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MULTIPLE REPRODUCTIVE BARRIERS MAINTAIN SPECIES BOUNDARIES IN STONE PLANTS OF THE GENUS *ARGYRODERMA*

Authors: Florian C. Boucher[1,2], G. Anthony Verboom[3], Laure Gallien[1,4] & Allan G. Ellis[2]

Authors affiliations:

1: Univ. Grenoble Alpes, Univ. Savoie Mont Blanc, CNRS, LECA, 38000 Grenoble, France

2: Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

3: Department of Biological Sciences, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

4: Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa

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Short running title: REPRODUCTIVE BARRIERS IN *ARGYRODERMA*

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ABSTRACT

Measuring the strength of different reproductive barriers across species pairs is key to reveal the mechanisms that have led to evolutionary radiations. Here we study one of these miniature plant genera, *Argyroderma*, which comprises 11 species restricted to a single plain of the Southern African desert. We measure different reproductive barriers in order to understand how species boundaries are maintained in this genus. Our results show that reproductive isolation is almost complete between all species pairs and relies on three powerful barriers: geographic isolation operating at spatial scales of c. 10 km, phenological isolation in flowering time, and habitat isolation operating at spatial scales of just a few metres, which is thought to be due to contrasting edaphic preferences between species. In comparison, post-mating isolation arising before seed formation is weak and does not restrict gene flow much between species. Interestingly, the high levels of both geographic and habitat isolation that we have measured between *Argyroderma* species might be due to their miniature size, which leads to restricted gene flow across space and to strong adaptation to micro-habitats.

Keywords: diversification – edaphic specialization – flowering phenology – Knersvlakte – local adaptation – plant size - pollination - reproductive isolation – speciation – Succulent Karoo

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INTRODUCTION

Biodiversity hotspots, areas which are remarkable for their extraordinarily high biodiversity per unit area, are a focus of conservation effort and of diversification research (Myers *et al.*, 2000; Mazel *et al.*, 2014). Some of these hotspots are the result of exceptionally rapid evolutionary radiations, as is the case for plants of the South American Paramo (Madriñán, Cortés, & Richardson, 2013) or the Mediterranean basin (Valente, Savolainen, & Vargas, 2010). In other cases, however, these evolutionary radiations have not broken speed records but rather unfolded over long periods of time (Linder, 2008), resulting in a high accumulation of species like in the Cape Floristic Region (Verboom *et al.*, 2014) and the seasonally dry neotropical forests (Pennington *et al.*, 2010).

Repeated speciation events, which ultimately lead to large evolutionary radiations and contribute to high species richness in a given ecosystem, require that reproductive barriers prevent gene flow between species (Mayr 1940, Coyne & Orr 1989). While reproductive barriers are increasingly being measured in plants, enabling preliminary generalizations, this evidence is still unevenly distributed among taxa and ecosystems (Lowry et al. 2008, Baack et al. 2015, Christie et al. 2022). First, the number of reproductive barriers at play seems to vary

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between study systems. Empirical studies have documented both cases in which gene flow is restricted by a few strong barriers or by various barriers of moderate strength (reviewed in Christie et al. 2022). Barriers are often distinguished based on when they act in an organism's life cycle and classified into pre-mating vs post-mating or pre-zygotic vs post-zygotic, depending on when exactly the temporal distinction is made. Theory predicts that barriers acting earliest in the life cycle will have the largest effect in reducing gene flow and are thus expected to evolve faster than barriers acting later (Coyne & Orr, 1989; Sobel *et al.*, 2010; Nosil, 2012). Empirical studies in plant support this hypothesis in finding pre-mating barriers to be generally stronger than post-mating ones (Lowry et al. 2008, Christie et al. 2022).

Interestingly, pre-mating barriers are often affected by extrinsic factors (Keller et al. 2016). For example, the spatial configuration of a given environment will affect geographic isolation, environmental gradients can lead to habitat isolation, and pollinator behavior will influence floral isolation in plants. These characteristics of the environment have the potential to lead to speciation across many clades, which could explain the formation of biodiversity hotspots. On the other hand, such extrinsic reproductive barriers might be more prone to change through time, potentially leading to speciation meltdown, and strong post-mating barriers could thus be necessary for completion of speciation (Coyne & Orr 2004, Seehausen et al. 2014).

Here we focus on an intriguing ecosystem, the winter rainfall desert of Southern Africa, also known as the Succulent Karoo (SK, Desmet & Cowling, 1999). Located within the Greater Cape Floristic Region, the Succulent Karoo hosts approximately 5,000 plant species in an area barely larger than 100,000 km^2 (Hilton-Taylor, 1996). This makes it the most species-rich arid ecosystem in the world relative to its area (Cowling *et al.*, 1998) and qualifies it as one of the world's top biodiversity hotspots (Myers *et al.*, 2000). The existence of such richness in an ecosystem with so little primary productivity goes against traditional expectations (Gillman *et al.*, 2015), and alternative explanations are required for how species originated and how species richness is maintained in the SK.

The study group that we use to tackle this question is one of the most impressive plant radiations of the Succulent Karoo: stone plants of the genus *Argyroderma*, which comprises 11 described species that are distributed in an area less than 100 km in extent. All these plants are miniature succulents up to a few centimeters tall growing among quartz pebbles of the Knersvlakte plain in South Africa. In addition to this extremely reduced spatial scale, the speed of this radiation is also noteworthy since it belongs to one of the plant clades with the highest diversification rate known to date, the core Ruschioideae (>1 species/lineage/million year, Klak, Reeves, &

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Hedderson, 2004; Valente et al., 2014). Interestingly, *Argyroderma* species are frequently found in sympatry. In many locations two or three species can be found growing together, although it is also frequent to observe complete turnover between these species over a few dozen metres (Eibes et al., 2021, F.C. Boucher, unpublished data). It is suspected that this drastic turnover reflects micro-edaphic variation along shallow elevation gradients: leached hilltops often have acidic soils, while flatter valley bottoms even a few metres below sometimes have strongly basic and saline soils due to the rapid evaporation of soil water in this desert environment (Schmiedel & Jürgens, 1999; Eibes *et al.*, 2021).

In order to better understand how an evolutionary radiation could take place over such small spatial and temporal scales, we set out to compare the strength of several reproductive barriers between multiple pairs of species in *Argyroderma*. These barriers are, in order of action during sexual reproduction: geographic isolation, phenological isolation (*i.e.*, separation of flowering times), habitat isolation (which influences pollen transfer but also hybrid survival, see Discussion), and finally post-mating isolation (measured as seed production in heterospecific hand crosses). We set out to test two alternative hypotheses. The first hypothesis reflects theory and previous empirical results in plants, and postulates that earlier-acting barriers are stronger than later ones. The second hypothesis is based on the specific biology of dwarf plants, which

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are expected to have both shorter scales of gene flow and to be able to adapt better to fine-scale environmental gradients (Aarssen et al. 2006, Boucher et al. 2017). Under this second hypothesis we expect that geographic and habitat isolation will be the strongest reproductive barriers between *Argyroderma* species. While this second hypothesis is not radically opposed to the first one in that geographic and habitat isolation are both pre-mating barriers (although habitat isolation also has some post-mating components, see Discussion), they still differ in their prediction of the relative importance of phenological vs habitat isolation. Finally, using a new phylogenetic hypothesis for the genus we then measured how these different reproductive barriers have evolved through time.

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MATERIAL AND METHODS

Study group and area

Our model system, the genus *Argyroderma* N.E.Br. (Aizoaceae), contains dwarf succulents up to a few centimeters in height that vary extensively in their growth form (degree of branching) and leaf morphology (photographs of each taxon are shown in Fig. S1) but share a uniform bowl-shaped floral morphology (Fig. 1) and thus appear adapted to the same pollinators, mainly solitary bees, small beetles, and thrips (Struck, 1995, A. G. Ellis pers. obs.). While only 11 species are currently recognized in *Argyroderma* (Hartmann, 1973; Van Jaarsveld, 1997), previous phylogenetic investigations along with the observation of differences in morphology and phenology suggest that the genus potentially contains as many as 13 species (Ellis, Weis, & Gaut, 2006). We do not attempt to resolve this taxonomic issue here, but we treat the genus as comprising 13 different putative taxa. We first distinguished two subspecies, *A. framesii* subsp. *framesii* and *A. f.* subsp. *hallii*, and distinguished between the two ecotypes of *A. delaetii*: an early flowering form with yellow/white/pale pink flowers and a late flowering form with magenta flowers (Ellis *et al.*, 2006). For simplicity, we hereafter refer to these 13 taxa as species.

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All *Argyroderma* species grow in a single plain of the Succulent Karoo, the Knersvlakte, which is about 100 x 80 km in extent and consists of a small number of separated drainage basins. Erosion of quartz-intruded shales, which, together with other less abundant rocks, form the bedrock of the area (de Beer *et al.*, 2002), has resulted in the formation of a unique landscape characterized by the presence of many gravel patches formed of quartz pebbles typically >1 cm in diameter. This quartz habitat, to which all but one *Argyroderma* species are restricted (*A. fissum* is also found outside of quartz patches), is extremely hostile as it reflects the intense UV radiation of the region, although nocturnal condensation of moisture on the quartz pebbles may "add to" the meagre rainfall input (typically between 100 mm and 175 mm per year, Schmiedel & Jürgens, 1999). It is also highly heterogeneous, particularly in terms of the chemistry of the soil found under quartz pebbles, which can vary strongly in pH, salinity, and ionic composition over spatial scales of a few metres (Schmiedel & Jürgens, 1999; Ellis & Weis 2006, Eibes *et al.*, 2021; Musker *et al.*, 2021).

Phylogenetic inference

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In order to clarify relationships and to infer relative divergence times between species pairs, we inferred a phylogeny for the genus *Argyroderma* using the AFLP dataset of Ellis et al. (2006). This dataset consists of 253 biallelic AFLP loci scored for 183 individuals belonging to 12 *Argyroderma* taxa, *i.e.,* all described taxa except the recently discovered *A. theartii* Van Jaarsv.. Details of the genotyping procedure can be found in the original publication describing this dataset (Ellis *et al.*, 2006). We used this dataset to infer a species tree for *Argyroderma* under the multi-species coalescent (Rannala & Yang, 2003), using the program SNAPP, which directly estimates a species tree from unlinked biallelic markers without explicitly reconstructing gene trees (Bryant *et al.*, 2012). All 12 taxa were treated as independent lineages for species tree inference. Due to computational limitations of the SNAPP software, we reduced the dataset to one individual per population when multiple populations were sampled, except in cases where only one population was sampled. In the final dataset, each taxon was represented by two to four individuals (see Table S3).

We used a Yule prior for the species tree, with an inverse prior for the speciation rate (Bryant *et al.*, 2012). We also used inverse priors for the forward and backward mutation rates and a gamma prior for effective population sizes on each branch of the tree. SNAPP infers rooted, ultrametric trees, whose branch lengths are measured in expected numbers of substitutions.

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Knowledge of substitution rate and generation time would enable transformation of these branch lengths to units of time (Bryant *et al.*, 2012). Unfortunately, neither generation time nor substitution rates have been measured in *Argyroderma* specifically. Direct calibration is not possible either since there are no *Argyroderma* fossils known and secondary calibration would be highly unreliable since larger dated phylogenies of Aizoaceae have included only one *Argyroderma* species (Valente *et al.*, 2014). Given these obstacles and since we are mainly interested in relative divergence times between species pairs, we kept the initial species tree with branch lengths measured in arbitrary time units. We then use this calibrated phylogeny to test whether each individual reproductive barrier ($RI_{geographic}$, $RI_{phenology}$, $RI_{habitat,}$ and RI_{seed}, see below) correlated with the relative divergence time of species pairs for which this barrier had been measured.

We ran two MCMC chains of 200,000 steps each, discarding the first 10% steps of each chain as burnin. We then verified that the model's prior, likelihood, posterior, and parameter estimates had reached effective sample sizes higher than 100 and that both MCMC chains had converged to the same solution. The two chains were combined and a maximum clade credibility tree built from the combined chains.

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Measures of reproductive isolation

We measured four different reproductive barriers. The first one, geographic isolation, was measured across the whole study area and between all species. The three other barriers (phenological isolation, habitat isolation and seed formation) aim at quantifying local processes that isolate species in sympatry and have thus only been measured between some pairs of species that are (partly) sympatric.

In order to be able to compare the strength of these barriers, we used the unified framework of Sobel & Chen (2014) with detailed equations from Keller et al. (2016). All of these measures are scaled so that complete reproductive isolation between a pair of species corresponds to a value of 1, equal levels of gene flow between and within species to a value of 0, and heterospecific gene flow only corresponds to a value of -1. All of these measures also have a directionality, meaning that the barrier to gene flow from species A into species B might differ from the barrier to gene flow from species B into species A.

Geographic isolation

Reproductive isolation (hereafter, RI) between *Argyroderma* species is often imposed by

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geographic isolation, which for miniature succulents in the Knersvlakte usually results from occupancy of different drainage basins at spatial scales of a few kilometers (Desmet *et al.*, 2002; Ellis *et al.*, 2006). Geographic overlap between the ranges of *Argyroderma* species was calculated from detailed field records of their distribution obtained by F.C. Boucher and A.G. Ellis. Following previous studies (Anacker & Strauss, 2014; Boucher, Zimmermann, & Conti, 2016), we estimated the geographical range of each species by placing a 0.01 ˚ (c. 1 km in the Knersvlakte) round buffer around each occurrence point and merging all overlapping circles obtained. Since *Argyroderma* grows almost exclusively on quartz patches, we produced a map classifying the Knersvlakte into dense quartz vs other land cover types using supervised image classification based on a MODIS image (see Supplementary Materials S2). We then cropped the geographic range of each species so as to retain only parts that fell onto dense quartz patches in the final range map. Geographic reproductive isolation between pairs of species was then measured as:

$$RI_{geographic}=1-area_{overlap}/area_{species}$$

In this equation $area_{species}$ is the area of the geographic range of the focal species and $area_{overlap}$ is the area over which its range overlaps the range of the other species in the species pair.

Phenological isolation

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When different *Argyroderma* species co-occur at the same site, gene flow reduction might be achieved through differences in flowering phenology (Ellis *et al.*, 2006). In order to measure this barrier to gene flow we used data from detailed surveys that took place in eight different sites of the Knersvlakte in which two or three species coexisted locally. In total, nine species-pairs were measured. Numbers of flowering individuals were recorded every week over the whole flowering season during the winters of 2000 and 2001, except in three cases where measures were only recorded in 2000. In three out of the nine species-pair comparisons, flowering time was recorded at two sites. In total, almost 3,000 flowers were counted. From these measures, we calculated phenological isolation as:

$$RI_{phenology} = 1 - 2 \sum_{weeks,i} \frac{A_i}{A_{total}} \cdot \frac{B_i}{A_i + B_i} \qquad RI_{phenology} = 1 - 2 \sum_{weeks,i} \frac{A_i}{A_{total}} \cdot \frac{B_i}{A_i + B_i}$$

In this equation, which quantifies the phenological isolation of species A from species B, for each week i of the flowering season, A_i/A_{total} is the proportion of flowers of species A that are open, and $B_i/(A_i+B_i)$ is the proportion of open flowers that belong to species B (Sobel & Chen, 2014; Keller *et al.*, 2016). Where phenological measurements for the same species pairs were repeated over multiple years or at multiple sites, measures of RI_{pheno} were averaged.

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Habitat isolation

While it is common to observe two or more *Argyroderma* species growing at the same site, they are often segregated at very small spatial scales of a few metres only, which might correspond to differences in the underlying soil chemistry. In order to measure how this micro-edaphic specificity might lead to habitat isolation between *Argyroderma* species, we surveyed transects across the Knersvlakte in which at least two different species occurred. These transects typically ran from a hilltop to its bottom, spanning a few metres in elevation only. They were between 48 and 76 m long and were sampled every 2 m. At each sampling point we took a soil core from which pH and electric resistance were measured by the Elsenburg facility of the Western Cape Department of Agriculture, South Africa. These two variables were chosen because they are relatively easy to measure and correspond to the two main axes of edaphic variability in the area (Schmiedel & Jürgens, 1999; Musker *et al.*, 2021). We also counted the number of individuals of each *Argyroderma* species, including putative morphological hybrids (*i.e.* individuals that looked morphologically intermediate between two *Argyroderma* species occurring locally), in a square of 1 m x 1 m centered on each of these sampling points. These transects were surveyed during the winter season, between June and August 2017.

To estimate habitat isolation of a species pair, we first estimated species individual habitat

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suitability, and then quantified the overlap in habitat suitability between the two species. For each species, the relationship between its abundance along transects and the soil variables was estimated by fitting a generalized linear model using a Poisson distribution for abundance. Based on preliminary examination of how *Argyroderma* species' abundance varied with soil variables we included both linear and quadratic terms for pH and log-transformed electric resistance in all models. Models were thus of the form: $Species\ abundance \sim pH + pH^2 + log(resistance)$. When data from multiple transects for the same species pair were combined, the transect location was treated as a random variable in the model. All species habitat suitability models were projected and compared on a unique environmental space made of a regular grid of 50 x 50 points spreading evenly from the minimum to the maximum of both edaphic variables observed across transects. Since *Argyroderma* species of different size differ in the maximum abundance they can attain within a square meter, we normalized the abundances predicted by the model for each species so that they summed to one, yielding relative abundances. Finally, we calculated the overlap between the predicted relative abundances of species pairs as the area under both predicted curves and obtained habitat isolation as:

$$RI_{habitat} = 1\text{-}overlap(species\ A, species\ B)$$

While we cannot envision which factors other than fine-scale edaphic variables could vary over

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spatial scales of a few metres as investigated here, we acknowledge that this measure quantifies the level of habitat isolation between two co-occurring species that is due to all micro-habitat factors.

Post-mating isolation

We measured post-mating isolation between different *Argyroderma* species using hand crosses. These controlled crosses were conducted *in situ* at three different sites in June and July of 2016 and 2017 and involved six different taxa: *A. congregatum, A. delaetii* 'late flowering'*, A. fissum, A. framesii* subsp. *framesii, A. framesii* subsp. *hallii* and *A. pearsonii*. Additional data from crosses between *A. fissum* and *A. pearsonii* conducted in May 2001 were also used. However, due to both logistical constraints and differences in flowering phenology between species, all reciprocal crosses between the six species were not possible. For each cross, flower buds were bagged ca. one week before flowering in order to ensure that they were free of pollen from other plants. When flowers opened they were hand pollinated using paintbrushes with a mix of pollen collected less than one hour earlier from either conspecific individuals growing at the same site (hereafter 'outcrossing' treatment) or individuals of a different species (hereafter 'interspecific crossing', either from the same or a different site). Finally, for each cross we also left some bagged flowers unpollinated in order to test the ability of *Argyroderma*

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species to autonomously self. The fruit eventually produced by each plant, a capsule, was collected when ripe in October to November of the same year. Due to the high number of tiny seeds produced by *Argyroderma* species (up to 2,600 per capsule according to our measurements) we only opened some of the locules of each capsule (usually from two to four of the 10 to 20 locules, depending on the species) and counted the number of well-formed seeds in them under a binocular lens before extrapolating this number to the total number of locules in the capsule. While we initially aimed for a minimum of 20 replicates for each pollen recipient species x treatment, sample sizes were reduced through herbivory and bag loss. In total, 324 pollen recipient plants were retrieved and their seed production measured over the three field seasons.

Intrinsic isolation between pairs of species that had been crossed was then measured as:

$$RI_{seed} = 1-2*(H/(H+C))$$

In this equation H is the mean number of seeds obtained in heterospecific crosses and C is the mean seed number in conspecific ones (Sobel & Chen, 2014; Keller *et al.*, 2016).

Overall reproductive isolation

There were two pairs of species for which we measured the intensity of all four reproductive

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barriers studied here: *A. delaetii* 'late flowering' / *A. framesii* subsp. *framesii* and *A. congregatum* / *A. framesii* subsp. *hallii*. For these two pairs we combined estimates of the different aspects of RI into a measure of the overall strength of reproductive isolation, RI_{tot}, following Sobel & Chen (2014).

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RESULTS

Phylogenetic inference

After checking for convergence of the MCMC chains, we combined them to build a maximum clade credibility species tree for *Argyroderma*. Clade support in the *Argyroderma* phylogeny was limited for most nodes but two strongly supported clades were recovered (Fig. 1, Fig. S3). The first one consisted of *A. subalbum* and *A. framesii* subsp. *hallii*, which were inferred to be sister taxa with 100% posterior support. Another large clade including all *Argyroderma* species except *A. fissum*, *A. subalbum*, and *A. framesii* subsp. *hallii* received 100% posterior support. Consistent with previous taxonomic treatments (Hartmann, 1973) and molecular evidence (Ellis *et al.*, 2006), we inferred *A. fissum* to be sister to the rest of the genus, although with low support (48% posterior support). Relative divergence times in the *Argyroderma* phylogeny showed that the two initial divergence events followed each other closely, with all further divergence events within *Argyroderma* being much younger (Fig. 1, Fig. S3).

Measures of reproductive isolation

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Geographic isolation

Thanks to detailed relevees across the Knersvlakte, geographic isolation could be measured for all species pairs in the genus *Argyroderma* and values covered the whole spectrum of reproductive isolation (Table 1). On the one extreme, $RI_{geographic} = 0$ for the small-ranged species, *A. theartii* and *A. subalbum,* that have ranges that are completely nested within the ranges of more widespread co-occuring species. On the other extreme, we found values of $RI_{geographic} = 1$ in many cases, including (among others) all comparisons involving *A. ringens*, which has a distribution that does not overlap with any other *Argyroderma* species. On average geographic isolation was high (mean $RI_{geographic} = 0.85$, sd = 0.26). A group of four species especially abundant in central Knersvlakte showed consistently low values of geographic isolation, these where: *A. delaetii* 'early flowering', *A. delaetii* 'late flowering', *A. fissum* and *A. framesii* subsp. *framesii* (Table 1).

Phenological isolation

Phenological isolation was measured in nine species pairs in *Argyroderma*. The flowering periods of *Argyroderma* populations that we surveyed lasted between four and seven weeks, depending on the species and the site considered. In five out of the nine comparisons there was

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no overlap between the flowering periods of the two species, leading to complete reproductive isolation ($RI_{phenology}$ = 1, Table 2). While this certainly testifies to strong phenological isolation, it does not imply that such a situation is repeated every year: indeed, in the winters of 2016 and 2017 during which we conducted cross-pollination experiments we did find some individuals of *A. congregatum* and *A. framesii* subsp. *hallii* flowering simultaneously at the same site, while they showed no overlap in flowering phenology at the same site in 2000 and 2001. In the four other comparisons, phenological isolation varied widely, ranging from 0.99 to -0.37. Overall, phenological isolation was high (mean = 0.84, sd = 0.35, Table 2).

Habitat isolation

Habitat isolation was measured in five species pairs in *Argyroderma*. In total we surveyed eight transects: in four of them *A. delaetii* 'late flowering' and *A. framesii* subsp. *framesii* co-occured, in three of them *A. congregatum* and *A. framesii* subsp. *hallii* co-occured, and in the last one *A. crateriforme, A. fissum*, and *A. theartii* co-occured. Across the various transects we did find some putative hybrids between *Argyroderma* species, but these were always at extremely low frequencies. Hybrids represented 0.3% of all individuals in transects with *A. delaetii* 'late flowering' and *A. framesii* subsp. *framesii* (3 hybrid individuals) and 0.2% of all individuals in transects with *A. congregatum* and *A. framesii* subsp. *hallii* (1 hybrid individual). No hybrids

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were recorded between *A. crateriforme, A. fissum*, and *A. theartii*.

In general, there was little overlap in the distribution of different *Argyroderma* species along transects at the 2 m scale studied here, likely due to large variations in soil pH and electric resistance. Indeed, in all eight transects surveyed, pH varied by at least three units (over a maximum distance of 76 m) and the most extreme variation was found at the 'Goeie Hoop' site, where pH varied from 3.7 to 8.1 over 72 m. Variations in soil electric resistance were also important. As a result, measures of $RI_{habitat}$ obtained from predicted abundances based on soil pH and electric resistance were rather high (average = 0.72, sd = 0.16, Table 2).

Post-mating isolation

Post-mating isolation at the seed formation stage was measured in six species pairs in *Argyroderma*. These hand pollination experiments confirmed that *Argyroderma* species are primarily outcrossers: selfed flowers yielded between 0 and 28% of the seed output produced by outcrossed flowers of the same species (average = 7.7%, see Fig. 2).

Interspecific crosses generally yielded lower numbers of seeds, their output being on average 48.9% of the seed produced by conspecific crosses (Fig. 2). There was high variability between different crosses with some interspecific crosses yielding almost no seeds (2.4% of the output of conspecific crosses when pollen from *A. fissum* was crossed with flowers of *A. pearsonii*),

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while in others heterospecific seed production was higher than conspecific seed set (134% of the output of conspecific crosses when pollen from *A. framesii* subsp. *hallii* was crossed with flowers of *A. congregatum*).

These measures translated into a wide range of reproductive isolation values (Table 2): ranging from -0.15 when pollinating *A. congregatum* with pollen from *A. framesii* subsp. *hallii* (*i.e.,* higher seed output in this heterospecific cross than in intraspecific crosses) to almost complete isolation when pollinating *A. pearsonii* with pollen from *A. fissum* (RI_{seed} = 0.95). Average RI_{seed} across all species pairs was 0.43 (sd = 0.34, see Table 2). Among the species-pairs for which we had enough heterospecific crosses in both directions, levels of asymmetry in RI_{seed} varied but were never pronounced (Table 2).

Overall reproductive isolation

When comparing the average strength of all four different reproductive barriers we found the following relationship: $RI_{geographic} > RI_{phenology} > RI_{habitat} > RI_{seed}$. All pairwise comparisons between barriers were significant (Wilcoxon rank sum test: all $p < 0.027$), except for the difference between $RI_{geographic}$ and $RI_{phenology}$ (Wilcoxon rank sum test: p=0.83). In the two species pairs for which all four barriers could be measured we found that the resulting total

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reproductive isolation was extremely high: it was complete (RI_{total} = 1) between *A. congregatum* and *A. framesii* subsp. *hallii* in both directions and was almost full between *A. deleatii* 'late flowering' and *A. framesii* subsp. *framesii* (RI_{total} = 0.94 in both directions). Finally, of the four barriers measured here, only $RI_{habitat}$ increased significantly with relative divergence time between the species pairs for which it was measured, as measured from our phylogeny (Mantel correlation r = 0.99, p-value = 0.018). For all three other reproductive barriers the correlation was non-significant.

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DISCUSSION

Measuring multiple reproductive barriers for several species pairs for which a phylogeny is known is key to understanding which reproductive barriers contribute most to speciation and how they evolved. Due to the extensive work required to measure reproductive barriers, such datasets are scarce and usually restricted to groups of model organisms like *Drosophila (Coyne & Orr, 1989; Turissini et al.*, 2017), *Heliconius* butterflies *(M*érot *et al.*, 2017), or plants of the genera *Streptanthus* (Christie & Strauss, 2018) and *Mimulus* (Sandstedt, Wu, & Sweigart, 2021), but similar datasets are increasingly being assembled for other non-model organisms (reviewed in Matute & Cooper, 2021). In this paper we add one example to this growing list, with measurements of four different reproductive barriers across multiple pairs of species in the genus *Argyroderma*.

Reproductive barriers in *Argyroderma*

We found that multiple reproductive barriers contribute to high overall reproductive isolation between *Argyroderma* species. These were, in decreasing order of importance: geographic isolation (average $RI_{geographic} = 0.85$), followed by phenological isolation (average $RI_{phenology} =$

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0.84) and habitat isolation (average $RI_{habitat}$ = 0.72). Finally, post-mating reduction of hybrid seed formation was the weakest reproductive barrier (average RI_{seed} = 0.43). The differences in strength between the different barriers were not significant, except for the most extreme ones. Nevertheless, their order of importance exactly matches the hypothesis that reproductive barriers acting first in an organism's life cycle are subject to stronger selection than later-acting ones, because they have a higher impact on gene flow and are likely subject to selection for reinforcement (Coyne & Orr, 2004; Hopkins, 2013; Matute & Cooper, 2021), and thus evolve faster (Coyne & Orr, 1989; Sobel *et al.*, 2010; Nosil, 2012). This is also in line with previous evidence found in plants for stronger pre-mating compared to post-mating barriers (Widmer, Lexer, & Cozzolino, 2009; Levin, 2012, Lowry et al. 2008, Christie et al. 2022).

However, the second hypothesis that we proposed (*i.e.*, geographic and habitat isolation are the strongest barriers), although it received less support, cannot be rejected either. Indeed, one barrier that has both pre- and post-mating effects is key in this system: at the scale of a few metres, different edaphic micro-habitats contribute to strong isolation between species. Habitat isolation results in the spatial aggregation of conspecific individuals at scales of a few metres only. While these distances are shorter than the typical cruising distance of *Argyroderma*'s main pollinators (solitary bees and beetles, Struck, 1995), the fact that these pollinators forage

optimally reduces pollen carryover across sequential floral visits and thus limits heterospecific relative to conspecific pollen transfer, resulting in pre-mating isolation. This effect has not yet been measured in *Argyroderma* but has been experimentally demonstrated between two species of Asteraceae from the SK (de Waal, Anderson, & Ellis, 2015). Additionally, strong habitat isolation between species pairs likely reduces the fitness of hybrid individuals across all habitat types, resulting in post-mating isolation. This is typical of scenarios of ecological speciation, in which ecologically-based divergent selection leads to speciation (Nosil, 2012). Given the microscale habitat isolation observed in *Argyroderma*, where pollen movement distances likely exceed the spatial structuring resulting from habitat filtering, habitat isolation might have a stronger influence on hybrid survival (post-mating) than on pollen movement (pre-mating), but this remains to be measured. Interestingly, habitat isolation was the only reproductive barrier to correlate with relative divergence times within *Argyroderma* (Fig. S4), which suggests it evolved in a clock-like manner along the phylogeny of the genus and possibly that it could be a rate-limiting step for speciation, *i.e.,* the aspect of reproductive isolation that is slowest to evolve among those needed for speciation to be complete (Rabosky & Matute, 2013).

The most striking result of our study is that overall reproductive isolation is high in all species pairs investigated. For the two species pairs in which all four barriers could be measured RI_{total}

was higher than 0.95, and for all 78 species pairs in *Argyroderma* where at least one barrier could be measured, average overall reproductive isolation was 0.89. As a result, the different species in *Argyroderma* are strongly isolated from one another, which explains both the scarcity of F1 hybrids encountered in the field (Hartmann, 1973, this study), and the fact that no hybrid zone is known between these species. Such high reproductive isolation is unexpected given the young age of the genus *Argyroderma*. Indeed, while it is not possible to accurately date this genus, broader phylogenetic studies suggest that *Argyroderma* must be much younger than the whole core Ruschioideae radiation to which it belongs, with an age estimate of c. 1.5 Myr (Valente *et al.*, 2014).

The measurements that we collected for this study cover multiple species pairs and several reproductive barriers, which allows for a global assessment of reproductive isolation in *Argyroderma*, but also has limitations. Firstly, while in most instances multiple population pairs and/or multiple years were surveyed when measuring habitat and phenological isolation, seed formation was only quantified between a single pair of populations and during a single flowering season for each species pair. This was due to time constraints but we bear in mind that levels of RI might change between different populations of each species pair and between years, as documented in many other cases (reviewed in Coyne & Orr, 2004; Nosil, 2012). More importantly, our measures of habitat isolation could be improved by measuring the fitness of

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different species and their hybrids in different micro-habitats. Reciprocal transplants have actually demonstrated local adaptation between *A. fissum, A deleatii* 'early' and *A. pearsonii* (Ellis & Weis, 2006), as well as between populations of *A. pearsonii* (Ellis *et al.*, 2007). Such measures, if generalized across multiple species pairs, could offer important insights into the mechanisms leading to habitat isolation between *Argyroderma* species.

Implications for diversification in *Argyroderma* and the buildup of the Succulent Karoo biodiversity hotspot

The main message to emerge from our results is that reproductive isolation between species of *Argyroderma* relies on multiple major barriers to gene flow, two of these being strictly extrinsic (geographic and habitat isolation) and the last one having both extrinsic and intrinsic components. Phenological isolation indeed has a strong intrinsic component, in that flowering time separation is retained in common garden (greenhouse) conditions (Ellis, A.G., pers. obs.), but the extent of separation in the field does to some degree depend on environmental conditions. In comparison, the only strictly intrinsic barrier that we measured (seed production) was much weaker (Table 2) and on its own does not restrict gene flow between species much (average RI_{seed} = 0.43).

Two aspects of the Knersvlakte environment underlie the two major reproductive barriers. Geographic isolation between *Argyroderma* species typically occurs between drainage basins, at already relatively small scales of c. 5-10 km (average $RI_{geographic}$ = 0.85). While the Knersvlakte has a smooth topography, gene flow between adjacent drainage basins appears to be limited for *Argyroderma* species (Ellis *et al.*, 2007), as well as other Ruschioideae (Musker *et al.*, 2021). Indeed, while pollinators might be able to cover these distances and thus transfer pollen, seed dispersal appears to be restricted within drainage basins. In *Argyroderma* as in all core Ruschioideae, seeds are contained in a capsule that only opens and ejects seeds during rain events. Seeds typically only fall a few centimeters away from their mother plant due to the low stature of the latter (<10 cm). Secondary dispersal of either individual seeds or of whole capsules fallen to the ground might occur during flash floods and cover larger distances but will typically be downstream from the mother plant and remain in the same drainage basin (Ihlenfeldt, 1994).

A large part of extrinsic RI in *Argyroderma* occurs on an even finer spatial scale of a few metres and seems to be due to adaptation to different edaphic conditions. Previous studies have already shown high edaphic variability within quartz patches of the Knersvlakte (Schmiedel & Jürgens,

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1999; Ellis & Weis, 2006; Eibes *et al.*, 2021; Musker *et al.*, 2021) and our study confirms these observations, especially for pH that frequently varies by several units over a few dozen metres. Such drastic edaphic changes are commonly echoed by sharp vegetation turnover in both *Argyroderma* and other species (Schmiedel & Jürgens, 1999; Eibes et al., 2021, F.C. Boucher, unpublished data). This edaphic variability probably leads to strong selection across small spatial scales. Short seed dispersal distances (see above) together with large population sizes (up to 66 individuals/m^2 for *A. delaetii* in our dataset) makes *Argyroderma* species perfectly suited to respond to these selective pressures. This combination of limited dispersal and large population sizes might be a general syndrome of small plants, one that enables them to show local adaptation on small spatial scales (Boucher *et al.*, 2017). In *Argyroderma*, reciprocal transplants between sites tens of metres apart have demonstrated local adaptation between *A. fissum* and *A. pearsonii* (Ellis & Weis, 2006), but also between different populations of *A. pearsonii* (Ellis *et al.*, 2007). Another study has directly tested the role of substrate in a common garden involving two species of Ruschiodeae from the Knersvlakte and found local adaptation to substrate in *Ruschia burtoniae*, but not in *Conophytum calculus (Musker et al.*, 2021). Our results, while not providing direct measures of local adaptation to the edaphic environment, suggest that such adaptation contributes high levels of reproductive isolation between five species pairs in *Argyroderma* (average $RI_{habitat}$= 0.74).

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Put together, these two observations confirm a model of diversification that had already been proposed for *Argyroderma*, with an early phase of the evolutionary radiation involving allopatric speciation between different drainage basins and a later phase involving ecological speciation in response to extremely fine-scale edaphic variability and associated phenological divergence (Ellis *et al.*, 2006). While being comparatively weaker, intrinsic reproductive incompatibilities at seed formation further act to restrict gene flow between species. This has led to a miniature evolutionary radiation taking place rapidly (likely in the last million years, see above) but most importantly over an extremely reduced area of c. 100 x 80 km.

Our measures of reproductive barriers in *Argyroderma* support the hypothesis that in small plants geographic and habitat isolation should be particularly strong reproductive barriers (Boucher *et al.*, 2017). While the genus *Argyroderma* probably ranks near the top of the charts for plants in terms of both diversification rate and diversification density (Boucher *et al.*, 2020), many other plant clades are exceptionally species rich within the Succulent Karoo. This is for example the case for other miniature plants in the genera *Conophytum, Crassula*, *Gibbaeum*, *Haworthia, Lapeirousia* or *Tylecodon,* which have diversified extensively in the Succulent Karoo, and on quartz fields in particular. All of these groups contain micro-endemics as well

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as edaphic specialists and might show diversification histories similar to the one we propose for *Argyroderma*. This raises the possibility that the extremely high diversity of the Succulent Karoo flora could be largely due to the fact that it contains a large proportion of dwarf species (Cowling *et al.*, 1998), small plant stature being selected for in desert environments (Ihlenfeldt, 1994), and to the exceptionally fine-scale edaphic variation found across the SK (Ihlenfeldt, 1994; Cowling *et al.*, 1998; Mucina *et al.*, 2006; Ellis *et al.*, 2014). Small plant stature would have resulted in a combination of restricted gene flow and adaptive divergence to fine-scale edaphic variation, two factors leading to lineage divergence (Boucher *et al.*, 2017). Importantly, unlike other aspects of the environment, like climate, that vary over relatively short timescales (e.g., ca. 10,000 yr), edaphic differences remain constant over periods long enough for divergence to accumulate and ultimately lead to speciation. The miniature size of both plants and edaphic micro-habitats in the Succulent Karoo might thus explain why so many species are packed in this region, making this arid ecosystem one of the world top biodiversity hotspots (Cowling *et al.*, 1998; Myers *et al.*, 2000).

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TABLES AND FIGURE LEGENDS

	CON	CRA	DEE	DEL	FIS	FRF	FRH	PAT	PEA	RIN	SUB	TES	THE
CON	-	0.93	0.94	1	0.72	1	0.64	0.88	1	1	0.97	1	1
CRA	0.89	-	0.95	0.58	0.44	1	1	0.67	0.75	1	1	0.94	0.78
DEE	0.98	0.99	-	0.57	0.09	0.72	1	0.76	0.69	1	1	0.82	1
DEL	1	0.88	0.43	-	0.20	0.55	1	0.63	0.78	1	1	0.89	1
FIS	0.94	0.91	0.44	0.63	-	0.79	0.91	0.80	0.69	1	0.99	0.92	0.96
FRF	1	1	0.20	0.03	0.04	-	1	0.58	1	1	1	1	1
FRH	0.56	1	1	1	0.43	1	-	0.99	1	1	1	1	1
PAT	0.93	0.86	0.52	0.44	0.35	0.71	0.99	-	0.86	1	1	0.99	1
PEA	1	0.91	0.48	0.71	0.13	1	1	0.88	-	1	1	1	1
RIN	1	1	1	1	1	1	1	1	1	-	1	1	1
SUB	0	1	1	1	0	1	1	1	1	1	-	1	1
TES	1	0.94	0.13	0.58	0.33	1	1	0.99	1	1	1	-	1
THE	1	0	1	1	0	1	1	1	1	1	1	1	-

Table 1. Geographic isolation between *Argyroderma* species. Values for all possible species pairs have been reported, with the potential gene flow recipients as rows and potential gene flow donors as columns. Different levels of shading show increasing isolation (white: 0-0.5 ; light gray: 0.5-0.8 ; gray: 0.8-0.95, dark gray: 0.95-1). Species names have been abbreviated as follows: CON – *A. congregatum*; CRA – *A. crateriforme*; *THE* – *A. theartii*; FIS – *A. fissum*; DEL – *A. delaetii* 'late flowering'; FRF – *A. framesii* subsp. *framesii*; PAT – *A. patens*; PEA – *A. pearsonii*; DEE – *A. delaetii* 'early flowering'; RIN – *A. ringens*; SUB – *A. subalbum*; TES – *A. testiculare*; FRH – *A. framesii* subsp. *hallii.*

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	$RI_{geographic}$	$RI_{phenology}$	$RI_{habitat}$	RI_{seed}
A. f. subsp. framesii → A. delaetii 'late'	0.55	0.25	0.48	0.63
A. delaetii 'late' → A. f. subsp. framesii	0.03	0.82	0.48	0.34
A. f. subsp. framesii → A. fissum	0.79	-	-	0.76
A. fissum → A. f. subsp. framesii	0.04	-	-	0.43
A. fissum → A. delaetii 'late'	0.20	-	-	0.28
A. delaetii 'late' → A. fissum	0.63	-	-	0.68
A. f. subsp. framesii → A. patens	0.71	1	-	-
A. patens → A. f. subsp. framesii	0.58	1	-	-
A. fissum → A. pearsonii	0.13	0.84	-	0.95
A. pearsonii → A. fissum	0.69	0.82	-	0.80
A. pearsonii → A. delaetii 'late'	0.78	0.89	-	0.01
A. delaetii 'late' → A. pearsonii	0.71	-0.37	-	0.28
A. fissum → A. theartii	0.00	-	0.93	-
A. theartii→ A. fissum	0.96	-	0.93	-
A. fissum → A. crateriforme	0.44	-	0.68	-

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A. crateriforme → *A. fissum*	0.91	-	0.68	-
A. crateriforme → *A. theartii*	0.00	-	0.81	-
A. theartii → *A. crateriforme*	0.78	-	0.81	-
A. f. subsp. hallii → *A. congregatum*	0.64	1	0.70	-0.14
A. congregatum → *A. f. subsp. hallii*	0.56	1	0.70	0.07
A. crateriforme → *A. delaetii 'late'*	0.93	1	-	-
A. delaetii 'late' → *A. crateriforme*	0.89	1	-	-
A. patens → *A. delaetii 'late'*	0.63	1	-	-
A. delaetii 'late' → *A. patens*	0.44	1	-	-
A. delaetii 'early' → *A. pearsonii*	0.48	0.99	-	-
A. pearsonii → *A. delaetii 'early'*	0.69	0.96	-	-
A. fissum → *A. delaetii 'early'*	0.09	1	-	-
A. delaetii 'early' → *A. fissum*	0.44	1	-	-
Distribution across all species (mean±sd)	0.85±0.27	0.84±0.35	0.72±0.16	0.43±0.34

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Table 2. Measures

Table 2. Measures of reproductive isolation for 12 species pairs in which at least two

reproductive barriers were measured. Each row represents a possible cross, with the arrow pointing towards the species receiving gene flow. Pairs of species are always presented with the two possible cross directions (species A → species B and species B → species A) as two consecutive rows and with the same shading. Note that since all species pairs shown in this table have overlapping ranges, they have low values of $RI_{geographic}$ compared to all possible species pairs in *Argyroderma* (Table 1). The bottom line gives the mean and standard deviation of each barrier across all species pairs for which it was measured.

Fig. 1. The genus *Argyroderma.* Pictures show three representative species. (A) *A. delaetii* 'early flowering', (B) *A. testiculare* and (C) *A. framesii* subsp. *framesii*. Pictures by F. C. Boucher. (D) Phylogeny of all *Argyroderma* species except for *A. theartii,* for which no molecular data are available. This phylogeny is the maximum clade credibility species tree obtained using SNAPP from 253 biallelic AFLP loci. Posterior support is shown at each node. For the two subspecies of *A. framesii*, the species name as been abbreviated as *A.f.*.

Fig. 2. Seed production in hand pollination experiments. Each boxplot shows the number of seeds produced by a given cross. Different panels show different pollen recipient species, with

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boxplots for different pollen donors. Lightgray shows interspecific crosses, white shows outcrossing, and darkgray shows selfing.

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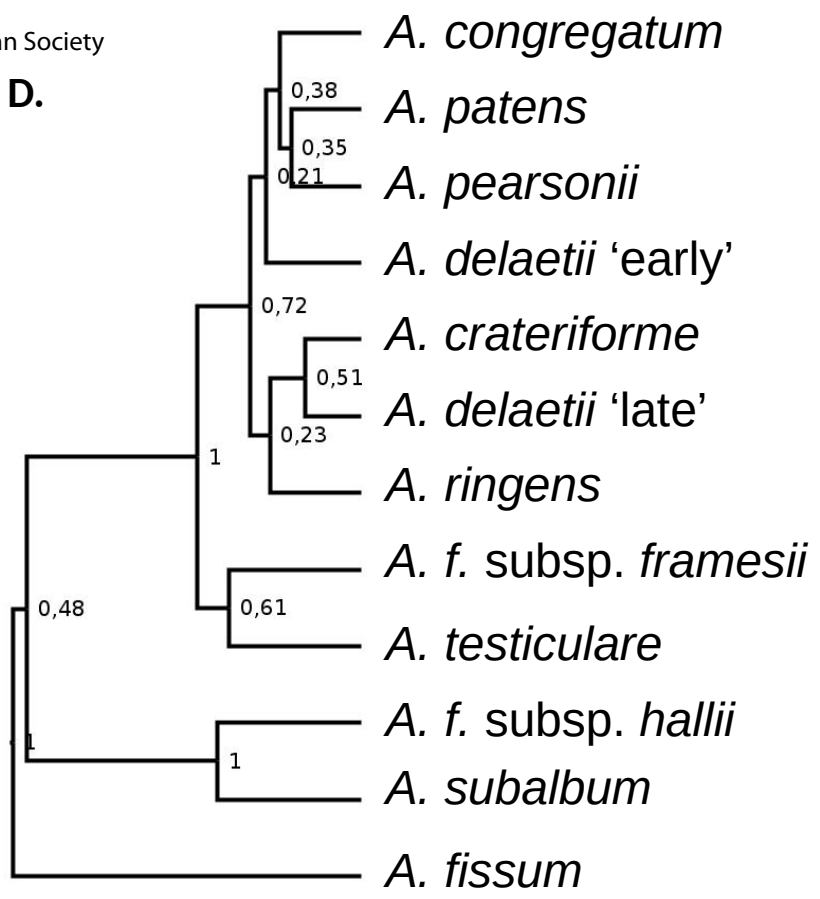
A.


B.


C.


D.
A. congregatum
A. patens
A. pearsonii
A. delaetii ‘early’
A. crateriforme
A. delaetii ‘late’
A. ringens
A. f. subsp. framesii
A. testiculare
A. f. subsp. hallii
A. subalbum
A. fissum
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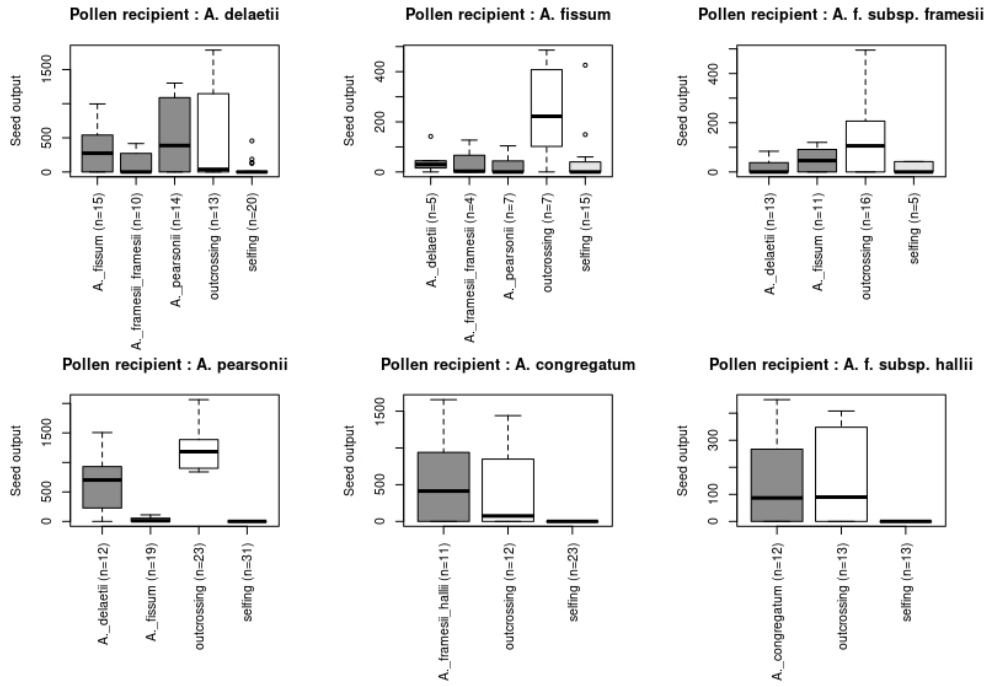

Fig. 2. Seed production in hand pollination experiments. Each boxplot shows the number of seeds produced by a given cross. Different panels show different pollen recipient species, with boxplots for different pollen donors. Lightgray shows interspecific crosses, white shows outcrossing, and darkgray shows selfing.

215x150mm (96 x 96 DPI)

SUPPLEMENTARY INFORMATION FOR

MULTIPLE REPRODUCTIVE BARRIERS MAINTAIN SPECIES BOUNDARIES IN STONE PLANTS OF THE GENUS *ARGYRODERMA*

by Florian C. Boucher, G. Anthony Verboom, Laure Gallien & Allan G. Ellis

S1. Morphological variability among *Argyroderma* species

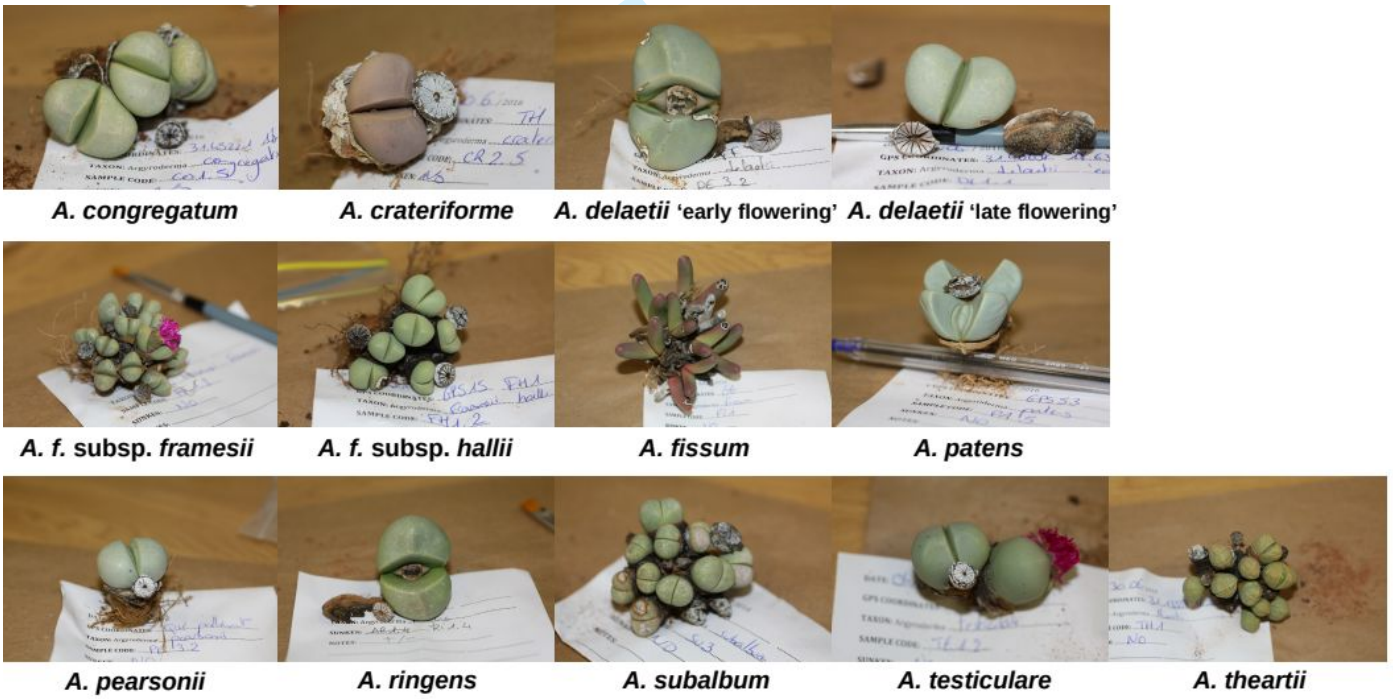

Fig. S1. Representative photographs of each *Argyroderma* taxon studied here. Paper labels on

each photograph are of the same size and can serve as an indication of scale. All photographs but one show the fruits of *Argyroderma* (capsules), the typical flower shape can be seen on the photographs in Fig. 1. The species name *Argyroderma framesii* has been abbreviated as '*A. f.*'. All photographs were taken by F. C. Boucher.

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S2. Distribution of quartz patches and *Argyroderma* species across the Knersvlakte

Since species of the genus *Argyroderma* always grow on quartz patches it was necessary to first produce a good map of quartz patches across the Knersvlakte, which we later used to constrain the ranges of the different *Argyroderma* species. This was done using supervised image classification in the R package Rstoolbox (réf?). Our classification was based on a MODIS image covering the whole study area taken on July 10th, 2016. We chose this date because the picture taken on this day was free of clouds and because this represents the middle of the wet season in the Knersvlakte, ensuring maximum contrast between quartz patches and surrounding loamy soils which harbor green shrubby vegetation in this season. We used bands 2 to 7 of the MODIS image, plus three other bands derived from the original images that enable good discrimination of geological backgrounds: the 'clay minerals', the 'ferrous minerals' and the 'iron oxyde' ratios (Drury, S. *Image Interpretation in Geology*. London: Allen and Unwin (1987), 243 pp.). The combined image was then segmented into two classes: dense quartz vs all other land cover types, the later including sparse quartz patches, bare sandy or loamy soils, bare slate, shrubby vegetation, and crops. We used a total of 130 points with known land cover (F.C. Boucher, pers. obs.), half of which were randomly sampled to train the classification algorithm while the remaining half were used for cross validation.

The accuracy of our classification of the Knersvlakte into dense quartz patches vs other land cover types was satisfying (cross-validation accuracy = 0.873). The resulting map shows most of the quartz patches being distributed alongside the course of the two main rivers of the

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Knersvlakte: the Sout and Geelbeks rivers (Fig. S2).

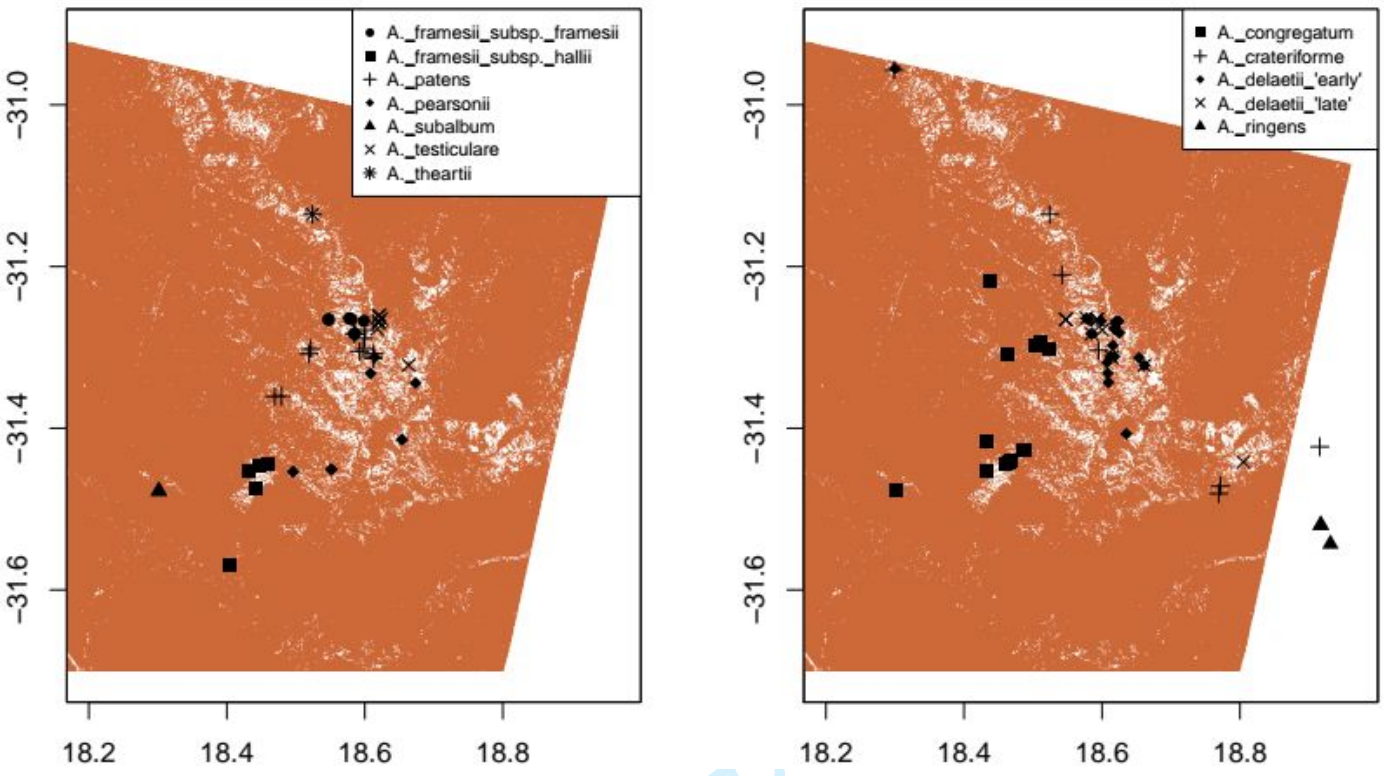

Figure S2. Distribution of quartz patches and of *Argyroderma* species. Both maps show the Knersvlakte region of South Africa, with quartz patches in white and other land cover classes in brown ; latitude and longitude are given in decimal degrees on the side of each map. Known populations of each taxon in the genus *Argyroderma* are shown with different symbols. Left map: species of the 'framesii group' as defined by Ellis et al. 2006. Right map: species of the 'delaetii group' as defined by the same authors. Note that we did not infer these two groups to be distinct clades (Fig. 2). *A. fissum*, which is widespread across the Knersvlakte, has been omitted for clarity.

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S3. Phylogenetic inference in *Argyroderma*

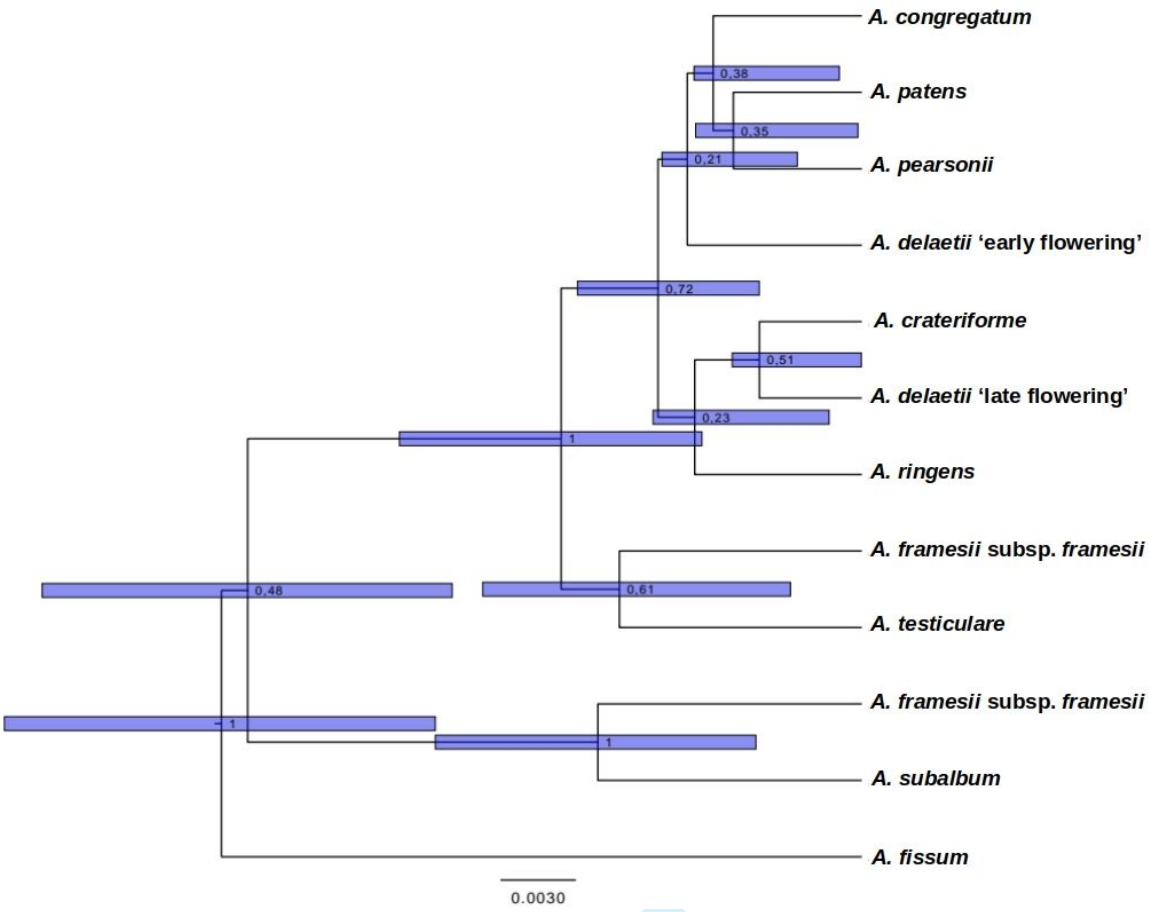

Fig. S3. Phylogeny of *Argyroderma*. This figure shows the maximum clade credibility species tree for 12 taxa in the genus *Argyroderma*, inferred from 253 biallelic AFLP loci and obtained by combining to runs of the SNAPP program of 200,000 steps each. Number at nodes show the posterior support of each corresponding clade and blue bars show the 95% high probability interval for the age of each node, in relative time units.

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Taxon	Population	Sample code
Argyroderma congregatum	DTG	A.co_DTG_88
	KBG	A.co_KBG_b042
	Koek	A.co_Koek_b056
	GM	A.co_G.M_b032b
Argyroderma crateriforme	DTG	A.cr_DTG_44
	Grd	A.cr_Grd_b006
	Ariz	A.cr_Ariz_b004
Argyroderma delaetii 'early flowering '	QK	A.de_QK_068b
	QK	A.de_QK_67
	QK	A.de_QK_69
Argyroderma delaetii 'late flowering'	Ariz	A.dl_Ariz_b010
	Gtrn	A.dl_Gtrn_71
Argyroderma fissum	Moed	A.f_Moed_97
	FV	A.f_FV_118
Argyroderma framesii subsp. *framesii*	FV1	A.ff_FV1_76
	Gtrn	A.ff_Gtrn_b022
Argyroderma framesii subsp. *hallii*	Moed	A.fh_Moed_82
	Koek	A.fh_Koek_b026
	Grd	A.fh_Grd_75
Argyroderma patens	FV2	A.pa_FV2_b016
	FV1	A.pa_FV1_112
Argyroderma pearsonii	S4	A.pe_S4_43
	S2	A.pe_S2_a03
	S1	A.pe_S1_14
	S3	A.pe_S3_19
Argyroderma ringens	VBG1	A.ri_VBG1_b030
	VBG2	A.ri_VBG2_59
	VDP	A.ri_VDP_85
Argyroderma subalbum	Koek	A.su_Koek_b043
	Koek	A.su_Koek_95
	Koek	A.su_Koek_94
Argyroderma testiculare	Ariz3	A.te_Ariz3_b027
	Ariz4	A.te_Ariz4_52

Table S3. Samples used for species-tree inference using SNAPP. For each taxon, between two and four different individuals were selcted to infer the species tree. When possible, these individuals were chosen in different populations. See Ellis et al. 2006 Evolution for details on the accessions corresponding to each individual, identified by a unique 'sample code'.

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S4. Evolution of reproductive barriers through time in *Argyroderma*

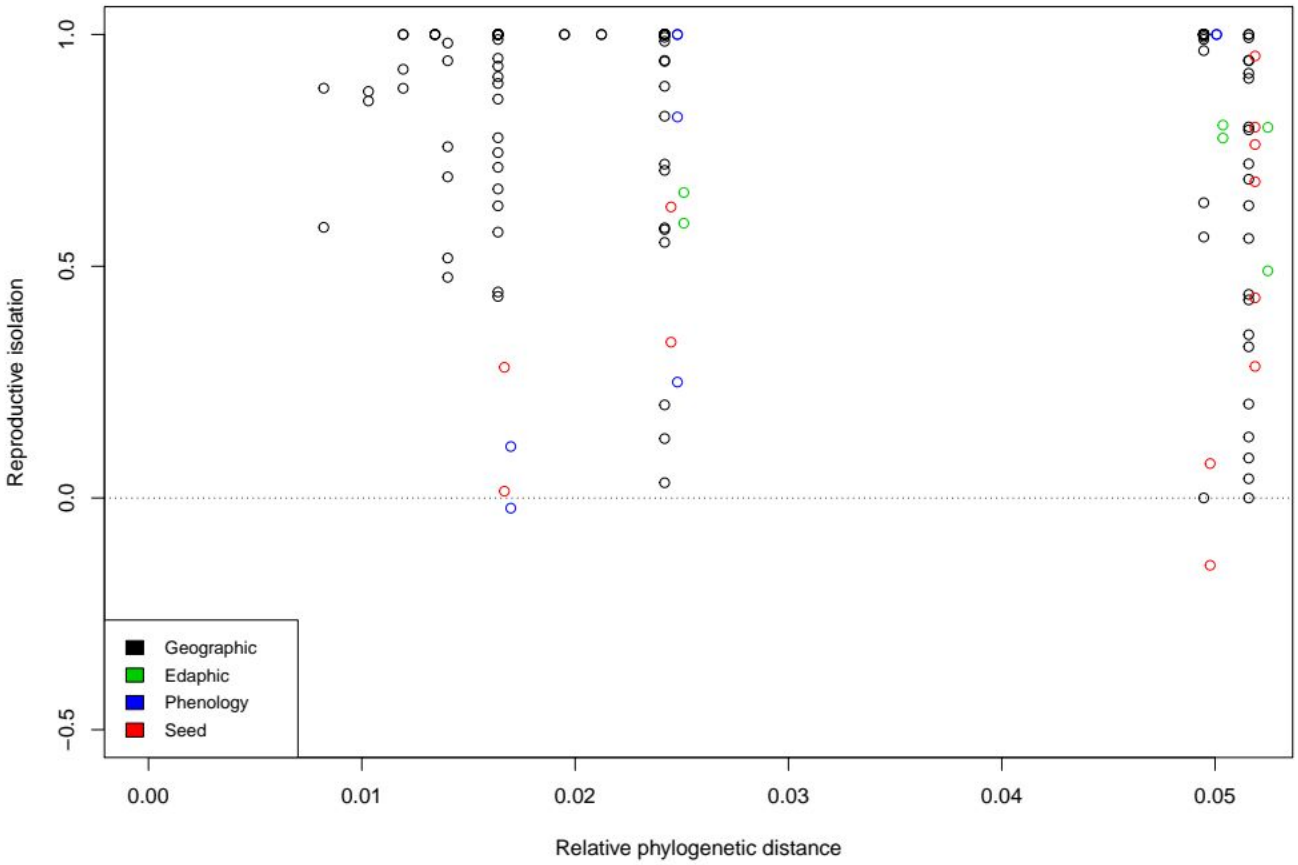

Fig. S4. Strength of different reproductive barriers as a function of relative phylogenetic distance for multiple species pairs in *Argyroderma.* 'Geographic' stands for $RI_{geographic}$. 'Edaphic' stands for $Ri_{habitat}$. 'Phenology' stands for $RI_{phenology}$. 'Seed' stands for Ri_{seed}. Of all four barriers, only $RI_{habitat}$ correlated significantly with divergence time (Mantel correlation r = 0.99, p-value = 0.018).