.441	0
	Marinha Grande	20	25	2.367	0.469	0
	Melides	15	28	2.696	0.542	0
	Moulay-Bousselham	6	27	3.123	0.666	0
	Praia de São Pedro da Maceda	20	25	2.572	0.479	0
	Troia	15	28	2.576	0.568	0
	Veirinhos	10	19	2.281	0.393	0

N, number of individuals used for the molecular study; A_n , number of alleles; AE_{ff} , number of effective alleles; H_t , total heterozygosity; PA, number of private allele.

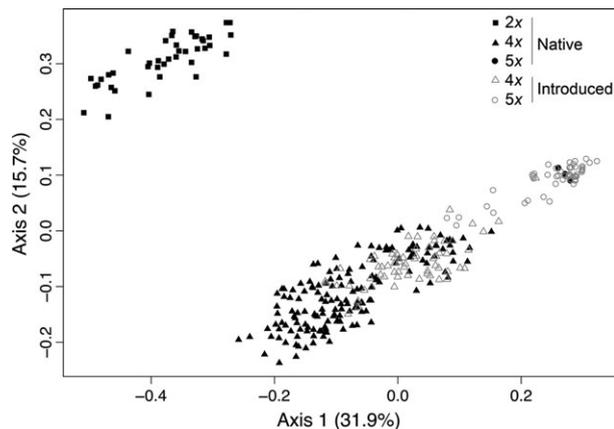


Fig. 3 Principal coordinate analysis (PCoA) of *Oxalis pes-caprae* individuals using the scored nSSR genetic phenotypes. Each sample is represented by a symbol depending on its geographical area of origin and ploidy level. Percentage of explained variance for each axis is given in parenthesis.

S-morph, but this difference was only significant for all three indices of genetic diversity when compared to native 4x populations. The presence of fertile 4x style morphs in invasive populations containing the 5x cytotype enables variable amounts of fruit set to occur (Table 1), and the survival and establishment of sexual offspring could increase genetic diversity in the introduced range. Despite the occurrence of the three style morphs in the introduced range, clonality predominated

in all populations through bulbil formation, and the 5x cytotype dominated in virtually all invasive populations that we sampled. Although populations in both the native and introduced ranges reproduce clonally, the balance between sexual and asexual reproduction differs dramatically between the regions as a result of a shift in reproductive mode associated with invasion.

Although the genetic diversity of asexual monomorphic 5x populations was lower than that of 4x populations, populations were by no means genetically uniform. A previous study of *O. pes-caprae* in Israel reported variability at AFLP loci within each of five asexual populations of the sterile 5x cytotype (Rottenberg & Parker 2004). The authors suggested that the variability may have arisen as a result of the accumulation of somatic mutations, which are known to accumulate in clonal plants (Klekowski 1988; Ally *et al.* 2008; Bobiwash *et al.* 2013), or through genome rearrangements, a common feature of polyploids (Soltis & Soltis 2000), resulting from somatic recombination (Puchta *et al.* 1994). These findings of genetic diversity in asexual populations caution against assuming that the absence of sexual reproduction in a population of *O. pes-caprae* necessarily means that it is composed of a single clonal genotype. Further work is required to determine the mechanism(s) responsible for generating diversity in *O. pes-caprae* and other putatively asexual plant populations.

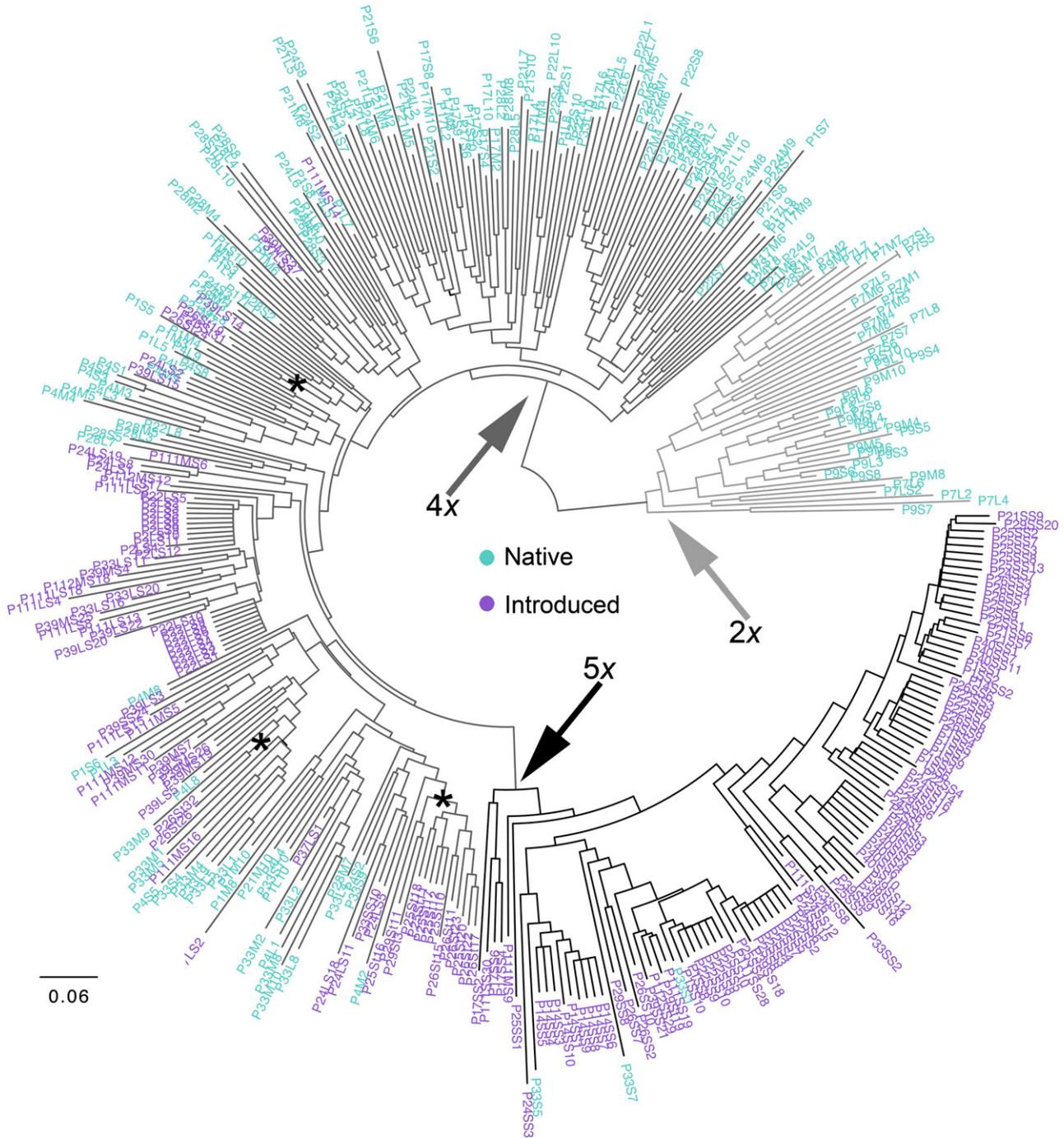


Fig. 4 Neighbour-joining tree based on the dissimilarity matrix of 380 individuals of *Oxalis pes-caprae* and seven SSR markers. The colours of samples refer to the geographical area where they were sampled. Branches of lineages showing the same ploidy level are coloured differently and marked with an arrow. Asterisks mark lineages with sterile double-flowered forms.

Origin of the forms newly found in invasive populations

Oxalis pes-caprae arrived in the central Mediterranean Basin towards the end of the eighteenth century (Henslow 1891; Clarke 1934). In its introduced range,

the species has most often been characterized, following Baker (1965), as an asexual form (5x S-morph), except in Australia where sexual tetraploids were also introduced and occasional hybrids apparently occur between 4x and 5x cytotypes (Michael 1964). The discovery of fertile 4x L-, M- and S-morphs in the Western Mediterranean

(Castro *et al.* 2007, 2013) raises the question of whether they arose through separate introduction(s) from South Africa and/or were generated *in situ* within the introduced range. The multiple introduction of tetraploids from South Africa could have occurred through horticulture and/or because populations of this cytotype are weedy in most regions of the native range (Krejčíková *et al.* 2013) thus increasing their likelihood of becoming naturalized and invasive. It is also plausible that L- and M- morphs could have originated in the introduced range by segregation following self-fertilization of the 5x form (Ornduff 1987). Because of the inheritance of tristyly (see Barrett 1993), selfing or intramorph crossing of an S-morph, heterozygous at the loci determining style length, should result in the segregation of both L- and M-morphs. Plants with odd ploidy levels (e.g. 3x, 5x) are known to produce viable gametes (Ramsey & Schemske 1998; Risso-Pascotto *et al.* 2003), and indeed, this has been observed in *O. pes-caprae* (Vignoli 1935, 1937). Moreover, a recent study (Costa *et al.* 2014) demonstrated that the 5x cytotype is capable of producing seeds in the Iberian Peninsula, which was also observed in our study, including several monomorphic populations of the 5x S-morph.

Our results support the multiple introduction hypothesis. The Neighbour-joining tree indicated that all 5x individuals, including the three found in the native area, constitute a single lineage derived from tetraploids. More importantly, the tetraploids collected in the Western Mediterranean region are more closely related to 4x individuals from the native area than to 5x individuals, and there are no 4x descendants from the 5x lineage. These results make it unlikely that the 4x forms in the introduced range originated by sexual reproduction of the 5x S-morph and support the occurrence of multiple introductions.

The sterile double-flowered form has been found in the Western Mediterranean region and other parts of the Mediterranean basin, including Italy and Israel (Castro *et al.* 2007; Signorini *et al.* 2014; S. C. H. Barrett, personal observations), and in some areas, it can even form monomorphic populations (Castro *et al.* 2007). In the native range, this form is rare and found only sporadically (Salter 1944; J. Suda and K. C. Oberlander, personal communications). The double-flowered mutant may arise when the sexual organs in the flower are replaced by petals through a mutation in the gene *agamous* that encodes for a protein responsible for tissue specification of stamen and carpel segments (Yanofsky *et al.* 1990). The double-flowered plants are commonly associated with horticulture, and no doubt gardening activities have hastened their spread, as it has been widely documented for a variety of other cultivated species (Reichard & White 2001; Dehnen-Schmutz *et al.*

2007). Our results indicate that the 4x double-flowered individuals do not group into a single lineage but rather are scattered across the cluster of 4x plants from the native range. This indicates that their occurrence in the introduced range is not associated with the 5x sterile cytotype and that they are likely to have resulted from separate introductions.

Origin and introduction history of the sterile 5x cytotype

In the native range, the 5x cytotype is very rare and until now has been restricted to the Cape Town area, including Kirstenbosch Botanical Garden (Michael 1964; te Beest *et al.* 2012; Signorini *et al.* 2014). Indeed, a recent large-scale survey in the Western and Northern Cape Provinces involving flow cytometry failed to find any 5x individuals (Krejčíková *et al.* 2013). We obtained similar results after sampling an additional 23 populations across the Cape region, further supporting the rarity of the 5x cytotype in the native range. However, our discovery of three 5x individuals at a new location in South Africa raises several intriguing questions concerning the origin of the 5x cytotype.

The most widely accepted interpretation of the origin of the 5x cytotype is that it originated in South Africa after the fusion of an unreduced gamete of a 4x plant and a reduced gamete of a 2x individual (Ornduff 1987). However, recently Krejčíková *et al.* (2013) rejected this idea because of the absence of 5x hybrids in contact zones between diploid and tetraploid populations. These authors proposed an alternative explanation for the origin of the 5x cytotype through hybridization between a 4x and a putative 6x plant, the latter supposedly originating from unreduced and normal gametes of the tetraploid. However, this scenario also lacks support for the same reasons pointed out by Krejčíková *et al.* (2013) in refuting the initial hypothesis, that is no hybrids have been detected in contact zones between cytotypes and, perhaps more importantly, the putative 6x cytotype has not been reported in South Africa or any other part of the range of *O. pes-caprae*.

Another hypothesis for the origins of the 5x cytotype has been proposed by Signorini *et al.* (2014) and Krejčíková *et al.* (2013), who suggested that 5x plants may not have originated in South Africa as proposed by Ornduff (1987). According to these authors, the 5x cytotype could have originated from 4x individuals in one or more regions of their introduced range, with 5x plants subsequently introduced to South Africa. Our results provide tentative support for the hypothesis of a possible introduction of 5x individuals to the Greater Cape Floristic Region from the introduced range. Pentaploids from South Africa clustered with those from

the Western Mediterranean region and, overall, individuals of the 5x cytotype were more related to tetraploids from the introduced range than they were to 4x individuals from the native range. This observation suggests the possible role of nurseries and botanical gardens in Europe in the spread of the invasive form (and see Michael 1964). Further surveys of ploidy level, style morph representation and patterns of genetic diversity in other Mediterranean regions where *O. pes-caprae* is a prolific weed (i.e. Australia, California and Chile) are necessary to fully understand the complex invasion history of this species.

To conclude, the results of our study have historical relevance coming as they do on the 50th anniversary of 'The Genetics of Colonizing Species' edited by Herbert G. Baker and G. L. Stebbins published in 1965. The reason is because *Oxalis pes-caprae* was highlighted in Baker's article (Baker 1965) concerned with the mode of origin of weeds as a classic example of a species possessing the 'general-purpose genotype strategy'. He argued that invasive populations of *O. pes-caprae* were composed of a sterile pentaploid genotype with wide environmental tolerance and an ability to grow in a multitude of climatic and edaphic conditions as a result of well-developed phenotypic plasticity. Our findings are not inconsistent with Baker's idea because the 5x cytotype certainly dominates in the Western Mediterranean and other parts of the introduced range. However, numerous 5x genotypes were evident in our survey, as well as sexual 4x L-, M- and S-morphs. Thus, recombination in sexual populations, and as yet undetermined genetic mechanisms generating variation in the 5x cytotype, may be more common in invasive populations than has generally been assumed.

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Data accessibility

Sampling locations, flow cytometry data, microsatellite genotypes and NJtree file are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.r69g1>.