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A tale of North and South America: time and mode of dispersal of the amphitropical genus *Munroa* (Poaceae, Chloridoideae)

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Plant disjunctions have provided some of the most intriguing distribution patterns historically addressed by biogeographers. We evaluated the three hypotheses that have been postulated to explain these patterns [vicariance, stepping-stone dispersal and long-distance dispersal (LDD)] using *Munroa*, an American genus of grasses with six species and a disjunct distribution between the desert regions of North and South America. The ages of clades, cytology, ancestral characters and areas of distribution were investigated in order to establish relationships among species, to determine the time of divergence of the genus and its main lineages, and to understand further the biogeographical and evolutionary history of this genus. Bayesian inference recovered the North American *M. pulchella* as sister species to the rest. Molecular dating and ancestral area analyses suggest that *Munroa* originated in North America in the late Miocene–Pliocene (7.2 Mya; 8.2–6.5 Mya). Based on these results, we postulate that two dispersal events modelled the current distribution patterns of *Munroa*: the first from North to South America (7.2 Mya; 8.2–6.5 Mya) and the second (1.8 Mya; 2–0.8 Mya) from South to North America. Arid conditions of the late Miocene–Pliocene in the Neogene and Quaternary climatic oscillations in North America and South America were probably advantageous for the establishment of populations of *Munroa*. We did not find any relationship between ploidy and dispersal events, and our ancestral character analyses suggest that shifts associated with dispersal and seedling establishment, such as habit, reproductive system, disarticulation of rachilla, and shape and texture of the glume, have been important in these species reaching new areas. © 2015 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2015, 00, 000–000.

Additional Keywords: American arid lands – amphitropical disjunction – historical biogeography – long-distance dispersal (LDD) – molecular phylogeny – stepping-stone dispersal – vicariance.

INTRODUCTION

For a long time, biogeographers have focused on understanding the causes that underlie the distribu-

tion patterns of disjunct taxa (Christenhusz & Chase, 2013). A remarkable number of examples with disjunct distributions are found in species or genera of plants from North America that are disconnected from their counterparts in South America (Humboldt, 1817; Johnston, 1940; Raven, 1963; Cruden, 1966; Barbour, 1969; Solbrig, 1972; Williams, 1975; Allred,

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1981; Carlquist, 1983; Simpson & Neff, 1985; Peterson & Columbus, 1997; Wen & Ickert-Bond, 2009; Donoghue, 2011; Moore *et al.*, 2012; Masson & Kadereit, 2013; González *et al.*, 2014; Linder & Barker, 2014). Several hypotheses have been postulated to explain the origin of these disjunctions, in particular: (i) the vicariance hypothesis, in which closely related taxa diverged from widely distributed tropical ancestors (Johnston, 1940; Barbour, 1969; Moore, Tye & Jansen, 2006; Escudero *et al.*, 2010); (ii) the stepping-stone dispersal hypothesis, in which diaspore(s) are dispersed over short distances through tropical zones using arid or semi-arid islands (i.e. high mountain corridors) or are dispersed directly via an old arid or semi-arid corridor close to the Pacific coast (Morrell, Porter & Friar, 2000; Winkworth *et al.*, 2002; Moore *et al.*, 2006; Gillespie *et al.*, 2012); and (iii) the long-distance dispersal (LDD) hypothesis, in which diaspore(s) are dispersed across the tropics, founding populations in distant areas (Raven, 1963; Cain, Milligan & Strand, 2000; Winkworth *et al.*, 2002; Blattner, 2006; Wen & Ickert-Bond, 2009; Donoghue, 2011; Gillespie *et al.*, 2012). The latter has been suggested as an explanation for the disjunct distribution of New World desert plants (Raven, 1963; Winkworth *et al.*, 2002; Wen & Ickert-Bond, 2009; Donoghue, 2011; Gillespie *et al.*, 2012; Linder & Barker, 2014).

Perhaps the best documented disjunctions of plants from North and South American deserts are those of *Larrea* Cav. (Lia *et al.*, 2001), *Tiquilia* Pers. (Moore & Jansen, 2006; Moore *et al.*, 2006), *Nitrophila* S. Watson (Masson & Kadereit, 2013) and *Erioneuron* Nash (Peterson & Ortíz-Díaz, 1998). For these taxa, South America is thought to be the centre of origin, with one or more subsequent northward dispersal events. In contrast, the distribution patterns of several grasses, such as *Lycurus setosus* (Nutt.) C.Reeder (Peterson & Morrone, 1997), *Muhlenbergia torreyi* Hitchc. ex Bush (Peterson & Ortíz-Díaz, 1998; Peterson, Columbus & Pennington, 2007) and *Scleropogon brevifolius* Phil. (Peterson & Columbus, 1997), probably originated instead in North America, and moved southwards (Peterson & Columbus, 1997; Peterson & Ortíz-Díaz, 1998; Peterson *et al.*, 2007). However, many biogeographers who have favoured dispersal are reluctant to accept that plants could, in a single event, travel between landmasses in different hemispheres (Winkworth *et al.*, 2002; Gillespie *et al.*, 2012). Instead, intermediate regions have often been suggested to be stepping stones in such movements, and the existence of a fossil record in these intermediate areas makes this hypothesis an enticing possibility (Winkworth *et al.*, 2002).

In turn, evidence of multiple and non-contemporaneous dispersal events in an amphitropi-

cally disjunct group would lend support to LDD as the most plausible mechanism explaining such disjunctions (Raven, 1963; Moore *et al.*, 2006; Wen & Ickert-Bond, 2009). However, although a single rare LDD event may indeed be impossible to predict, an understanding of the mechanisms involved in LDD over extended (evolutionary) time periods can lend predictability to the process. For example, understanding the different modes of LDD (dispersal by wind, birds and ocean currents) and associated dispersal adaptations in the context of geological, palaeontological, evolutionary and ecological data can lead to predictions concerning the origin, frequency of arrival and location of establishment of dispersed organisms (Cain *et al.*, 2000; Winkworth *et al.*, 2002; Gillespie *et al.*, 2012).

Without knowledge of continental drift, most early biogeographers hypothesized that dispersal was the historical mechanism behind such disjunctions (de Queiroz, 2005). However, the acceptance of plate tectonics in the 1960s and 1970s caused a shift towards the idea that most plant disjunctions resulted from the fragmentation of earlier, larger landmasses, such as Gondwana; the Gondwanan breakup seems to have occurred between 160 and 30 Mya (Nelson & Platnick, 1981; Wiley, 1988; de Queiroz, 2005; Upchurch, 2008). These vicariance explanations remained dominant until the advent of molecular systematic techniques, particularly molecular-based dating of lineage divergences (de Queiroz, 2005). Using these techniques, most research has shown that numerous plant disjunctions are far too young to have resulted from vicariance, again leaving dispersal as the only plausible alternative (Sanmartín & Ronquist, 2004; de Queiroz, 2005; Renner, 2005; Wen & Ickert-Bond, 2009; Donoghue, 2011; Popp, Mirré & Brochmann, 2011; Moore *et al.*, 2012; Christenhusz & Chase, 2013; Masson & Kadereit, 2013; González *et al.*, 2014; Linder & Barker, 2014).

The disjunct taxa distribution patterns have been explained by stepping-stone dispersal, LDD or vicariance, and the relative importance of these explanations in shaping current distributions may vary, depending on historical backgrounds or the biological characteristics of particular taxa (Raven, 1963; Winkworth *et al.*, 2002; Wen & Ickert-Bond, 2009; Donoghue, 2011; Gillespie *et al.*, 2012; González *et al.*, 2014; Linder & Barker, 2014). There has been substantial research focusing on extrinsic environmental factors influencing dispersal; in particular, it has been suggested that habitat similarity (Cain *et al.*, 2000; Crisp *et al.*, 2009; Gillespie *et al.*, 2012; Linder & Barker, 2014), dominant wind directions (Wright *et al.*, 2000; Winkworth *et al.*, 2002; Muñoz *et al.*, 2004; Blattner, 2006; Sanmartín, Wanntorp & Winkworth, 2007; Gillespie *et al.*, 2012) or bird migration routes

(Winkworth *et al.*, 2002; Coleman *et al.*, 2003; Blattner, 2006; Gillespie *et al.*, 2012) might determine dispersal routes and events. Although intrinsic attributes, such as seed morphological structures, were explored in the last century (Ridley, 1930), in the past decade these factors have not received much attention (Cain *et al.*, 2000). Indeed, Higgins, Nathan & Cain (2003), in a review of whether morphology relates to dispersal, concluded that the relationship between morphologically defined dispersal syndromes and dispersal is poor. With regard to ploidy, an association between polyploidy and dispersal events has been reported for diverse plant groups (Coleman *et al.*, 2003; Kadereit, Mucina & Freitag, 2006; Linder & Barker, 2014), and it is now known that polyploids are more successful at dispersal than diploids, and that the frequent polyploidy in the grasses might have facilitated the extensive dispersal among continents, thus contributing to their evolutionary success (Linder & Barker, 2014).

It is clear that several hypotheses may explain the disjunct distribution patterns of New World plants (stepping-stone dispersal, LDD or vicariance) and that estimates of the time of divergence of independent lineages need to be considered when testing specific hypotheses regarding the role of orogenic and/or climatic events in the separation or extinction of populations of plants in certain areas (Winkworth *et al.*, 2002; Wen & Ickert-Bond, 2009; Escudero *et al.*, 2010; Jakob *et al.*, 2010; Donoghue, 2011; Popp *et al.*, 2011; Gillespie *et al.*, 2012; González *et al.*, 2014). Moreover, information on plant dispersal mechanisms, floral biology and ploidy is useful for understanding dispersal events. Indeed, it is only through the integration of data from multiple sources that plant disjunctions will be properly understood (Raven, 1963; Cain *et al.*, 2000; Blattner, 2006; Donoghue, 2011; Linder & Barker, 2014).

In this study, we use the grass genus *Munroa* Torr. (Chloridoideae, Scleropogoninae) to test further the hypotheses associated with plant disjunct distribution patterns. *Munroa* has an amphitropical distribution, with two species [*M. squarrosa* (Nutt.) Torr. and *M. pulchella* (Kunth) Amarilla] occurring in the deserts from southern Canada to northern Mexico and four species (*M. andina* Phil., *M. decumbens* Phil., *M. mendocina* Phil., *M. argentina* Griseb.) occurring in the South American deserts, extending from Peru, Bolivia and Chile to north-western Argentina (Anton & Hunziker, 1978; Amarilla *et al.*, 2013). *Munroa* spp. grow in arid and semi-arid regions in open areas of plains and mountains from mid (1000 m) to high (> 4000 m) elevations. Plants grow on alluvial and sandy-stony soils, often in dry creeks and river-beds (Amarilla *et al.*, 2013). Furthermore, *Munroa* spp. have C₄ metabolism and display variable morphology,

especially in attributes associated with dispersal (elongated stolons), reproductive system (andromonoecy, gynomonoecy or monoecy), life cycles (annual or perennial), articulation of the rachilla (disarticulating or non-disarticulating) and glume texture (membranous or coriaceous) (Anton & Hunziker, 1978; Amarilla *et al.*, 2013). With regard to cytology, early counts by Covas (1949) for *M. mendocina* and Reeder (1977) for *M. squarrosa* and *M. pulchella* [= *Dasyochloa pulchella* (Kunth) Willd. ex Rydb.] indicated a chromosome base number of $x = 8$ ($2n = 16$). However, ploidy in most *Munroa* spp. is unknown.

Based on phylogenetic and biogeographical analyses, we test whether the current disjunct distribution of *Munroa* is explained by any of the above-mentioned hypotheses. In a spatio-temporal framework, we examine whether shifts in attributes, such as ploidy (diploidy to polyploidy; Linder & Barker, 2014), habit (perennial to annual; Jakob *et al.*, 2010), reproductive system (monoecy to gynomonoecy; Mamut *et al.*, 2014) or a number of vegetative and floral characters that favour dispersal or establishment in new habitats, occurred at the nodes at which dispersal events happened.

MATERIAL AND METHODS

TAXON SAMPLING

This study included 167 accessions (species) representing all *Munroa* spp., 157 species of Chloridoideae and four outgroups selected after Peterson, Romaschenko & Johnson (2010): *Chasmanthium latifolium* (Michx.) H.O.Yates, *Aristida scribneriana* Hitchc., *Rytidosperma pictum* (Nees & Meyen) Nicora and *Danthonia compressa* Austin. The broad sampling of taxa was necessary to properly place the calibration points for molecular dating. GenBank accession numbers for the sequences generated for the present study (30) and for the previously generated sequences extracted from Peterson *et al.* (2010) are listed in Supporting Information (Table S1). We report voucher information for the six new collections of *Munroa* (Table S1).

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Genomic DNA was extracted from silica-dried or herbarium material using cetyltrimethylammonium bromide (CTAB) (Doyle & Doyle, 1987). DNA extractions were used to amplify four plastid DNA regions (*ndhF*, *rps16-trnK*, *rpl32-trnL* and *ndhA*) and one nuclear gene (ITS). Amplification reactions were performed in 25- μ L reaction solutions with volume 1.13 ReddyMix™ PCR Master Mix (2.5 mM MgCl₂) (ABgeneH, Thermo Fisher Scientific Inc., UK) following the manufacturer's instructions and using the

following primers: *ndhF*2091R and *ndhF*1311F for *ndhF* (Romaschenko *et al.*, 2010), *rpS16*-900F and 3914PR for *rps16-trnK* (Peterson *et al.*, 2010), *ndhAx4* and *ndhAx3* for the *ndhA* intron (Peterson *et al.*, 2010), *trnL* (UAG) and *rpl32-F* for *rpl32-trnL* (Shaw *et al.*, 2007) and ITS5a and ITS4 for ITS (White *et al.*, 1990; Stanford, Harden & Parks, 2000). Amplification products were visualized under UV light after electrophoretic separation on a 1% agarose TBE gel stained with SYBR Safe gel stain (Invitrogen, Carlsbad, California, USA). Amplified products were sent to Macrogen Inc. (Seoul, South Korea) for purification and sequencing with the BigDye™ terminator kit and run on an ABI 3730XL.

PHYLOGENETIC ANALYSES

Sequences were assembled and edited using Sequencher v4.1 (Gene Codes Corporation, Ann Arbor, MI, USA), pre-aligned in Muscle (Edgar, 2004), implemented in MEGA 5 (Tamura *et al.*, 2011) and manually adjusted. Nucleotide substitution models were determined with jModelTest 0.1.1 (Posada, 2008) using the Akaike information criterion and default search values for each marker. The best-fit models of evolution obtained are indicated in Table 1. To address the level of congruence among data partitions and their influence on combined datasets of plastid and nuclear ribosomal DNA (nrDNA), the incongruence length difference (ILD) test (Farris *et al.*, 1994) and parametric bootstrapping (Huelsenbeck, Hillis & Jones, 1995; Swofford *et al.*, 1996; Goldman, Anderson & Rodrigo, 2000) were performed. The ILD test with 1000 permutations and a significance level of $P < 0.01$ was conducted in TNT (*ILD.run* script). Parametric bootstrapping was carried out separately for the plastid and nrDNA datasets using GARLI (Zwickl, 2006). The maximum likelihood (ML) bootstrap (BS) analysis was performed with 1000 replicates, with ten random addition sequences per replicate. A majority rule consensus for each replicate was obtained by PAUP* 4.0b10 (Swofford, 2000). ML analyses yielded trees with possible incongruence between plastid DNA and nrDNA (see Results). To test this incongruence statistically, a pair of reciprocal BS analyses was conducted in which the nrDNA tree was constrained to have the plastid DNA topology with respect to this incongruence, and vice versa. ML searches with monophyly constraints consistent with the alternative hypothesis (plastid DNA vs. nrDNA) were carried out with GARLI. The ML BS was performed with identical parameters as mentioned previously. A majority rule consensus for each replicate was obtained by PAUP*. The constraint topology and model parameters were used to simulate 100 data matrices equal in size to the original matrix using Mesquite v. 2.75 (Maddison &

Table 1. Summary of four plastid regions and nuclear ribosomal DNA (nrDNA) (ITS) used in this study

	<i>ndhA</i> intron	<i>ndhF</i>	<i>rpl32-trnL</i>	<i>rps16-trnK</i>	Plastid	ITS	Combined plastid + ITS
Number of taxa	159	160	159	165	167	167	167
Average sequence length	950	725	675	730	3100	650	3850
Average aligned sequence length	1161	744	1178	894	3977	817	4794
Tree length obtained by parsimony analyses	1500	1741	1103	1200	7011	5083	18587
Consistency index	71	67	70	72	67	70	65
Retention index	80	75	81	82	85	80	80
Preferred model of evolution, Akaike information criterion (AIC)	GTR + G	GTR + G + I	T92 + G	T92 + G	GTR	K2 + G + I	GTR + G + I

Maddison, 2011). These parameters were then used in PAUP* to find the most parsimonious trees constructed under topological constraints and the most parsimonious unconstrained trees. Differences in tree length for constrained and unconstrained searches for each of the 100 simulated matrices were calculated and plotted as histograms using Mesquite v. 2.75. The distribution of tree length differences between two potential topologies was estimated. If the difference between constrained and unconstrained topologies fell outside the 95% confidence interval of this distribution ($P < 0.05$), the alternative hypothesis was rejected.

Based on these incongruence tests, we were able to decide that nuclear and plastid matrices can be combined (see Results). Parsimony analyses were performed in TNT ver. 1.1 (Goloboff, Farris & Nixon, 2008). DNA regions were concatenated and characters were treated as unordered, independent and equally weighted. Gaps were treated as missing data. A full parsimony search was performed using the following specifications: 5000 replicates with random sequence addition, tree bisection–reconnection (TBR) branch swapping, keeping all multiple shortest trees found during branch swapping (MulTrees = on) and other parameters using the default options. Clade support was estimated using non-parametric bootstrapping with 1000 replicates, 10 random addition sequence replicates with a maximum of 10 trees saved per replicate, and all other settings used in the full search.

Bayesian inference analyses were employed to infer the phylogeny using the plastid DNA and nrDNA datasets separately and subsequently using a combined dataset that included all sequences. Bayesian inference was performed using the NSF teragrid applications of MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) employing the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2010). There were two separate chains for each run, with three hot and one cold chain, running for 10 000 000 generations, sampling a tree every 1000 generations. Stationarity was determined on the basis of the convergence of likelihood scores, and sample points generated prior to stationarity were eliminated as burn-in (25%). The posterior probabilities (PPs) of the clades were determined by a 50% majority rule consensus of the trees retained. To decide data partitioning strategies, three analyses (ITS + combined plastid DNA, ITS + partitioned plastid DNA and total combined evidence) were performed using Bayes factors. Bayes factors were calculated from the estimated results of the harmonic mean likelihood of each partitioning data matrix, using the 'sump' command in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001). Decisions to partition data matrices were based on the $2\ln$ Bayes factor (B_{10}) criterion, in which a value of ≤ 0 is negative and ≥ 10 is positive (Kass & Raftery, 1995).

DIVERGENCE TIME ESTIMATION

Divergence times for *Munroa* and the lineages therein were estimated using BEAST v. 1.6.1 (Drummond & Rambaut, 2007), and the same data matrix was used for the Bayesian inference of phylogeny. For this analysis, Chloridoideae and *Distichlis* Raf. were constrained as monophyletic, following Bouchenak-Khelladi *et al.* (2009, 2010). The Chloridoideae node was constrained using a log-normal prior distribution [offset, 30; log (mean), 0; log (SD), 1; range, 35.18–30.19], following earlier estimates of Bouchenak-Khelladi *et al.* (2009, 2010). The *Distichlis* node was calibrated using fossil evidence of epidermal cells with round silica bodies and triangular stomata subsidiary cells (Dugas & Retallack, 1993). This node was constrained using a minimum age of 14 Mya and a log-normal prior distribution [offset, 14; log (mean), 0; log (SD), 1; range, 19.18–14.19] (Bouchenak-Khelladi *et al.*, 2010). Analyses were run using a molecular clock model with uncorrelated rates, assuming a log-normal distribution of rates (UCLD). Models of sequence evolution for each partition were the same as for those used in the MrBayes analyses. The Yule process was selected as the tree prior and a single overall UCLD model was applied for all partitions. Four Markov chain Monte Carlo (MCMC) analyses were run, each with 100 million generations and sampling every 10 000th generation. Time series plots of all parameters were analysed in Tracer v. 1.5 (Rambaut & Drummond, 2009) to check for adequate effective sample sizes ($ESS > 200$) and convergence of the model likelihood and parameters between each run. Trees were combined in Log Combiner v. 1.6.1 (Drummond & Rambaut, 2007), setting the burn-in to 25% of the initial samples of each MCMC run. After burn-in, samples were summarized using the maximum clade credibility tree (MCC) option in Tree Annotator v. 1.6.1 (Drummond & Rambaut, 2007). The single tree was visualized with Figtree v. 1.5.4 (Drummond & Rambaut, 2007).

CYTOLOGICAL STUDIES

Mitotic chromosomes were examined in squashes of root tips that were obtained from germinating seeds. Root tips were fixed in a 3 : 1 ethanol–acetic acid mixture, after pretreatment in a solution of 2 mM 8-hydroxyquinoline for 24 h, and were stained with alcoholic hydrochloric acid–carmin (Snow, 1963). At least ten metaphases per species were photographed with a phase contrast optic Axiophot microscope. The photomicrographs were used to take measurements for each chromosome pair: short arm (s), long arm (l) and mean total chromosome length (c). The arm ratio ($r = l/s$) was then calculated and used to classify the chromosomes according to Levan, Fredga & Sandberg

(1964). In addition, the total haploid chromosome length of the karyotype (tl), average chromosome length (C) and average arm ratio (R) were calculated. Idiograms were based on the mean values for each species. The chromosomes were first arranged into groups according to their increasing arm ratio and then according to the decreasing length within each group. Karyotype asymmetry was estimated using the intrachromosomal (A_1) and interchromosomal (A_2) indices of Romero Zarco (1986).

ANCESTRAL AREA RECONSTRUCTIONS

Two analyses for inferring the ancestral areas and ancestral ranges were performed: Bayesian binary MCMC (BBM) and statistical dispersal-vicariance analysis (S-DIVA) methods, implemented in RASP software (Nylander *et al.*, 2008; Yu, Harris & He, 2010; 2011). These analyses were developed for the Scleropogoninae clade; for this, some sequences were pruned from the combined (*ndhA* intron, *ndhF*, *rpl32-trnL*, *rps16-trnK*, ITS) data matrix and 12 terminal taxa [*Scleropogon brevifolius* Phil., *Swallenia alexandrae* (Swallen) Soderstr. & H.P.Decker, *Blepharidachne kingii* Hack., *B. benthamiana* Hitchc., *Erioneuron avenaceum* (Kunth) Tateoka, *E. nealleyi* (Vasey) Tateoka (only two *Erioneuron* spp. were included because we lacked adequate material from other species and varieties; however, we included representatives occurring in North and South America, thus covering the complete disjunct distribution area of the genus) and all *Munroa* spp.] were included. Two areas were defined for the biogeographical analysis based on the extant distribution patterns of *Munroa* and the geological history of the Americas (Taylor, 1991; Posadas, Estévez & Morrone, 1997; Gregory-Wodzicki, 2000; Morrone, 2006; Antonelli *et al.*, 2009), as follows: (1) temperate North America; (2) South American Transition Zone (SATZ). The input file for RASP consisted of the 7500 post-burn-in trees from the Bayesian inference analyses run in MrBayes (GTR model). Tree files were combined in RASP to estimate PPs for each node. MCMC chains were run simultaneously for 5 000 000 generations and the reconstructed state was sampled every 1000 generations. The fixed model JC + G (Jukes-Cantor + Gamma) was used for BBM analysis with a null root distribution.

ANCESTRAL CHARACTER STATE RECONSTRUCTIONS

Ancestral character state reconstruction with the ML criterion (Mk1 model) was conducted in Mesquite v. 2.75 (Maddison & Maddison, 2011) to the Scleropogoninae clade; for this, some sequences were pruned from the combined (*ndhA* intron, *ndhF*, *rpl32-trnL*, *rps16-trnK*, ITS) data matrix and 12 terminal taxa (*Scleropo-*

gon brevifolius, *Swallenia alexandrae*, *Blepharidachne kingii*, *B. benthamiana*, *Erioneuron avenaceum*, *E. nealleyi* and all *Munroa* spp.) were included. The input file for Mesquite consisted of the 7500 post-burn-in trees from the Bayesian inference analyses run in MrBayes (GTR model). The 50% majority rule was calculated in Mesquite. Morphological characters and character states were coded from observations of herbarium vouchers (Supporting Information, Table S1) and complemented by information from the literature (Anton & Hunziker, 1978; Valdés-Reyna & Hatch, 1995; 1997; Valdés-Reyna, 2003; Amarilla *et al.*, 2013). Five morphological features were coded as discrete characters: life form (perennial 0, annual 1); rachilla (non-disarticulating 0, disarticulating 1); reproductive mode (andromonoecy 0, gynomonocoe 1, monoecy 2); glumes (absent 0, present 1); glume texture (membranous 0, coriaceous 1) (Supporting Information, Table S2).

RESULTS

RECONSTRUCTION OF PHYLOGENETIC RELATIONSHIPS

Phylogenetic hypotheses were largely congruent between partitions according to the ILD test ($P = 0.44$). Our results mostly agree with previous phylogenetic relationships for Chloridoideae reported by Peterson *et al.* (2010). A single incongruence based on plastid DNA vs nrDNA data matrices involved the phylogenetic position of *Sohnsia filifolia* (E.Fourn.) Airy Shaw; using the plastid DNA data matrix, *S. filifolia* was retrieved as sister to the Muhlenbergiinae clade (BS = 65%), whereas nrDNA retrieved *S. filifolia* as sister to Muhlenbergiinae + Scleropogoninae (BS = 50%). Parametric bootstrapping analyses did not, however, reject the alternative, constrained topologies for the plastid DNA and nrDNA trees ($P > 0.05$ in both), indicating a statistically insignificant incongruence between the two genomes in the placement of these taxa. The 2ln Bayes factor (B_{10}) results indicated positive values for all strategies: ITS + combined plastid DNA ($B_{10} = 2.5$), ITS + partitioned plastid DNA ($B_{10} = 3.0$) and total combined evidence ($B_{10} = 2.8$). Based on these tests and to compare our results with those of Peterson *et al.* (2010), we conducted parsimony analysis with total combined evidence and Bayesian analysis using the ITS + partitioned plastid DNA data matrix ($B_{10} = 3.0$). The alignment of the 167 accession dataset consisted of 4794 aligned positions (Table 1). The strict consensus tree resulting from parsimony analyses shows the same topology as the 50% majority rule consensus tree resulting from Bayesian analyses, and all nodes were strongly to moderately supported (> 75% BS, Fig. 1).

Our Bayesian results (Fig. 1) corroborate previously reported phylogenetic relationships for Chloridoideae

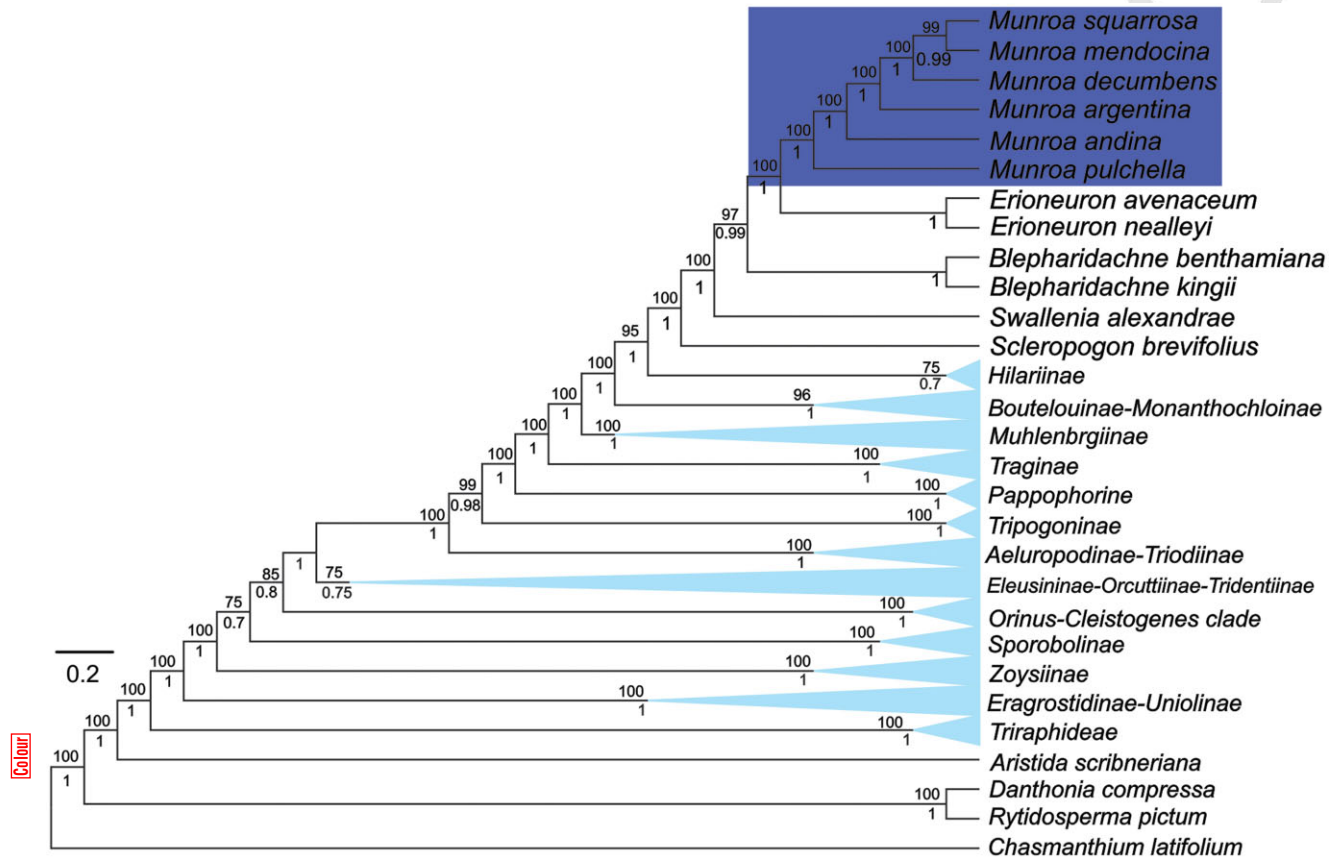


Figure 1. Fifty per cent majority rule Bayesian consensus of the molecular phylogenetic analyses of Chloridoideae with emphasis on *Munroa*. Posterior probabilities are indicated below the branches. Bootstrap supports are indicated above the branches. Condensed branches represent all subtribes included in this study.

(Peterson *et al.*, 2010). The Scleropogoninae clade (BS = 100, PP = 1.0) is monophyletic and includes *Munroa*, *Erioneuron*, *Swallenia alexandrae*, *Blepharidachne* and *Scleropogon brevifolius*. *Munroa* was recovered as monophyletic with six species (BS = 100, PP = 1.00). *Munroa pulchella* is sister to the rest of *Munroa*, with *M. squarrosa* and *M. mendocina* appearing as the last diverging clade. Further details on the degree of support and relationships recovered are shown in Figure 1.

DIVERGENCE TIME ESTIMATES

The maximum clade credibility chronogram for Chloridoideae is included in Figure 2. Our results show that the diversification of Scleropogoninae began in the early Miocene, at *c.* 17.5 Mya (19.5–15.5 Mya). *Munroa* diverged from the *Erioneuron* clade in the mid-Miocene, at *c.* 11 Mya (13–9.5 Mya), and the diversification of the crown node of *Munroa* began in the late Miocene, at *c.* 7.2 Mya (8.2–6.5 Mya). The divergence between *M. squarrosa* and *M. mendocina* began in the mid-Pleistocene, at *c.* 1.8 Mya (2–0.8 Mya).

CYTOLOGICAL STUDIES

All *Munroa* spp. were diploid with $2n = 16$ ($x = 8$). Karyotypes were uniform, exclusively composed of small-sized metacentric chromosomes with no visible secondary constrictions. In general, considering centromere position and chromosome size variation, karyotypes were symmetrical. The chromosome morphological data are shown in Supporting Information, Table S3.

ANCESTRAL AREA RECONSTRUCTIONS

The reconstructions of BBM are presented in Figure 3. The most recent common ancestor (MRCA) of *Munroa* (node N1) probably occurred in North America (Temperate North America area; $P = 88\%$). The ancestral distribution of nodes N1–N4 was reconstructed as ‘South America’ (SATZ, $P = 82\%$), whereas the MRCA of *M. mendocina* and *M. squarrosa* (N5) may have had a distribution in South America ($P = 91\%$). Two dispersal events between North and South America (i, between nodes N1 and N2; ii, following the split

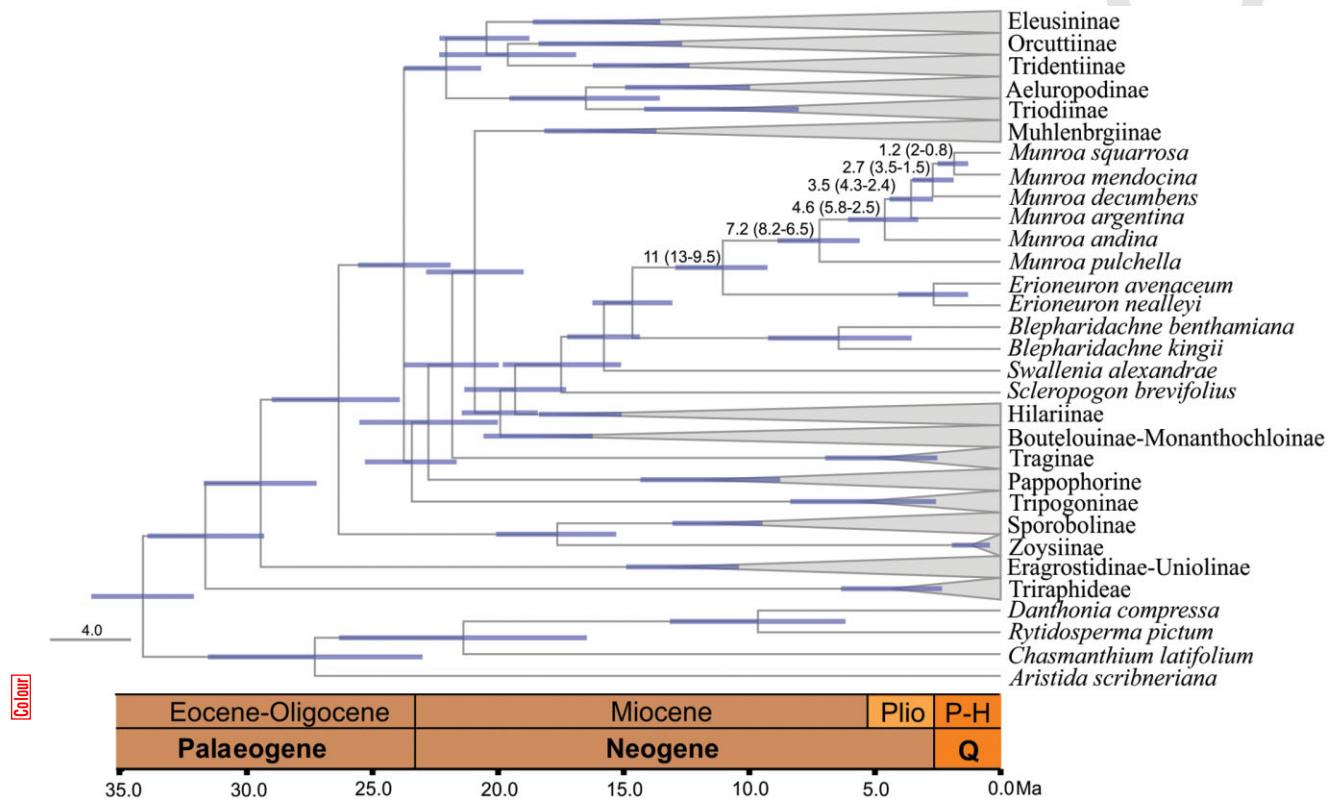


Figure 2. BEAST chronogram of the combined dataset of 163 representatives of Chloridoideae. Estimated ages are presented in millions of years (Myr) and 95% confidence intervals are given at the nodes. Condensed branches represent all subtribes included in this study.

between *M. mendocina* and *M. squarrosa*, along the branch leading to the terminal *M. squarrosa*) were reconstructed in all analyses (BBM and S-DIVA). In addition, vicariance was inferred at nodes N1 and N5. S-DIVA optimizations (not shown) were similar to those presented in Figure 3.

ANCESTRAL CHARACTER STATE RECONSTRUCTIONS

The evolution pattern of selected morphological traits is summarized in Figure 4. The ancestor of *Munroa* is inferred to have been a perennial herb (PP = 0.92), with a disarticulating rachilla (PP = 0.52), monoecious reproductive system (PP = 0.70) and membranous glumes (PP = 0.99). The putative ancestor of the South American clade is inferred to have been an annual herb (PP = 0.99), with a disarticulating rachilla (PP = 0.58), gynomonocious reproductive system (PP = 0.77) and membranous glumes (PP = 0.99). *Munroa pulchella* has a perennial habit, disarticulating rachilla, monoecious reproductive system and membranous glumes. All the other taxa are annual and gynomonocious, except for *M. argentina* (monoecious), *M. andina*, *M. decumbens* and *M. squarrosa*, which have disarticulating rachillas and membranous glumes. *Munroa*

argentina has a non-disarticulating rachilla and coriaceous glumes, whereas *M. mendocina* has a non-disarticulating rachilla and lacks glumes.

DISCUSSION

Our phylogenetic reconstruction recovered *Munroa* as monophyletic, and the North American *M. pulchella* is recognized as sister to the remaining species, which form a clade of mixed South and North American taxa. We present a well-supported and well-resolved phylogenetic hypothesis which is appropriate to evaluate biogeographical movements and possible associations between morphological shifts with dispersal events. Our results are congruent with previously proposed divergence times for Chloridoideae and with the radiation of C_4 grasses in the Americas (Christin *et al.*, 2008, 2009; Vicentini *et al.*, 2008; Osborne & Freckleton, 2009; Edwards *et al.*, 2010; Edwards & Smith, 2010; Strömberg & McInerney, 2011). The ancestral area reconstructions and age estimations indicate that *Munroa* probably originated in North America ($P = 88\%$) during the time period in which the expansion of arid grassland in North America was correlated with global cooling and aridification (Retallack, 1997).

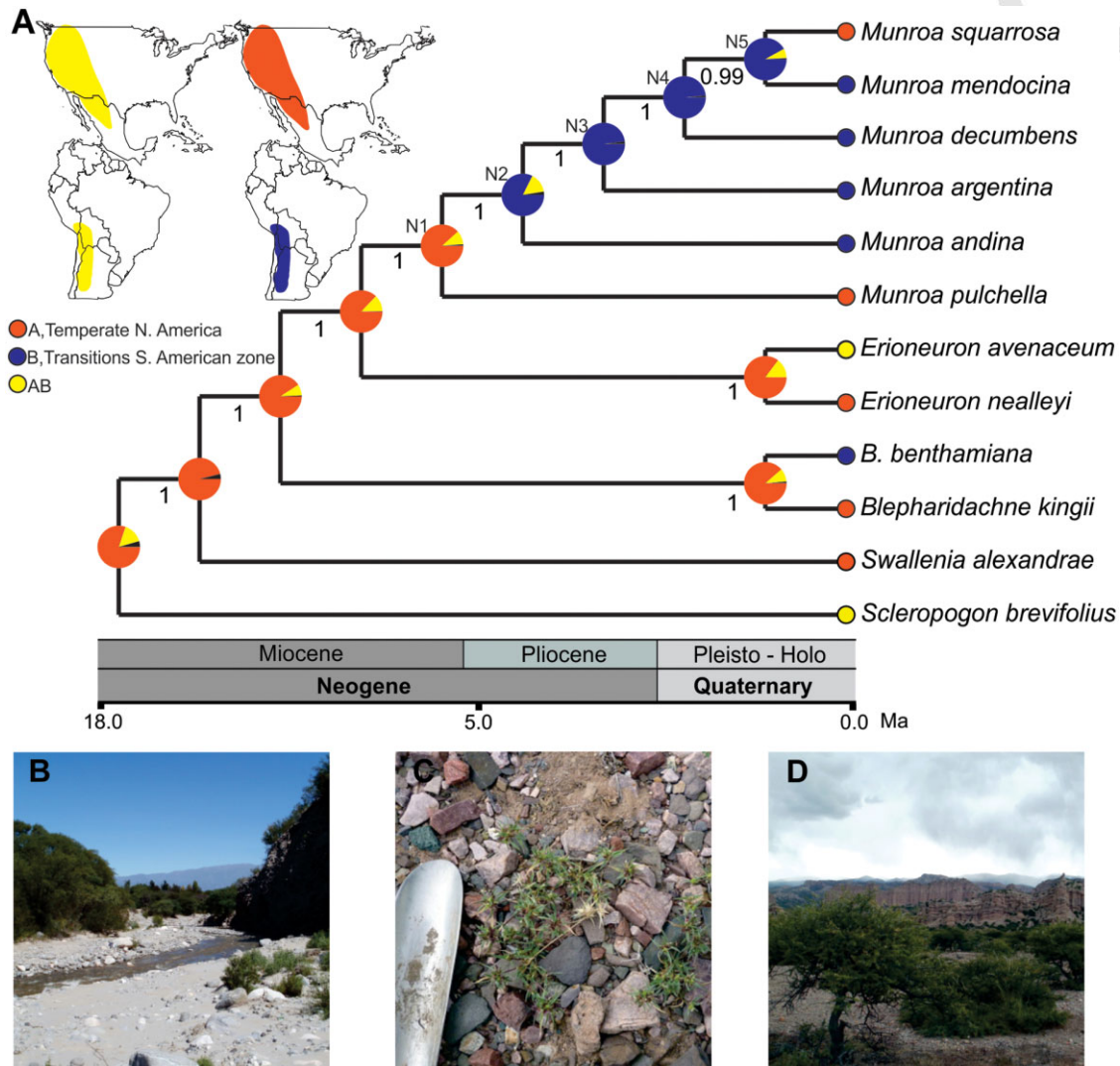


Figure 3. A, Bayesian binary Markov chain Monte Carlo (MCMC) (BBM) reconstruction of ancestral areas for *Munroa*. Posterior probabilities are indicated below the branches. Current distributions are indicated before the species names and pie graphs report probabilities after BBM analysis for reconstructions with $P > 0.01$; other reconstructions are collectively indicated in black. N1–N5 indicate the node names. B, D, Habitats in which *Munroa* grows in South America: Santiago del Estero (Argentina) and Potosí (Bolivia), respectively. C, *Munroa argentina* in Salta, Argentina.

The split between North American *M. pulchella* and its common ancestor with South American *M. andina* took place at *c.* 7.2 Mya (8.2–6.5 Mya), when arid and semi-arid environments were available to receive trans-tropical migrants (Graham, 1999; Hartley & Chong, 2002; Wen & Ickert-Bond, 2009; Rech *et al.*, 2010; Masson & Kadereit, 2013). Moreover, the radiation of *Munroa* in South America took place at *c.* 4.6 Mya (5.5–3.2 Mya) ($P = 82\%$), during the onset of hyper-aridity of the Atacama Desert (Rech *et al.*, 2010). The split between *M. mendocina* and *M. squarrosa* took place at *c.* 1.2 Mya (2–0.8 Mya). The MRCA of the *M. mendocina* + *M. squarrosa* clade occurred in South America ($P = 91\%$), which is another example of an

amphitropical desert disjunction that originated in South America and reached North America during the early Pliocene–late Pleistocene (Raven, 1963; Wen & Ickert-Bond, 2009).

Although vicariance is a hypothesis often used to explain the disjunctions between North and South American plants (Raven, 1963; Wen & Ickert-Bond, 2009), our results do not suggest this hypothesis as the most explanatory. As in *Munroa*, divergence time estimates of various lineages found that a number of plant disjunctions originated much later than the split of the various landmasses (de Queiroz, 2005; Upchurch, 2008; Wen & Ickert-Bond, 2009; Donoghue, 2011; Gillespie *et al.*, 2012; González *et al.*, 2014;

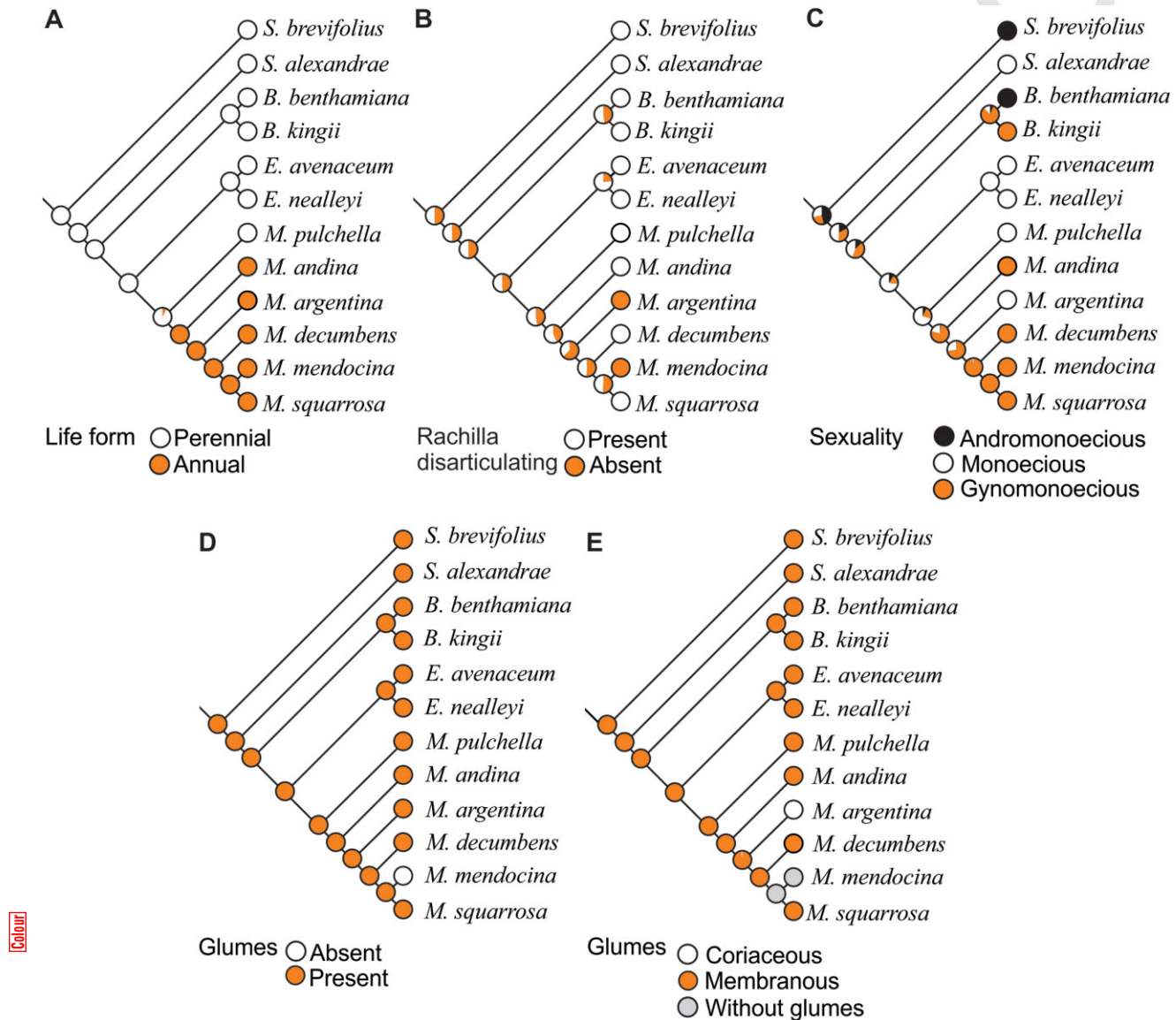


Figure 4. Maximum likelihood ancestral character state reconstructions of selected characters associated with dispersal; pie diagrams indicate the probability of each ancestral character state.

Linder & Barker, 2014). Further, the hypothesis of vicariance implies the existence of intermediate tropical taxa and near-identical arid-adapted taxa from widely distributed tropical ancestors (Johnston, 1940; Barbour, 1969; Moore *et al.*, 2006); this condition is not met by *Munroa*.

Under the stepping-stone dispersal hypothesis, we would expect that the dispersal events occurred when arid and semi-arid environments (or arid and semi-arid patches along the Pacific coast of the American continent) were available to receive trans-tropical migrants (since the late Miocene; Raven, 1963; Solbrig, 1972; Williams, 1975; Graham, 1999; Cain *et al.*, 2000; Hartley & Chong, 2002; Winkworth

et al., 2002; Moore *et al.*, 2006; Gillespie *et al.*, 2012). The diversification of the *Munroa* clade began in the late Miocene (*c.* 7.2 Mya), which is consistent with the timeline and the climatic conditions derived from this hypothesis. However, molecular dates indicate that contemporary taxa were present during the Quaternary, thus making it difficult to explain why only contemporary taxa and not the stepping-stone forms survived through the climatic instability. Such a result would suggest that the stepping-stone hypothesis is improbable (Winkworth *et al.*, 2002). Further, there are no fossils of *Munroa* or other evidence to verify its occurrence in intermediate areas to determine gradual migration.

Munroa has endemic species in both North and South America, with an ancestor originating in the arid–semi-arid areas of North America, which expanded to South America. Later, another South American ancestor (the putative ancestor of *M. mendocina* and *M. squarrosa*) expanded in the opposite direction. Therefore, two, non-contemporaneous dispersal events must have occurred in the *Munroa* clade, and LDD appears to be a plausible explanation for its current distribution, as has been proposed for other amphitropical disjuncts (Xiang & Soltis, 2001; Winkworth *et al.*, 2002; Sanmartín & Ronquist, 2004; Bell & Donoghue, 2005; de Queiroz, 2005; Renner, 2005; Blattner, 2006; Moore *et al.*, 2006; Inda *et al.*, 2008; Escudero *et al.*, 2010; Jakob *et al.*, 2010; Donoghue, 2011; Popp *et al.*, 2011; Gillespie *et al.*, 2012; Linder & Barker, 2014). The LDD hypothesis has been considered as difficult to prove (Winkworth *et al.*, 2002; de Queiroz, 2005; Donoghue, 2011; Gillespie *et al.*, 2012); however, as a result of the diversification time of *Munroa* and the current lack of fossil evidence (potential finding of fossils would change the situation), it is not possible to accept the vicariance or stepping-stones hypothesis.

Based on our results, we suggest two dispersal events in *Munroa* to explain its current distribution pattern; these events occurred in opposite directions, the first from north to south and the second from south to north. The limitation of *M. mendocina* to Argentina can be attributed to a secondary diversification process, as suggested by Nie *et al.* (2012) and Loera, Sosa & Ickert-Bond (2012) for *Ampelopsis* Michx. and *Ephedra* L., respectively. Dispersal events have occurred several times in other taxa, as in grasses such as *Lycurus setosus* (Peterson & Morrone, 1997), *Muhlenbergia torreyi* (Peterson & Ortíz-Díaz, 1998), *Scleropogon brevifolius* (Peterson & Columbus, 1997) and *Hordeum* L. (Blattner, 2006), and in other plants including *Lycium* L./*Grabowskia* Schltdl. (Miller, 2002; Levin & Miller, 2005), *Ephedra* (Ickert-Bond & Wojciechowski, 2004; Huang, Giannasi & Price, 2005), *Hoffmannseggia* Cav. (Simpson, Tate & Weeks, 2005) and *Tiquilia* (Moore *et al.*, 2006).

In Poaceae, many of the attributes involved in adaptations for dispersal and seedling establishment (texture of glume and awns, pubescence, number and size of florets per spikelet, elongated stolons, disarticulating rachilla, reproductive system, annual life cycle, etc.) are relatively prone to parallelism, convergence and even reversals (Barkworth & Everett, 1987; Davidse, 1987; Soreng & Davis, 1998; Soreng, Davis & Voionmaa, 2007), and our ancestral character state reconstructions indicate that the shift from perennial to annual life form and monoecy to gynodioecy occurred at a key node in the phylogenetic tree in the disjunct clades that were correlated with dispersal

events. Our counts show the chromosome base number of $x = 8$ ($2n = 16$) for all *Munroa* spp. Recently, Linder & Barker (2014) proved that successful dispersal is more likely in polyploid than in diploid lineages, but we did not find changes in chromosome number in this disjunct taxon; therefore, we cannot associate the dispersal process with polyploidization events.

The putative ancestor of *Munroa* (North American) was perennial, whereas the putative ancestor of *M. andina* (South American) was annual; it is possible that the shift of life form may have speeded up the establishment of seedlings in South America after the dispersal processes. This result is in agreement with the hypothesis of Stebbins (1982), who proposed that the perennial habit is an ancestral character. An identical pattern of shift has been reported for *Hordeum* (Jakob *et al.*, 2010). Our ancestral character state reconstruction showed that the reproductive mode monoecy shifted to gynodioecy; this shift occurs in the nodes involved in the dispersal event with directions north to south. It has been postulated that gynodioecy (plants have both female flowers and hermaphroditic flowers) benefits outcrossing, as hermaphroditic flowers promote more seeds, whereas female flowers counterbalance the loss of male function through better seed quality (Mamut *et al.*, 2014). It is possible that this shift in reproductive mode may have speeded up the establishment of seedlings after the dispersal processes.

All *Munroa* spp. have small propagules, comprising either single florets or a portion of the inflorescence (Anton & Hunziker, 1978; Amarilla *et al.*, 2013); these propagules might increase the probability of dispersal and establishment in new habitats, but our results were not sufficiently conclusive to discern whether the putative ancestors involved in the dispersal events had a disarticulating or non-disarticulating rachilla. All putative ancestors of *Munroa* have membranous glumes with awns; this trait might have increased the probability of dispersal, as they can attach to animal fur or plumage, as reported for *Hordeum* (Jakob *et al.*, 2010). The only exception is *M. mendocina*, in which the glumes are absent and it has a restricted distribution in South America. *Munroa argentina* is a unique taxon with coriaceous and geniculate glumes, and it has a wide distribution in South America.

The mobility of propagules by wind (anemochory), water (hydrochory) and birds (epizoochory) has been documented in *Munroa* spp. (Anton & Hunziker, 1978; L. D. Amarilla, pers. observ.) and, as has been suggested for other amphitropical disjunct taxa (Raven, 1963; Cain *et al.*, 2000; Winkworth *et al.*, 2002; Blattner, 2006; Wen & Ickert-Bond, 2009; Donoghue, 2011; Popp *et al.*, 2011; Gillespie *et al.*, 2012), these vectors could have helped the dispersal of *Munroa*. Moreover, local and transoceanic whirlwinds are

known for the Americas (Virji, 1981) and could carry the small anthecious or partial inflorescences of *Munroa*, as proposed for *Hordeum* (Blattner, 2006) and several herbaceous plants with a desert amphitropical distribution in the Americas (Raven, 1963; Wen & Ickert-Bond, 2009).

CONCLUSIONS

The amphitropical disjunction in *Munroa* involved an initial dispersal event from North America to South America, expansion of its distribution area to the east and west of the Andes, and dispersal back from South America to North America. The time of diversification of the *Munroa* clade appears to be the late Miocene at *c.* 7.2 Mya. During the course of its range expansion and colonization of South American habitats, the diversification of *Munroa* in South America started at *c.* 4.6 Mya. This radiation and last dispersal event (south to north) are associated with the arid conditions of the late Miocene–Pliocene and with the Quaternary climatic oscillations. All *Munroa* spp. are diploid, and therefore we did not find any relationship between ploidy and dispersal events. Shifts from perennial to annual life forms in *Munroa* and shifts from monoecy to gynomoecy may have been important for dispersal and seedling establishment.

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SUPPORTING INFORMATION

13 Additional Supporting Information may be found in the online version of this article at the publisher's website:

14 **Table S1.** Taxa sampled, voucher information (herbarium), laboratory number and GenBank accession numbers
 15 (first, ITS; second, *ndhA* intron; third, *ndhF*; fourth, *rpl32-trnL*; fifth, *rps16-trnK*). Asterisk, sequences newly
 16 generated for this study; –, absent sequence. Sequences without collection information were downloaded from
 17 GenBank.

18 **Table S2.** Morphological data matrix used in ancestral character state reconstructions.

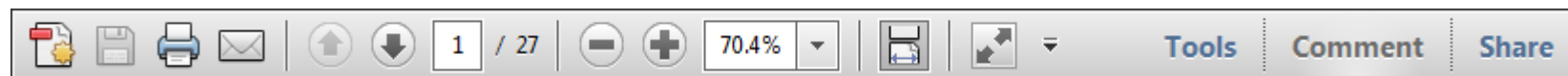
19 **Table S3.** Morphometric data obtained from cytological study. Karyotype formulae; total haploid chromosome
 20 length (tl); arm ratio (*r*); mean chromosome length (*C*); ratio between the largest and smallest chromosomes in
 21 the complement (*R*); intrachromosomal asymmetry index (*A*₁); and interchromosomal asymmetry index (*A*₂).

USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

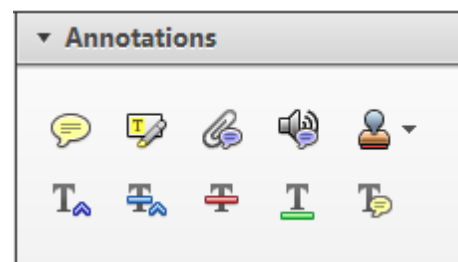
Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)

The latest version of Acrobat Reader can be downloaded for free at: <http://get.adobe.com/reader/>

Once you have Acrobat Reader open on your computer, click on the [Comment](#) tab at the right of the toolbar:



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1. Replace (Ins) Tool – for replacing text.

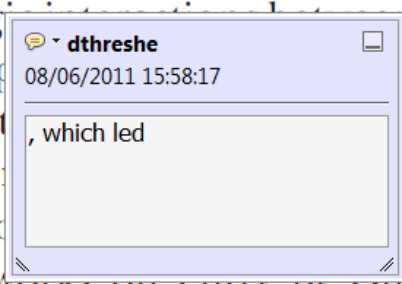


Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
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- Type the replacement text into the blue box that appears.

standard framework for the analysis of microeconomics. Nevertheless, it also led to the emergence of a new paradigm of strategic behavior. The number of competitors in the industry is that the structure of the industry is a key component of the competitive environment. At the industry level, are externalities an important work on entry by Shirasaka (1987) and henceforth) we open the 'black b



2. Strikethrough (Del) Tool – for deleting text.



Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the [Strikethrough \(Del\)](#) icon in the Annotations section.

there is no room for extra profits and the number of competitors are zero and the number of competitors (net) values are not determined by the number of firms. Blanchard and Kiyotaki (1987), in their paper on perfect competition in general equilibrium, show that the effects of aggregate demand and supply shocks in the classical framework assuming monopoly are not different from an exogenous number of firms

3. Add note to text Tool – for highlighting a section to be changed to bold or italic.



Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the [Add note to text](#) icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

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4. Add sticky note Tool – for making notes at specific points in the text.

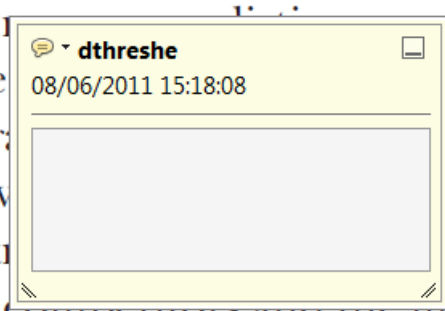


Marks a point in the proof where a comment needs to be highlighted.

How to use it

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- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

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USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

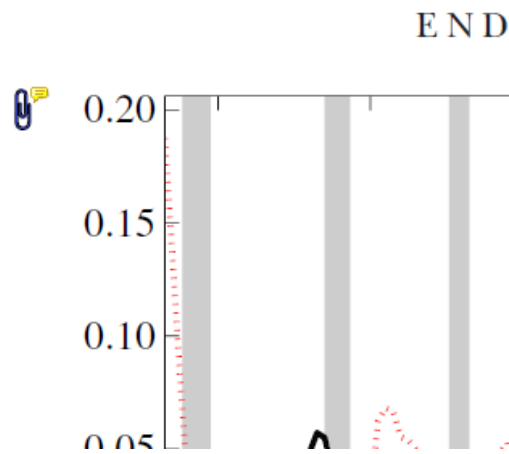
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Inserts an icon linking to the attached file in the appropriate place in the text.

How to use it

- Click on the [Attach File](#) icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
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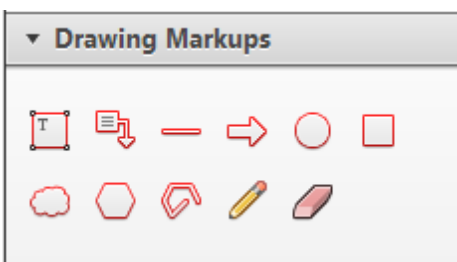


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How to use it

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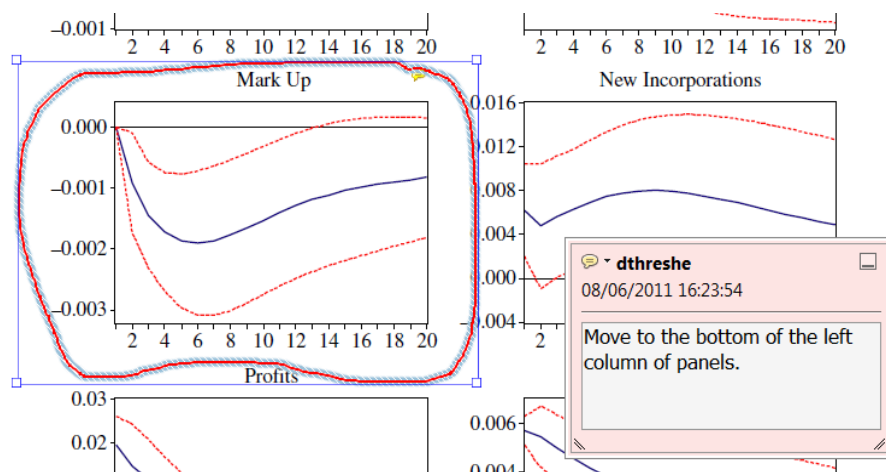


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