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ORIGINAL PAPER

Molecular systematics and ecology of invasive Kangaroo Paws in South Africa: management implications for a horticulturally important genus

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Abstract Most legislation pertaining to non-native organisms is implicitly focussed at the individual species level. However, in some cases interspecific hybrids can be more invasive than any of the parent species. This is problematic for policy makers, and for horticulturists developing or trading in new ornamental cultivars. We explore these issues in the context of the need to manage naturalized populations of Kangaroo Paws (*Anigozanthos* species) in South Africa. Self-sustaining, dense populations of naturalized Kangaroo Paws occur at several localities and are highly attractive to local nectar-feeding birds. The populations show high levels of seed set with or without bird pollination. Given the known propensity of Kangaroo Paws to hybridise in their native range in Australia, and confusion about the species identity of naturalized populations in South Africa, it was essential to resolve some key taxonomic issues in the group. We constructed the first molecular phylogeny for *all* species of the Kangaroo Paw group (genera *Anigozanthos* and *Macropidia*; family Haemodoraceae). As previously determined by taxonomists working on herbarium specimens, naturalized populations were identified as *A. flavidus*. In addition, we also identified a second species, *A. rufus*. Relative genome size estimates for *Anigozanthos* species

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indicated that small inter-specific differences in genome sizes are positively correlated to hybrid fitness. *Anigozanthos flavidus* and *A. rufus* have relatively 'compatible' genomes and may produce fertile hybrids under field conditions. However, for species whose genome size differ more than ~30%, there is little inter-specific compatibility and consequently a very low risk of producing fertile hybrids. In conclusion, we recommend that trade in Kangaroo Paws in South Africa should be temporarily restricted and that particular cultivars should first be subjected to a careful risk assessment.

Keywords Biological invasions · Kangaroo Paws · *Anigozanthos* · *Macropidia* · Systematics · Pollination · Breeding system · Self-compatibility · Hybridisation

Introduction

Managing invasive populations by responding rapidly, even in the absence of insights from detailed natural history studies, is cost-effective (Simberloff 2003). This is because the cost and likelihood of success of an eradication programme scales exponentially with the size of infestation (Rejmánek and Pitcairn 2002). However, any action taken, or planned, must be justifiable. This is particularly important when the invasive species has commercial or other value. Increasingly, the justification for intervention needs to include objective verification that the putative invader has a high risk of spreading and/or causing damage. A key task in objective risk analysis for biological invasions is the accurate determination of the taxonomic identity of the subject and an assessment of its biogeographic status: native versus alien (sensu Pyšek et al. 2004). When the taxonomic identity of an organism is clear and the species is known to be invasive elsewhere in the world, initial risk assessment may require little additional information (Pheloung et al. 1999). However, if the taxonomy is uncertain (e.g., due to a lack of local expertise, cryptic invasions, or possible hybridisation), elucidation of the taxonomic status is crucial for legislation. Moreover, well-resolved taxonomy on its own is of little value if no additional information is available.

There have been several attempts to define which parameters and traits are pertinent to invasion risk for plants. For example, Richardson et al. (2000) suggested that spread of alien plants may be limited by a lack of suitable pollinators, and that pollinators in the introduced range may play an important role in maintaining reproductive fitness and in facilitating spread. Therefore, studies investigating the breeding system and/or pollination ecology of naturalized plants can both aid risk assessment, and help estimate the potential for hybridisation (Ellstrand and Schierenbeck 2000). Such basic natural-history studies should run concurrently with management programs. This dual approach, whereby evidence is gathered without compromising timely action to limit invasions, is particularly important if there is a conflict of interest or if co-operation needs to be encouraged or enforced to achieve eradication (Simberloff 2009).

South Africa is developing new legislation to regulate the use, sale, and control of non-native species, as part of the National Environment Management: Biodiversity Act (NEM:BA). NEM:BA follows on from the Conservation of Agricultural Resources Act (CARA) in listing regulated species (Nel et al. 2004). Much of the legislation relies on species being defined entities, but the legislation also attempts to deal with cultivars and hybrids. For example: "all seed producing species or hybrids of *Lantana* that are non-indigenous to Africa" are regulated. This specification, and prolonged engagement with horticulturists, resulted in an agreement to destroy all stock of the lantana cultivar 'Sundancer' when it was shown to produce fertile seeds, but to continue trading with the species *Lantana montevidensis*, which does not produce fertile seeds in South Africa (although Czarnecki and Deng (2009) showed some cultivars of *L. montevidensis* produce fertile pollen and seed in experimental trials in the USA). The regulations thus aim to be pro-active, but rely on focussed research to provide key biological information on which to base decisions. Continued support from horticulturists will depend on the understanding that trade restrictions will only apply to taxa with a high risk of invading, based on objective and transparent criteria.

In this paper we describe a case-study in South Africa of a group of Australian plants called Kangaroo Paws (the genera *Anigozanthos* and *Macropidia*). We assess the current extent of invasion of known

naturalized populations, place this in a phylogenetic context, and, by exploring genome compatibility, assess the likelihood of hybridisation. Based on these findings we provide recommendations on how to accommodate this group of plants in developing regulations.

Materials and methods

Study system

Kangaroo Paws are perennial herbs native to Western Australia from the genera *Anigozanthos* and *Macropidia* (Haemodoraceae). Adaptations to bird pollination, unusual flower morphology, and colouration (Fig. 1) make them popular and important horticultural species in many parts of the world (Tsror et al. 2005). Twelve species of Kangaroo Paws are currently recognized (Hopper 1987); 11 in the genus *Anigozanthos* and a single species in the genus *Macropidia* (*M. fuliginosa*). The taxonomy of Kangaroo Paws is problematic and has been the subject of numerous studies (Anderberg and Eldenäs 1991; Hopper 1980; Hopper and Campbell 1977; Hopper et al. 1999, 2009; Simpson 1990). In particular, the validity of *Macropidia* as a monotypic genus has been discussed in detail, with some authors supporting its monotypic status (Hopper 1980; Hopper and Campbell 1977; Hopper et al. 1999, 2009) while others have argued in favour of it being lumped within *Anigozanthos* (Anderberg and Eldenäs 1991; Simpson 1990). Relationships between closely-related taxa within *Anigozanthos* are also unclear (Hopper 1980). Contributing to this taxonomic obscurity is the ease with which some species within *Anigozanthos* hybridise (Hopper 1977a, b, 1978, 1980; Hopper and Burbidge 1978; Shchori et al. 1995).

Kangaroo Paws are cultivated commercially in several countries around the world and at least 26 cultivars are registered with the Australian Cultivar Registration Authority (http://www.anbg.gov.au/acra/acra-list-2009.html#a). In its native country, populations of *A. flavidus* have naturalized and are spreading in New South Wales and South Australia (Australian Native Plants Society 2009; Martin O'Leary, personal communication 2009) and are considered serious environmental weeds (Hoskins et al. 2007). Hopper (1993) noted that *A. flavidus* is extremely competitive and indeed the most invasive and robust species of Kangaroo Paw. Despite this, *A. manglesii* is the only taxon listed in Randall's (2007) "The introduced flora of Australia and its weed status".

In South Africa, Kangaroo Paws have been traded in the horticultural industry since the 1990s and

Fig. 1 **a** Native nectarivorous birds such as the Orange-breasted Sunbird are attracted to kangaroo paw flowers. **b** Protruding stamens allow deposition of pollen on foreheads (*black arrow*) for cross-pollination, while **c** other birds (Cape Sugarbird) sometimes feed on pollen (photographs by Sjirk Geerts)

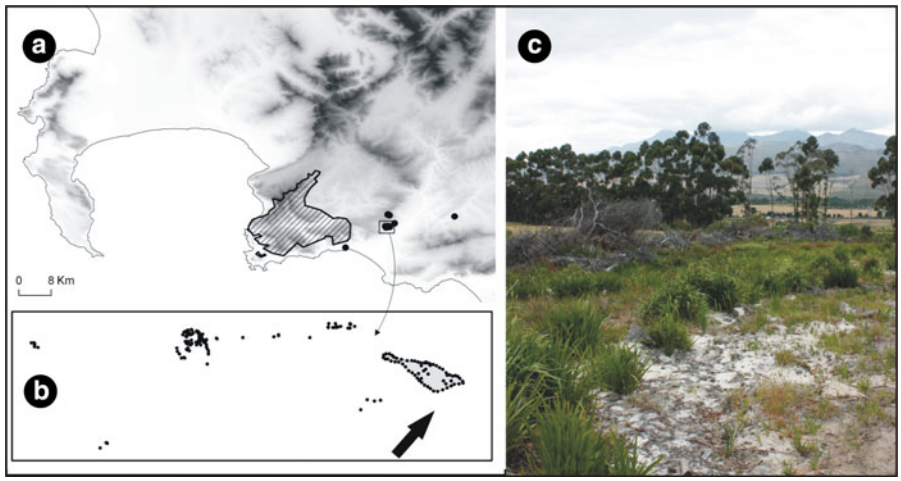

Fig. 2 **a** Map illustrating the locations of *Anigozanthos* populations in South Africa (Western Cape). One large population (ca. 180,000 individual sprouts) was found on Honingklip farm (enlarged in **b**) and contained areas infested by monotypic stands of *Anigozanthos* (**c**) indicated by *black arrow* in **b**. The polygon in **a** depicts the location of the Kogelberg Biosphere Reserve (photograph by Sjirk Geerts)

currently about ten commercial hybrids are available (Jacques Malan, MalanSeuns Nursery, personal communication). Data from Compton Herbarium in Cape Town indicated that areas around Kleinmond, in South Africa's Western Cape Province, are the only known locations where Kangaroo Paws occur (Fig. 2). These records identified naturalized populations as *A. flavidus* and, potentially, *A. manglesii*. These populations presumably are derived from plants introduced to a local flower farm (Honingklip) somewhere between 1960 and 1969. The record for this population in the South African Plant Invaders Atlas gives the taxon as *A. flavidus* (Henderson 2007), although Stephen Hopper (personal communication), the world authority on the group, has suggested that these populations represent "*A. flavidus* and hybrids". The current invasion occurs within 10 km of one of South Africa's most pristine and important biodiversity-hotspot conservation areas, the Kogelberg Biosphere Reserve (Fig. 2).

Mapping of invasive populations and population size estimates

All known naturalized populations were systematically surveyed by walking parallel lines extending ~50 m beyond the most isolated plant found (see Zenni et al. 2009 for details). For smaller outlying populations the geographic position of each plant found was marked using a hand-held Global Positioning System (GPS Garmin® GPSmap 60CSx, maximum resolution of 3 m). For large populations (>20,000 plants) the tracklogs from the tracking lines recorded in the GPS were used as the basis for drawing a polygon of the surveyed area in ArcView GIS v. 3.2. Three plots of 15 m × 15 m within these larger populations were then subdivided into quarters and the percentage plant coverage (rhizome mats) visually determined independently by two persons. We also used twelve 2 m × 2 m random plots within large populations to determine the number of sprouts/unit area covered by monotypic stands. We used sprouts because in most cases it was impossible to distinguish individual plants from sprouts (clusters of fans [leaves]) from the same rhizome in dense stands. Using the spatial analyst tool in ArcGIS we determined the total area of the polygon (population) for large populations. Using data for coverage and density estimates we were able to extrapolate estimates for population size/density and the percentage area covered.

Pollination biology

Flowers in the largest patch of Kangaroo Paws (Fig. 2) were observed for bird visitation for 1 h during peak activity (morning) before and after flower removal. Pollinator species, number of flowers visited and behaviour were also recorded. To determine accessibility for Sunbirds and Sugarbirds, flower depth was measured in 13 randomly selected flowers.

As an additional estimate of the attractiveness to pollinators, we estimated the standing crop of nectar. This is influenced by the rate of production, consumption, and evaporation of nectar and gives an

indication of the actual resource available to birds at any time. Nectar standing crop was measured in the morning (when birds were still very active) from 15 randomly selected young flowers. We determined nectar volume in the field with a 5 µl capillary tube and nectar concentration with a handheld 0–50% Bellingham and Stanley refractometer (Tunbridge Wells, Kent, UK).

Seedset

To determine the importance of pollinators on individual fitness, we compared capsule set and number of seeds set per capsule for bagged and unbagged flowers. Twenty clumps were selected from the largest population and inflorescences were bagged in fine-mesh pollinator exclusion bags while in bud phase, and kept bagged throughout the flowering period (a total of 748 flowers; 10 inflorescences). A nearby inflorescence in the same clump was marked and used as a control (flowers were open to all flower visitors, with a combined total of 912 flowers; 10 inflorescences). The proportion of capsules that set seed was determined by comparing the number of filled capsules with the number of stem scars left by aborted flowers after 12 weeks. Capsules were dissected and numbers of seeds counted. All statistical analyses were conducted in R (R Development Core Team 2009). Differences in capsule set between open and bagged flowers were tested using generalised linear models with binomial errors. Differences in seed set were tested using both generalised linear models with a variety of error structures, to explore the impact of over-dispersion, and with generalised linear mixed-effect models, to account for between-plant variation.

Kangaroo Paw taxonomy

Taxon sampling and DNA extraction

Leaf samples were collected from South Africa and Australia, dried and kept on silica gel (Table 1). Accessions of all *Anigozanthos* species and *M. fuliginosa* in Australia were obtained from collections held at Kings Park and Botanic Garden in Perth, Western Australia. Invasive taxa in South Africa were collected by JLR. Total genomic DNA was extracted using the CTAB extraction protocol described by Doyle and Doyle (1990).

PCR amplification and DNA sequencing

The spacer and intron regions of the plastid *trnL-F* region were amplified using the universal primers "c" and "f" (Taberlet et al. 1991). Each 50 µl PCR reaction contained approximately 50 ng of genomic DNA, 200 µM of each dNTP (AB gene, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 25 pmol of each primer, 5 U *Taq* DNA polymerase (Super-Therm JMR-801, Southern Cross Biotechnologies, Cape Town, South Africa), 1× PCR reaction buffer, 1.5 mM $MgCl_2$. PCR consisted of a thermocycle of initial denaturation of 95°C for 5 min; 35 cycles at denaturation at 94°C for 30 s, annealing at 58°C for 60 s, elongation at 72°C for 90 s; and final extension at 72°C for 10 min. All PCR amplifications were done in a Multigene gradient cycler (Labnet International, Inc., NJ, USA). Amplified DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Southern Cross Biotechnologies, Cape Town, South Africa) and sequenced in both directions using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an automated ABI PRISM 377XL DNA sequencer (PE Applied Biosystems, Foster City, California, USA).

DNA sequence alignment and analysis

Contiguous sequences were constructed, edited and aligned using BioEdit version 7.0.5.3 (Hall 1999). All edited sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov; Table 1). We also included data for other Kangaroo Paw species that were available on GenBank. We used *Blancoa canescens*, a known sister species to Kangaroo Paws (Hopper et al. 1999), as outgroup taxon.

The dataset was analyzed using maximum-likelihood search criteria with parameter estimates obtained from the program MODELTEST version 3.06 (Posada and Crandall 1998). We estimated base frequencies and the transition/transversion ratio from the data. Heuristic searches were carried out with TBR, MULTREES, and COLLAPSE options in effect and performed with PAUP* 4.0beta10 (Swofford 2002); 1,000 bootstrap replicates (Felsenstein 1985) were used to assess branch support. Trees were visualised in TreeEdit version 1.0a1-19 (http://evolve.zoo.ox.ac.uk/software/TreeEdit/main.html).

Table 1 Details of specimens of *Anigozanthos* and *Macropidia* species collected in this study

Specimen ID	Species	Country	Lat/long	GenBank accession
JLR033	Unknown	South Africa	−34°20′19.02″, +19°2′16.56″	GU223383
JLR034	Unknown	South Africa	−34°17′31.74″, +19°8′10.08″	GU223384
JLR035	Unknown	South Africa	−34°17′31.74″, +19°8′10.08″	GU223385
JLR036	Bush Pearl (*A. humilis* × *A. bicolor* × *A. flavidus*)	Nursery stock South Africa	NA	GU223392
JLR038	Unknown	South Africa	−34°17′31.74″, +19°8′10.08″	GU223387
JLR158	*A. manglesii*	Australia	−31°43′19.30″, +115°51′43.70″	GU223388
JLR159	*A. manglesii*	Australia	−31°50′37.98″, +116°19′32.34″	GU223389
JLR160	*A. humilis*	Australia	−32°0′47.28″, +116°36′0.18″	GU223390
JLR161	*A. kalbarriensis*	Australia	−31°57′59.13″, +115°50′17.56″	GU223391
JLR162	*A. flavidus*	Australia	−34°57′30.30″, +117°48′29.40″	GU223386
JLR163	*A. viridis*	Australia	−31°57′59.13″, +115°50′17.56″	GU223393
JLR164	*A. gabrielae*	Australia	−31°57′59.13″, +115°50′17.56″	GU223394
JLR165	*A. preissii*	Australia	−34°51′15.40″, +116°56′13.50″	GU223395
JLR166	*M. fuliginosa*	Australia	−31°57′59.13″, +115°50′17.56″	GU223407
JLR167	*A. pulcherrimus*	Australia	−31°57′59.13″, +115°50′17.56″	GU223404
JLR168	*A. flavidus*	Australia	−34°56′4.00″, +117°56′38.30″	GU223396
JLR169	*A. flavidus*	Australia	NA	GU223397
JLR171	*A. humilis*	Australia	−32°1′11.70″, +115°58′55.62″	GU223398
JLR172	*A. rufus*	Australia	−34°19′41.50″, +117°50′13.50″	GU223399
JLR173	*A. rufus*	Australia	NA	GU223400
JLR174	*A. preissii*	Australia	−34°51′15.40″, +116°56′13.50″	GU223401
JLR175	*A. bicolor*	Australia	−32°12′36.80″, +116°18′41.80″	GU223402
JLR176	*A. bicolor*	Australia	NA	GU223403
JLR252	Bush Gold (*A. humilis* × *A. flavidus*)	Nursery stock South Africa	NA	GU223405
JLR254	Bush Pearl (*A. humilis* × *A. bicolor* × *A. flavidus*)	Nursery stock South Africa	NA	GU223406

Genome size estimates and hybrid reproductive output and fertility

Relative genome sizes of silica-dried specimens were determined by flow cytometry using a Partec PA II instrument (Partec GmbH., Münster, Germany) equipped with a mercury arc lamp for UV excitation. The methodology generally followed the two-step procedure (without centrifugation) described by Suda and Trávníček (2006). Otto I buffer (0.1 M citric acid, 0.5% Tween 20) was used for nuclei isolation and Otto II buffer (0.4 M $Na_2HPO_4 \times 12\ H_2O$), supplemented with AT-selective fluorochrome DAPI (at final concentration 4 µg/ml) and ß-mercaptoethanol (2 µl/ml), was used to stain the nuclear suspension. *Bellis perennis* L. (2C = 3.38 pg) was selected as an appropriate internal reference standard and flow histograms were evaluated using the Partec FloMax software ver. 2.4d.

Flow cytometry analysis was conducted on all *Anigozanthos* species except *A. onycis* due to a lack of tissue material for this species (Table 2). A matrix of genome size ratios (differences) was constructed for all pairwise species combinations for which we had estimates. Similarly, we constructed matrices for numbers of seed set, seed germination success, and pollen fertility for the same pairwise species combinations (hybrids), using data from Hopper (1980). These datasets allowed us to explore how differences in parental genome sizes affect their resulting hybrids' fitness (seed set, germinability, pollen fertility). We used linear regression to examine the effect of the ratio

Table 2 Relative genome size estimates (mean ± SD) for selected *Anigozanthos* species

Species	Relative genome size (pg)	No. of samples
A. bicolor	0.615	1
A. flavidus	0.796 ± 0.010	3
A. gabrielae	0.609	1
A. humilis	0.700	1
A. kalbarriensis	0.712	1
A. manglesii	0.639 ± 0.010	3
A. preissii	0.897 ± 0.074	2
A. pulcherrimus	0.753	1
A. rufus	0.773 ± 0.017	2
A. viridis	0.643	1

Bellis perennis, 2C = 3.38 pg, was used as a unit value

of genome sizes (larger over smaller, log-transformed) on the number of seeds per capsule produced by hybrids (also log-transformed). We also tested a regression weighted by the number of capsules investigated, as this varied from 2 to >200, see Appendix 1 in Hopper (1980). The effect of the ratio of genome sizes on the probability of hybrid seed germination was tested using a generalised linear model with binomial and quasi-binomial errors. Finally, the effect of the ratio of genome sizes on the percentage pollen fertility of the F_1 hybrids (logit transformed and zeros excluded, as data on sample size was not available) was tested using a linear model. In this case, we also tested to see whether there was an effect of weighting by the number of hybrids the pollen was collected from. We assumed that different parental crosses are distinct entities (although obviously with the same ratio of genome sizes). This may inflate the significance level of any results, but given the strength of the relationships obtained this should not affect the qualitative conclusions.

Results

Invasive Kangaroo Paw populations in South Africa

Population mapping and size estimates

We found one large population that covered around 6,676 m^2 on Honingklip farm, the original point of introduction into the Western Cape (indicated by black arrow in Fig. 2). Within this stand, percentage cover by monotypic stands of plants in subplots ranged from 15 to 85% (average: 42%). These monotypic stands contained between 102 and 462 individual sprouts (average 265). Using these data we estimated that this area is currently infested by monotypic stands of Kangaroo Paws roughly corresponding to around 180,000 individual sprouts. This population was surrounded by scattered individuals up to 700 m away.

We also found two small populations further away. The first population had around 59 individuals in an urban area about 5.5 km from the main Honingklip population. It appears to have spread from a deliberate planting. The second population was about 7.0 km from the main Honingklip population in a mountainous area of natural vegetation and consisted of around 227 individuals. The origin of this population is unknown, but is probably the result of accidental, human-mediated, long-distance dispersal.

Floral visitors

The high density of Kangaroo Paws provided a nectar-rich environment that attracted high numbers of native nectarivorous birds, especially Sugarbirds (*Promerops cafer*). We observed 425 Sugarbird visits in our 1 h observation period (0.44 visits/flower/hour, with a maximum of ten observed at any one time). We also observed incidental visits of the Orange-breasted Sunbird (*Anthobaphes violacea*) (Fig. 1a) and Malachite Sunbirds (*Nectarinia famosa*) at smaller flowering patches. The longer billed Malachite Sunbirds and Sugarbirds carry pollen on their beaks whereas the shorter-billed Orange-breasted Sunbirds carry pollen on their head feathers (Fig. 1b). Sometimes pollen was also consumed by birds feeding on nectar (Fig. 1c). In comparison, only three Sugarbirds and one Malachite Sunbird were observed during 1 h following the removal of all inflorescences.

Flower morphology and nectar properties

Kangaroo Paws have tubular flowers with tube lengths of 27.2 ± 1.8 mm (±1 SD throughout) making nectar accessible to all Sunbirds and Sugarbirds (Geerts and Pauw 2009a). The protruding

stamens ensure pollen placement on the head or upper part of the bill (Fig. 1). Standing nectar crop was relatively low at 8.4 ± 9.9 µl but within the range previously reported for *Anigozanthos* species during dry, non-rainy, seasons (Hopper and Burbidge 1978). Sucrose concentrations were 16.8 ± 2.7%, typical for bird pollinated taxa, and once again within the range previously reported for *Anigozanthos* species (Hopper and Burbidge 1978).

Capsule set and seed production

Bagging flowers did not affect the proportion of flowers that set capsules ($P = 0.25$ from a Chi-squared test comparing generalised linear models with and without bagging as a factor, a similar result is obtained if non-parametric tests are used). Both bagged and unbagged plants showed a high proportion of capsule set (88–100%, except for one bagged plant with a capsule set of 66%).

Bagged flowers, however, tended to have fewer seeds per capsule (Fig. 3). When capsules were treated as replicates, capsules that resulted from bird pollination contained significantly more seeds (41%, 95% C.I. of 13–76%; $LR_{1,180} = 9.16$, $P(\text{Chi}) = 0.0025$, from the model with negative binomial errors, there was substantial over-dispersion if Poisson errors were used). This is similar to the findings of Hopper (1980) who showed a 40% reduction in selfed vs outcrossed populations of *A. flavidus*. However, if plant was included as a grouping factor in a mixed effects model, then there was no significant effect of bagging on the number of seeds set of capsules ($Chisq_{2,4} = 0.88$, $P = 0.64$).

Kangaroo Paw taxonomy

Sequence variation and phylogenetic analysis

The aligned *trnL-F* matrix contained 1,076 characters. All DNA sequences have been deposited in GenBank (accession numbers GU223383–GU223407). The alignment matrix constructed using data generated in this study and additional sequence data obtained from GenBank required nine gaps (indels), ranging from 1 to 17 characters in size. The best-fit maximum likelihood model was a Hasegawa, Kishino, and Yano (1985) (HKY) model. This model with base frequencies determine from the data ($A = 0.3296$; $C = 0.1326$; $G = 0.1530$; $T = 0.38480$), ts/tv = 2.0887, Rates = equal. The score of the optimal tree was −ln likelihood = 1,774.31.

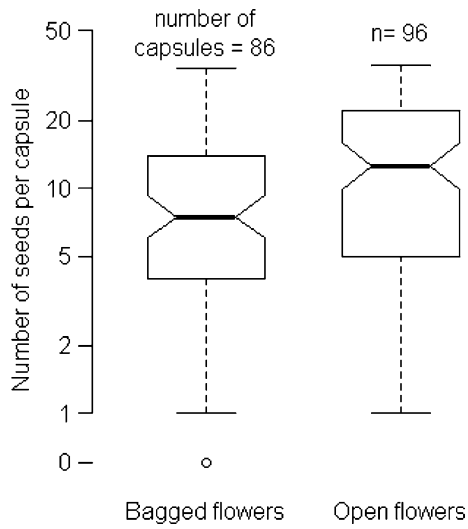

Fig. 3 A boxplot of the effect of bagging flowers on the number of seeds per capsule. In one instance no seeds were found per capsule, as the *y*-axis is logarithmic this is plotted separately. The *bold lines* show the median, the *boxes* the quartile ranges, and the *lines* show either 1.5 times the interquartile range or the point furthest from the median, whichever is less. Outliers outside this range are plotted individually

The ML tree revealed that Kangaroo Paw species from the genus *Anigozanthos* form a monophyletic clade joined by *Macropidia fuliginosa* as the sister lineage, with moderate support (70% BS) (Fig. 4). Within *Anigozanthos* some species of section Anigozanthos (branched species) were more closely related to all species within section Haplanthesis (unbranched species) than to other species within section Anigozanthos. For example, *A. preissii* and *A. onycis* (section Anigozanthos) showed closer phylogenetic relationships to species within section Haplanthesis (e.g., *A. gabrielae*) than to other species within section Anigozanthos (Fig. 4). These findings are somewhat in agreement with Hopper (1980) who found *A. onycis* more capable of hybridising with species from section Haplanthesis than those from section Anigozanthos. Not surprisingly, a constrained maximum likelihood analysis enforcing monophyly for sections Haplanthesis and Anigozanthos resulted in a less likely tree topology (−ln likelihood = 1,824.39).

Our phylogenetic analysis identified invasive populations in South Africa to be a mixture of two species: *A. flavidus* and *A. rufus*. The commercial hybrids, Bush Pearl and Bush Gold, had plastid parental lineages that corresponded to *A. humilis*. This is in agreement with the known hybrid origins of these cultivars (*A. humilis* × *A. bicolor* × *A. flavidus* and *A.humilis* × *A. flavidus*, respectively).

Flow cytometry, hybridisation and fitness

We obtained estimates of relative nuclear DNA amounts for 19 samples (see Fig. 4; Table 2). Inter-specific genome sizes varied 1.59-fold (min. value in *A. gabrielae* JLR 164, max. value in *A. preissii* JLR 165) while the intraspecific variation was usually low (<4.5%, except for *A. preissii* where there was 1.18-fold variation between the two accessions). Superimposing flow cytometry results on the phylogenetic tree (Fig. 4) showed a good agreement between genome size values of *A. flavidus* and one sample from Kleinmond (JLR 33) and two samples from Honingklip (JLR 34, JLR 35). In addition, a clade with a low nuclear DNA amount (comprising *A. bicolor*, *A. manglesii*, and *A. viridis*) was revealed. Genome size values in *A. flavidus* and *A. rufus*, the two species found to be invasive in South Africa, were similar and so we could not identify any hybrid individuals from the flow cytometry data.

The ratio of genome sizes between parental species significantly affected the reproductive biology of hybrids. There was a marked decline in seed set of hybrids with an increase in genome size differences between the parents ($F_{1,63} = 27.7$, $P < 0.01$, Fig. 5a, weighting by number of capsules investigated did not significantly affect the parameter estimates). However, the resulting seeds did not show any difference in germination probability, with an average germination of around 14% (P [$\text{Chi}_{1,60}$] = 0.61, quasi-binomial errors provided a much better fit given the strong over-dispersion, Fig. 5b). The fertility of pollen in the F_1 declined sharply with an increase in genome size differences between the parental stock ($F_{1,34} = 28.6$, $P < 0.01$, Fig. 5c, weighting by number of hybrids used to collect pollen from again did not significantly affect the parameter estimates). It is important to note that some of the data on seed germination percentage and much of the data on F_1 pollen fertility are censored in the original experiment. If it was difficult to grow F_1 hybrids through to pollen fertility then the data are missing (and so not counted as zeros in the analysis).

Discussion

The potential for hybridisation in the introduced range

Hybridisation often results in increased invasiveness for plants (Ellstrand and Schierenbeck 2000; Prentis et al. 2008). Previous taxonomic work putatively identified *A. flavidus* and *A. manglesii* as naturalized in South Africa. In addition to confirming the identity of *A. flavidus*, our phylogenetic approach also identified the second species as *A. rufus*. Our single plastid-gene phylogeny precluded any assessment of whether hybrids are currently present within naturalized populations in South Africa. However, there are several reasons to suspect that there is a potential risk of hybridisation. Firstly, there should be pollen exchange, as the populations comprise mixes of two species with overlapping flowering times and a high frequency of visitation by pollinators. Secondly, most species of Kangaroo Paws are capable of inter-specific outcrossing (Hopper 1980; Shchori et al. 1995), and *A. flavidus* and *A. rufus* are known to produce hybrids in experimental crosses, although these have very low pollen fertility (Hopper 1980). With *A. flavidus* as the paternal lineage, hybrid seeds have a 29.0% germination success, and mature hybrids show 2.9% pollen fertility (Hopper 1980). Unfortunately, reciprocal comparisons of parental crosses between *A. flavidus* and *A. rufus* are not available (the success of Kangaroo Paw hybrids varies with the identity of the paternal and maternal lineages; Hopper 1980). Thirdly, the small difference in genome size between *A. flavidus* and *A. rufus* (3.0%) suggests these species could produce some fertile hybrids. In Australia, populations of *A. manglesii* and *A. humilis* (with 8.5% difference in genome size) readily hybridise and back-cross in the wild (Hopper 1977a, b). Moreover, it should also be noted that the variation in genome size between two accessions determined as *A. preissii* can most easily be explained by the incidence of interspecific hybridisation. Overall, the likelihood of fertile hybrids in Kangaroo Paws appears to be strongly affected by the genome size difference between parental strains, with no fertile hybrids produced above a cut-off of ~30%

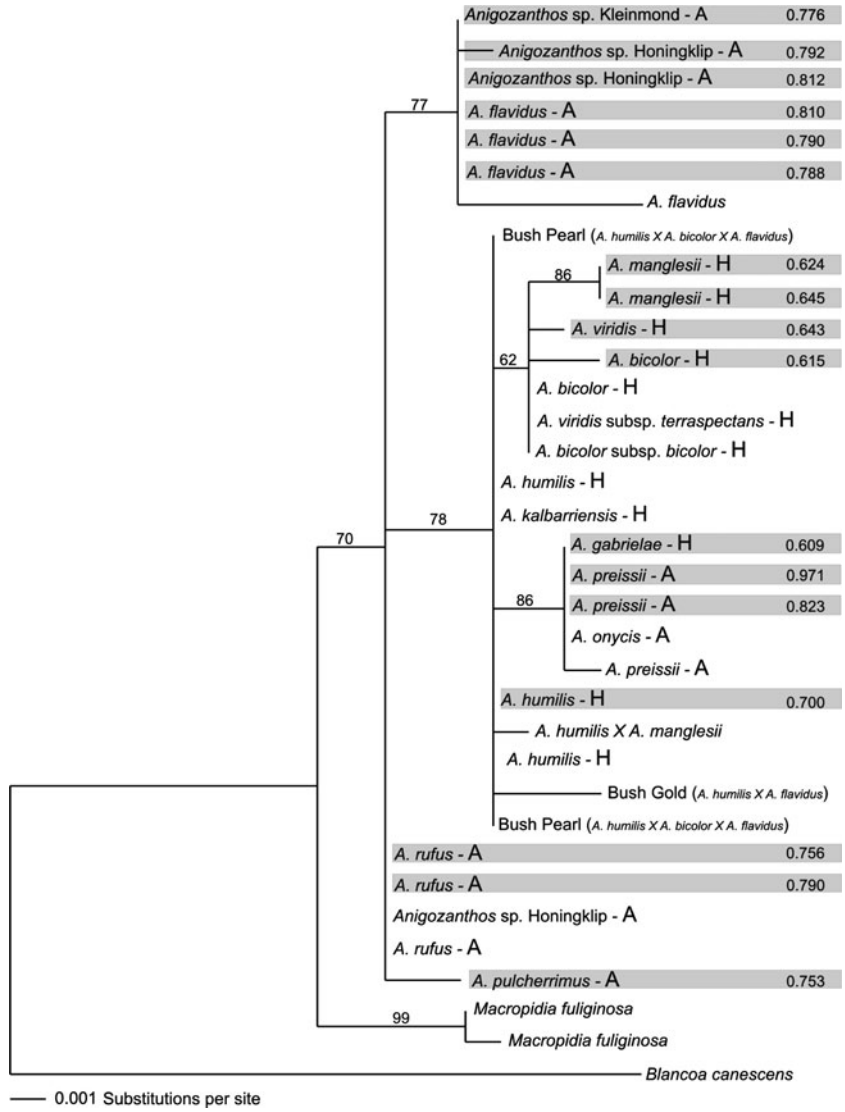

Fig. 4 The maximum likelihood tree for Kangaroo Paws based on trnL-F DNA sequence data. Taxonomic subclassification into branched (section Anigozanthos) and unbranched (section Haplanthesis) is indicated as "A" and "H", respectively. Relative genome sizes are also shown for those taxa where estimates are available (*shaded*). Branch support is indicated as bootstrap values (1,000 replicates)

(Fig. 5). These data support the notion that differences in nuclear DNA amount can serve as a strong barrier for successful hybridisation (Magdalena Kubešová et al. unpublished data; Petr Bureš, personal communication 2009).

The need to control naturalized populations before they spread further

We also showed that *Anigozanthos flavidus* can set seed in South Africa in the absence of pollinators, and so might produce self-sustaining outlying foci after long-distance dispersal. Kangaroo Paws are a predominantly outcrossing group and selfing, on average, results in a 90% decrease in seed set (Hopper 1980). Interestingly, compared to other conspecifics, both *A. flavidus* and *A. rufus* have relatively higher levels of self-compatibility and display relatively high levels of seed set when selfed (Hopper 1980). We found only a 40% reduction in seed set in selfed vs bird-pollinated individuals. This may explain the existence of small outlying populations surrounding the main infestation site on Honingklip farm, and clearly increases the potential for the plants to spread rapidly.

Although the naturalized populations have not yet spread widely, plants can clearly spread and form mono-specific stands. The Early Detection and Rapid Response (EDRR) program in South Africa was

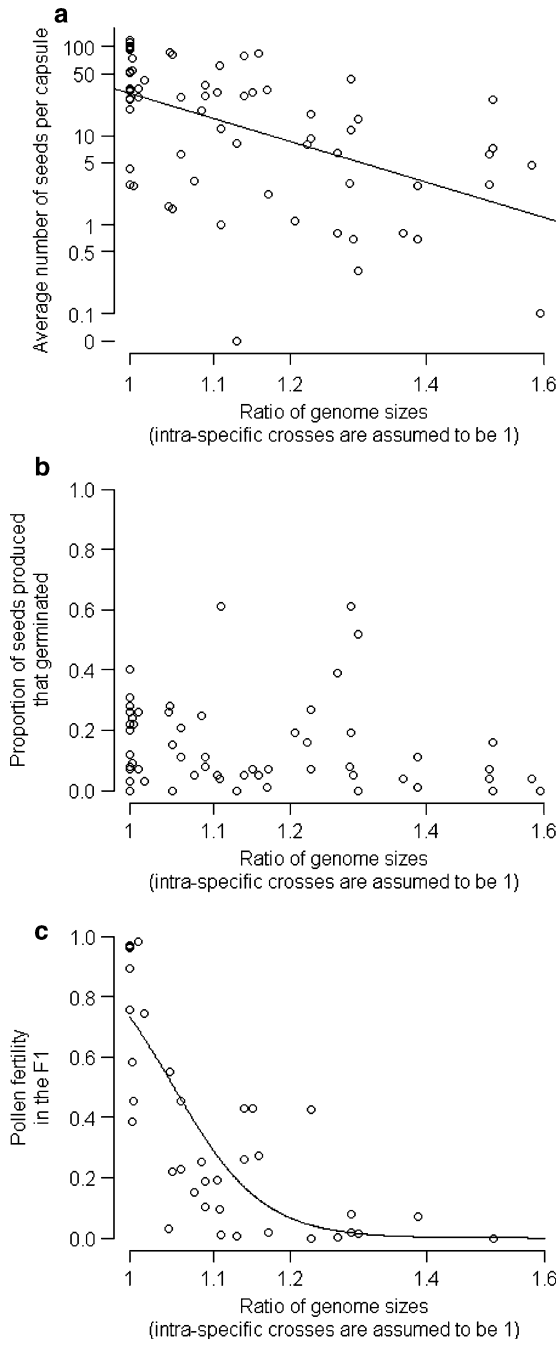

Fig. 5 The effect of relative parental genome size on **a** the number of seeds per capsule, **b** the germination probability of resulting seeds, and **c** F_1 pollen fertility of various hybrids of Kangaroo Paw species. Seed set and pollen fertility data were obtained from Hopper (1980). Statistically significantly fitted relationships are shown **a** $r^2 = 0.37$; **c** $r^2 = 0.46$. In one instance no seeds were found in a capsule, and so this point was excluded from the log-linear regression and, as the *y*-axis is logarithmic, it is plotted separately on **a**

established in part to deal with species before they become widespread. *Anigozanthos* populations are still containable at a relatively low cost, and it is prudent to act while control costs are small.

Anigozanthos species also show the potential to change pollination webs. The floral morphology of invasive *Anigozanthos* species present in South Africa (Armstrong 1979) closely matches that of many native plants in the Cape Floristic Region. Kangaroo Paws provide Sunbirds and Sugarbirds with a rich source of nectar at a time of nectar scarcity (late summer), in return being rewarded by increased reproductive output due to increased outcrossing. Kangaroo Paws could therefore ultimately alter Sunbird and Sugarbird abundance in South Africa (Geerts and Pauw 2009b).

Another important consideration is that invasive populations of Kangaroo Paws in South Africa occur in fynbos, an evergreen hard-leafed shrubland that occurs along the southwestern coastal belt (100–200 km wide) of South Africa. Fire is a crucial ecological factor in the functioning of fynbos ecosystems. As an adaptive response to wild fires in Australia, flowering, branching, seed production, seed viability and seedling establishment of Kangaroo Paws are stimulated by smoke and heat (Lamont and Runciman 1993; Tieu et al. 2001). The frequent occurrence of fires in the currently invaded areas is likely to act as a stimulus for increased reproductive output, spread, and invasiveness. Fire is used as an integral part of management for well-established woody invasive species in fynbos, to kill seedlings and stimulate seed germination following mechanical clearing (van Wilgen et al. 1994). We cannot see any practical role of utilizing fire in an integrated management plan for Kangaroo Paws in fynbos. Even though these two species (or hybrids) currently occupy a relative small area, our studies indicate they have considerable invasive potential and should be immediately controlled.

Although the naturalized populations have not yet spread widely, plants can clearly spread and form mono-specific stands. Current infestations are within 10 km of one of South Africa's most pristine and important biodiversity-hotspot conservation areas, the Kogelberg Biosphere Reserve, and so management is regarded as a priority.

Recommendations made from results of this study have already led to the clearing of one of the smaller

outlying populations of Kangaroo Paws in the Western Cape (59 individuals 5.5 km away from the main infestation) which was flagged as the highest priority for intervention (since isolated populations of invading species are known to contribute disproportionally to population growth and invasion rates; Higgins and Richardson 1999). The results will also be used to formulate a longer-term strategy for dealing with the larger populations. The landowner has undertaken the initial "holding action" of removing, through mechanical brush cutting, the flower heads of as many mature plants as possible in the biggest populations to decrease pollinator abundance, reduce seed set, the potential for hybridisation, and the establishment of additional satellite foci.

Kangaroo Paw taxonomy

This is the first attempt to reconstruct a molecular phylogeny for *all* species of Kangaroo Paws (*Anigozanthos* and *Macropidia*). Given the taxonomic uncertainties within this group (Anderberg and Eldenäs 1991; Hopper 1978, 1980; Simpson 1990), the use of molecular systematics is particularly relevant here. While previous molecular phylogenies of the Haemodoraceae supported *Macropidia* as the monotypic sister group of *Anigozanthos* (Hopper et al. 1999, 2009), a molecular phylogeny that includes all Kangaroo Paw species would render indisputable support for the placement of *Macropidia*.

Our molecular phylogeny supports the view of Hopper et al. (1999, 2009) in suggesting that *Macropidia fuliginosa* is indeed the monotypic sister lineage of *Anigozanthos*. However, the subdivision of *Anigozanthos* into sections *Haplanthesis* (unbranched species) and *Anigozanthos* (branched species) (Hopper 1980) has little phylogenetic support and remains unresolved. Indeed, Hopper (1980) suggested that the division based on branched and unbranched stems is "to some extent artificial". For example, here, the branched species *A. preissii* and *A. onycis* showed a closer phylogenetic relationship to the unbranched species *A. gabrielae*, than to other branched species (also see Hopper et al. 2009). Interestingly, Hopper (1980) reported that only slight crossing barriers exist between *A. onycis* and members of section Haplanthesis. The reconstruction of a molecular phylogeny using more variable gene regions is currently underway and should render better resolution of these relationships (Rhian Smith, personal communication 2009).

Implications for legislation

The South African nursery industry currently trades in various horticultural hybrids of *Anigozanthos* species. We included the 'Bush Pearl' and 'Bush Gold' varieties in our molecular analysis (*A. humilis* × *A. bicolor* × *A. flavidus* and *A. flavidus* × *A. humilis*, respectively). Inter-specific hybrids of *A. flavidus* are often produced in the horticultural industry for their increased vigour, longevity and floriferous properties (Hopper 1980). Hybridisation often results in sterility as afforded by chromosomal rearrangements and/or factors under direct genetic control (Rieseberg 2001). Previous work illustrated that in Kangaroo Paws, seed set, germination success and pollen fertility of back-crossed F_1 hybrids approximate or equal those of inter-specific hybrids (Hopper 1980). Our results indicate that 'genome compatibility' (genome size similarity) may be one of the underlying mechanisms that is correlated with this phenomenon. Several Kangaroo Paw species hybridise in disturbed habitats in their native range (Hopper 1977a, b) with some hybrid combinations being fertile (Hopper 1980). Even though horticulturists claim that commercial hybrids are 'mostly' sterile (Angus Stewart, personal communication 2009) our results illustrate that these hybrids could have invasive potential. Consequently we propose that the trade in all species of Kangaroo Paws and their hybrids in South Africa should be restricted until detailed studies can show complete and stable sterility in horticulturally important cultivars.

Conclusions

Naturalized populations of *Anigozanthos flavidus* and *A. rufus* in South Africa represent a threat to the biodiversity of the Cape Floristic Region, both by creating dense monocultures and potentially by altering pollination networks. The population appears to be spreading, and there is a high probability that the two species will hybridise if they have not already done so. This will have unknown and potentially undesirable consequences. One population has

already been cleared, and the plans for treating the others areas as well as follow-up work are on-going.

Our study also highlights the importance of phylogenetic assessments in addressing plant invasions. By clarifying taxonomic issues of the Kangaroo Paw group, we have identified areas of potential concern. Parental species with similar-sized genomes (up to 30% difference) should not be grown together, and an assessment of fertility and risk of invasiveness needs to be done for different cultivars (hybrids). While some cultivars of Kangaroo Paw might be deemed 'safe', we would caution against allowing any taxa in this genus to be grown or sold until a thorough assessment, using both molecular and ecological data, has been conducted. These studies should investigate pollen fertility, outcrossing success and seed set ability of individual cultivars.

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