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Mycorrhizal compatibility and symbiotic reproduction of *Gavilea australis*, an endangered terrestrial orchid from south Patagonia

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Abstract *Gavilea australis* is a terrestrial orchid endemic from insular south Argentina and Chile. Meeting aspects of mycorrhizal fungi identity and compatibility in this orchid species is essential for propagation and conservation purposes. These knowledge represent also a first approach to elucidate the mycorrhizal specificity of this species. In order to evaluate both the mycorrhizal compatibility and the symbiotic seed germination of *G. australis*, we isolated and identified its root endophytic fungal strains as well as those from two sympatric species: *Gavilea lutea* and *Codonorchis lessonii*. In addition, we tested two other strains isolated from allopatric terrestrial orchid species from central Argentina. All fungal strains formed coilings and pelotons inside protocorms and promoted, at varying degrees, seed germination, and protocorm development until seedlings had two to three leaves. These results suggest a low mycorrhizal specificity of *G. australis* and contribute to a better knowledge of the biology of this orchid as well as of other sympatric Patagonian orchid species, all of them currently under serious risk of extinction.

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Introduction

Nearly 87 genera with 271 orchid species are cited for Argentina, with the highest species diversity confined to the warmer and humid subtropical northeastern provinces, where epiphytic species widely prevail (Schinini et al. 2008). Nevertheless, in the cold Patagonia grow several orchid species, exclusively terrestrial, that are well adapted to the harsh temperate to subantarctic climate.

At least 23 species belonging to three genera (*Chloraea* 13 species, *Gavilea* 9, and *Codonorchis* 1) were cited for the Chilean and Argentinian Patagonia and surrounding islands (Correa 1969; Novoa et al. 2006; Schinini et al. 2008; Chemisquy 2012). Although most of these orchids grow sympatrically in this region, the different species vary widely in their distribution: some species are confined to Tierra del Fuego and surrounding small islands, others are well distributed along de Andean range up to more than 2,000 km to the north. All southern populations grow in areas with seasonal climate, mainly in humid grassy meadows of the subantarctic rain forest (Chemisquy and Morrone 2012; Pridgeon et al. 2003).

Among Patagonian orchids, *Gavilea australis* (Skottsb.) MN Correa is one of the species with the most restricted distribution. It is only found in the south and west region of the Tierra del Fuego Island (Argentina and Chile) and a few populations located in the Malvinas islands (Argentina) (Novoa et al. 2006; Broughton and McAdam 2002). The plant is 30–60 cm high, with a multi-flowered inflorescence carrying 7 to 20 small white-greenish to pale yellow flowers that blooms from December to February (summer southern hemisphere). The habitat where *G. australis* grows is between 500

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and 1,500 m asl, in humid soils near the edges of the Andean patagonic forest dominated by *Nothophagus* species (*Fagaceae*), among low shrubs such as *Empetrum rubrum* (*Ericaceae*), or in grasslands exposed to direct sunlight (Fig. 1). It forms small concentrated populations and is rarely associated with other sympatric orchid species (Chemisquy 2012).

Symbiotic orchid propagation has been stated as the preferred method towards plant multiplication related to conservation and reintroduction programs (Swarts and Dixon 2009). To this purpose, orchid seeds, protocorms, and roots were shown to be appropriate sources to isolate promoting fungal strains (Zelmer et al. 1996). In the last decade, effective symbiotic protocols were established for a growing number of terrestrial orchid species worldwide (Wu et al. 2010; Chutima et al. 2011; Fracchia et al. 2013).

Moreover, it has been proposed that rarity or even a restricted distribution or habitat in orchid species could be associated to highly specialized mycorrhizal interactions, thus limiting orchids to relatively narrow ranges of environmental conditions (McCormick et al. 2006). In line with this, Swarts et al. (2010) suggested the spatial distribution of germination-efficacious mycorrhiza (GEM) as the main driver of rarity in several Australian *Caladenia* species. A narrow fungus specificity was also observed in other rare terrestrial orchid species from the genus *Cypripedium*, supporting the hypothesis of rarity coupled with mycorrhizal specialization (Shefferson et al. 2005). However, specific mycorrhizal symbiosis does not appear to be the cause of rarity in species from the terrestrial Australian genus *Drakaea*, suggesting other ecological interactions to be involved (Phillips et al. 2011). Low density or absence of pollinators (Neiland and Wilcock 1998), specificity of ecological niche (Tremblay et al. 1998), or low reproductive success due to grazing pressure (Yan et al. 2006) has also been proposed as main causes explaining rarity in orchid species.

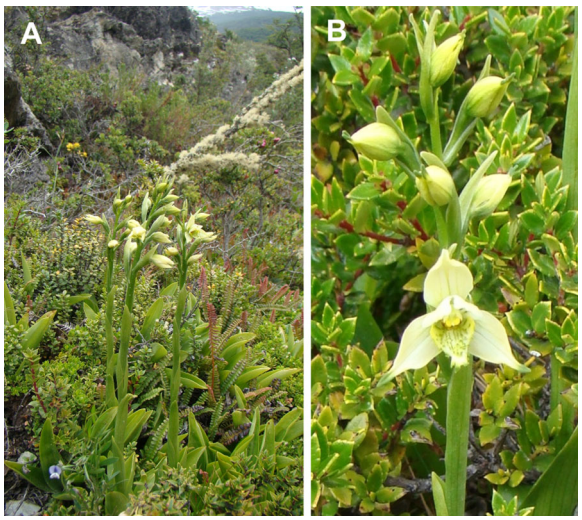

Fig. 1 *G. australis*. **a** Typical habitat of *G. australis* on the edge of the Patagonian forest in Tierra del Fuego Province (Argentina). **b** Spike with flowers

Compared to orchids from other latitudes, the species from south Patagonia have been poorly studied. Although some works have been published focusing on their taxonomy and distribution (Chemisquy 2012; Novoa et al. 2006), the conservation status, pollinator biology, as well as protocols for symbiotic/asymbiotic propagation or mycorrhizal fungal identity of any of these species are still outstanding.

The present study aimed to isolate mycorrhizal fungi associated with *G. australis* and to determine the ability of the orchid to germinate and grow symbiotically in in vitro culture with self and non-self mycobionts, in order to discuss this trait regarding the restricted distribution of the species. To this purpose, we (1) isolated root-fungal strains from the species *G. australis*, and the sympatric species *Gavilea lutea* and *Codonorchis lessonii*; (2) performed the molecular taxonomic identification of the isolates; and (3) tested symbiotic seed germination and protocorm development of *G. australis* in dual cultures with the isolated strains and two other fungi of the allopatric terrestrial species *Aa achalensis* and *Sacoila lanceolata*.

Materials and methods

Orchid source

Whole plants at various developmental stages of *G. australis*, *G. lutea*, and *C. lessonii* were collected from their natural habitat (Ushuaia, Tierra del Fuego Province, Argentina) in February 2010. The identification of the *Gavilea* species was performed following the morphological descriptions of Correa (1996) and later by description and keys of Chemisquy (2012). Seeds of *G. australis* were collected from mature capsules belonging to different individuals of the same population during February–March 2010 and pooled together in plastic vials with silica gel. Viability of seeds was determined within 10 days using the tetrazolium test (Van Waes and Bebergh 1986). The remaining seeds were stored in silica gel at −5 °C in the dark until used in the germination assays. No significant viability changes were observed after being stored for longer periods (4 months).

Fungal collection and conservation

Fungal isolation was carried out on detached healthy roots from the three orchid species (*G. australis*, *G. lutea*, *C. lessonii*) as described by Fracchia et al. (2013). The isolated strains were stored in glass tubes at 0 °C and included with a strain number in the fungal collection at the CRILAR (Centro Regional de Investigaciones Científicas, La Rioja, Argentina). Previously isolated fungi from *A. achalensis* and *S. lanceolata*

were also chosen to test their effect on *G. australis* seed germination. Cultures were conducted in a sterile mixture of wheat bran–pot soil–perlite (2:1:1 *v/v*), as we have previously found that *Rhizoctonia*-like fungi growing in this substrate and stored at −5 °C allowed us to keep their viability for longer time periods (>2 years) without subculturing (Fracchia, personal communication).

Fungal molecular characterization

DNA isolation, amplification, and sequencing Total genomic DNA from the fungal isolates was extracted with the DNeasy Plant Mini Kit (Qiagen, Valencia, California), and used as template for the PCR amplification of the intergenic spacer region from the nuclear ribosomal DNA (ITS hereafter), including ITS1, the 5.8S subunit, and ITS2. Amplification and sequencing were carried out using the primers ITS4 and ITS5 (White et al. 1990). PCR reactions were performed in 25 μl final volume with 50–100 ng of template DNA, 0.2 μM of each primer, 25 μM of DNTPs, 4 mM $MgCl_2$, 1× buffer, and 1.5 units of Taq polymerase provided by Invitrogen (Brazil). The reaction conditions were as follows: a first period of denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min 30 s. Final extension at 72 °C for 7 min terminated the reactions. PCR products were run out on a 1 % TAE agarose gel stained with ethidium bromide. Purification of PCR products and automated sequencing were performed by Macrogen, Inc. (Korea). Sequences from isolates CC31, CC33, CC34, CC35, and CC36 were deposited at GenBank (accession numbers).

Sequence alignment and phylogenetic analyses Assembly and editing of sequences were performed with BioEdit version 5.0.9 (Hall 1999). All sequences were submitted to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments were performed using MAFFT program version 6 (Katoh and Toh 2008) available on line (http://mafft.cbrc.jp/alignment/server/). We performed phylogenetic analyses to assign isolates to a specific fungal group using ITS representative sequences available in GenBank. Sequences with at least 97 % similarity were chosen. The analysis included sequences from Ceratobasidiales, Tulasnellales, Sebacinae, and one from *Multiclavula corynoides* in order to root the tree (GenBank accession number U66440).

The phylogenetic analyses were performed under the parsimony criterion using TNT version 1.1 (Goloboff et al. 2008). Parsimony-uninformative characters were excluded and gaps were considered as missing data. Search strategy consisted of heuristic searches performed using 1,000 series of random addition sequences (RAS) followed by TBR branch rearrangements, retaining 10 trees per series. Trees found were saved in memory and additionally TBR swapped retaining a maximum 10,000 total trees.

A strict consensus tree was generated from the most parsimonious trees. Branch support was calculated by bootstrapping, performing 1,000 resampling iterations and a heuristic search strategy of five addition sequences swapped with TBR with two trees saved per replicate.

Seed sowing and fungal inoculation

G. australis seeds were surface-sterilized following the procedure of Dutra et al. (2009). Seeds (150–200) were plated in 9-cm diameter Petri plates containing 20 ml of sterile oat meal agar (OMA) medium (2 g l^{-1} rolled oats, agar 0.7 %, pH 6.5 measured prior to autoclaving). The plates were inoculated 2–3 days previously with a 1×1-cm plug of each fungal inoculum taken from the hyphal edge after culturing on potato dextrose agar (PDA). Each treatment consisted of eight replicates inoculated with a single fungal strain. Uninoculated plates served as a control treatment. Petri plates were sealed with ParafilmM® and stored in the dark at 25±2 °C for 2 weeks. After this period, plates were exposed to a 14-h white light photoperiod with cool white fluorescent tubes at 80 μmol m^{-2} s^{-1} measured at the plate surface. Seed germination and protocorm development were monitored weekly and scored on a scale of 0–5 (Table 1, Fig. 2a). Percent seed germination and protocorm development was calculated by dividing the number of seeds in each developmental stage by the total number of viable seeds. Seedlings with two leaves (>10 weeks) were transplanted to 200 ml glass flasks with fresh OMA to promote further root growth.

Visualization of the mycobiont structures inside protocorms (n=20) was evaluated at week 4 after overnight staining with trypan blue (0.05 % trypan blue–water solution) at room temperature and cleared in water for 1 day. The stained protocorms were mounted in water and observed under light microscope (Leica DMLB) at×100–×400 magnification.

The symbiotic assay was performed three times.

Table 1 Seed germination and protocorm developmental stages of symbiotic in vitro culture of *G. australis*. Adapted from Stewart and Kane (2006)

Stage	Description
0	Ungerminated seed, testa intact
1	Enlarged embryo, testa ruptured
2	Appearance of rhizoids (=germination)
3	Appearance of protomeristem
4	Emergence of first leaf (=seedling)
5	Presence of second leaf

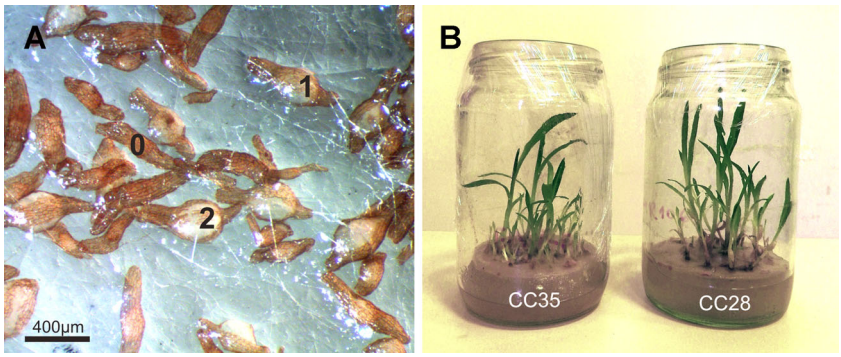

Fig. 2 **a** Symbiotic seed germination of *G. australis* (stages 0–2, strain CC28). **b** Transplanted seedlings in 200 ml glass flasks

Statistical analysis

We used one-way analysis of variance (ANOVA) to test for differences in the percentage of germinated seeds between treatments. Previous to the analysis, percentage data were square root transformed to meet the assumption of homogeneity of variances. Post hoc comparisons were performed using Duncan's multiple range test ($p=0.05$). The statistical analyses were conducted with STATISTICA 7.0 (Statsoft, Tulsa, OK).

Results

Fungal isolation and culture

Four endophytic fungal isolates were recovered from the roots of *G. australis* ($n=2$), *G. lutea* ($n=1$), and *C. lessonii* ($n=1$). Table 2 shows fungal isolates data, including the additional strains CC28 and CC36 that were previously isolated from *A. achalensis* and *S. lanceolata*. All isolates developed monilioid cells in PDA culture. Sclerotial masses were evident after 25–35 days of culture at 25 °C. In the long-term storage culture method, the development of sclerotia was observed in all strains also inside the perlite particles. Thereby, the strains were stored in this substrate and in sterile plastic tubes at −5 °C for long-term conservation. Two years after storage, all fungal strains recovered mycelial growth on PDA medium at 25 °C.

Molecular identification

BLAST analyses revealed that sequences from isolates CC31, CC34, CC35, and CC36 were similar (99, 96, 99, and 98 %, respectively) to uncultured Ceratobasidiaceae/uncultured *Ceratobasidum*/*Rhizoctonia* sp. Sequence from isolate CC33 was 99 % similar to uncultured Tulasnellaceae/*Tulasnella* sp./*Tulasnella calospora*. Isolate CC28 (GB number KF151201) had been already identified as *Thanatephorus cucumeris*/*Rhizoctonia solani* with 98–99 % similarity.

The ITS data set comprises 28 sequences. From a total of 782 characters, 456 were phylogenetically informative. The analysis of the aligned matrix resulted in a unique most parsimonious tree (length=1,312; CI=0.64; RI=0.83) (Fig. 3).

The phylogenetic analysis revealed two highly supported clades (1 and 2). Clade 1 is composed of two subclades: one including sequences of Sebacinaceae (bootstrap value (BS)=99) and the other gathering together sequences of *Tulasnella*/*Epulorhiza* (BS=100). Within the latter, isolate CC33 of orchid mycorrhizal fungi was recovered as sister of the reference sequence AY373298, identified as *Tulasnella calospora*.

In clade 2, the remaining five fungal isolates (CC28, CC31, CC34, CC35, CC36) were gathered together with reference sequences of *Thanatephorus cucumeris*, *Ceratobasidium* spp., and uncultured Ceratobasidiaceae (BS=100) (Fig. 3).

Seed viability and symbiotic germination

The tetrazolium test revealed that 74 % ($n=100$) of the harvested *G. australis* seeds were viable. Although the remaining

Table 2 Fungi used in the germination of *G. australis*

Isolate code	Host	Close relative from GenBank	GenBank accession	Collection date
CC28	*A. achalensis*	*Thanatephorus cucumeris*	KF151201	December 2010
CC31	*G. lutea*	*Ceratobasidiaceae*	KJ713697	March 2010
CC33	*C. lessonii*	*Tulasnella calospora*	KJ713701	March 2010
CC34	*G. australis*	*Ceratobasidiaceae*	KJ713698	March 2010
CC35	*G. australis*	*Ceratobasidium albasitensis*	KJ713699	March 2010
CC36	*S. lanceolata*	*Ceratobasidium sp.*	KJ713700	October 2009

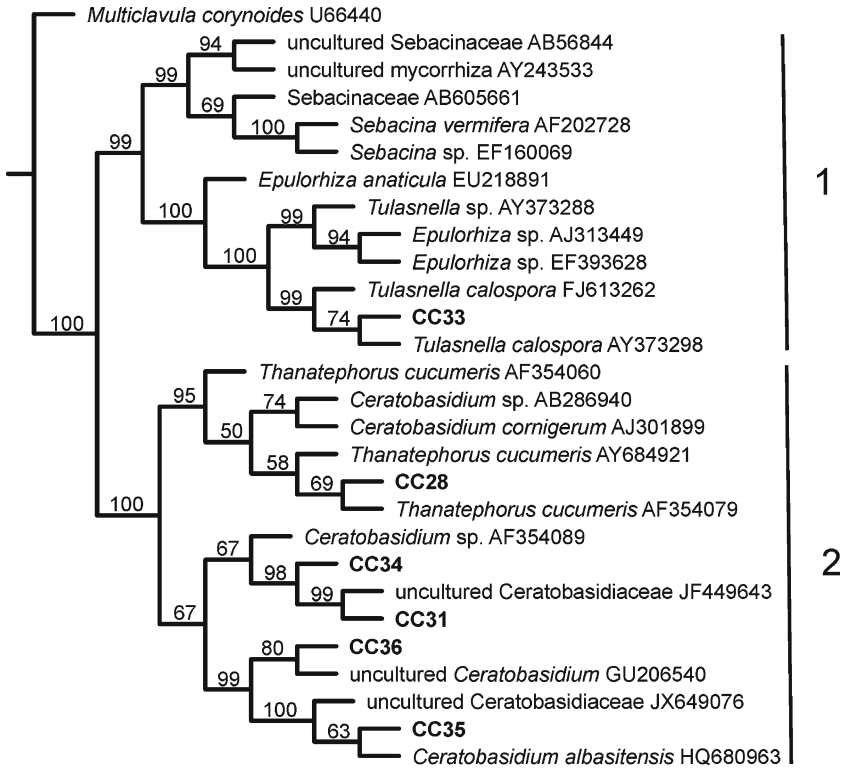

Fig. 3 Single most parsimonious tree (length=1,312 steps; CI=0.64; RI=0.83) resulting from the ITS data matrix analysis. *Numbers above branches* refer to bootstrap values. *Bars* indicate main clades discussed in the text

seeds were stored in silica gel at −5 °C, the viability decreased significantly in the following 2 years to values of 58 % in the first year and of 29 % in the second year.

All tested endophytic fungal strains promoted seed germination and protocorm development regardless their taxonomic identity or host origin (Fig. 4). Six days after sowing, the embryos from all treatments swelled breaking the testa. On the other hand, rhizoids appeared was evident after 20 days of dual culture (Fig. 2a). In the control treatment, rhizoid development (stage 2) was significantly lower than in the symbiotic treatment, and no further growth was observed after this stage. At week 4, careful examination of protocorms after trypan blue staining revealed typical pelotons and coiling hyphae in all the inoculated treatments.

The strains CC31, CC35, and CC28 were the most effective in promoting seed germination and plant development. After 11 weeks of culture, more than 50 % of the seeds germinated, and between 2 and 15 % of them further developed to plantlets with two leaves (stage 5) (Fig. 4). In the first 5 weeks, the fungal strains CC36 and CC33 were also highly effective in promoting seed germination (44 and 45 %, respectively), but seedling with two leaves were not seen until week 15. On the other hand, only erratic results were observed with the strains CC34 and CC33, where a few small seedlings in only one or two Petri plate replicates developed to stage 5. Once the seedlings developed two leaves in each treatment, we proceeded to axenic transplantation to fresh sterile OMA in 200 ml glass flasks. These plantlets were maintained (>22 weeks) at the same temperature, light, and photoperiod conditions as stated above, to allow further root development and to program harden of plants in peat-based substrates (Fig. 2b).

Discussion

This is the first research describing successful symbiotic seed germination and protocorm development of a *Gavilea* species. Although commercial and amateur growers, as well as scientific researchers, have reported successful symbiotic propagation of Patagonian orchids, including *Gavilea* and *Chloraea* species, these valuable research have not been published so far on peer-reviewed scientific journals and are only available in the internet (e.g., Novoa and Maissner, http://www.ewetel.net/~frank.meissner2/Homepage/ChloraeainChile.pdf).

In our research, we observed that all tested fungal isolates promoted, to varying degrees, in vitro seed germination and protocorm development in *G. australis*. Considering the fungal isolates identities, both *Tulasnella*- and *Ceratobasidium*-related species were able to promote symbiotic germination. Even the strains CC28 and CC36, isolated from allopatric terrestrial orchids with a remote distribution related to the concerned species, promoted plant growth. This behavior has also been reported for other orchid species, where fungal strains isolated from a specific terrestrial orchid promoted efficiently seed germination and protocorm development in

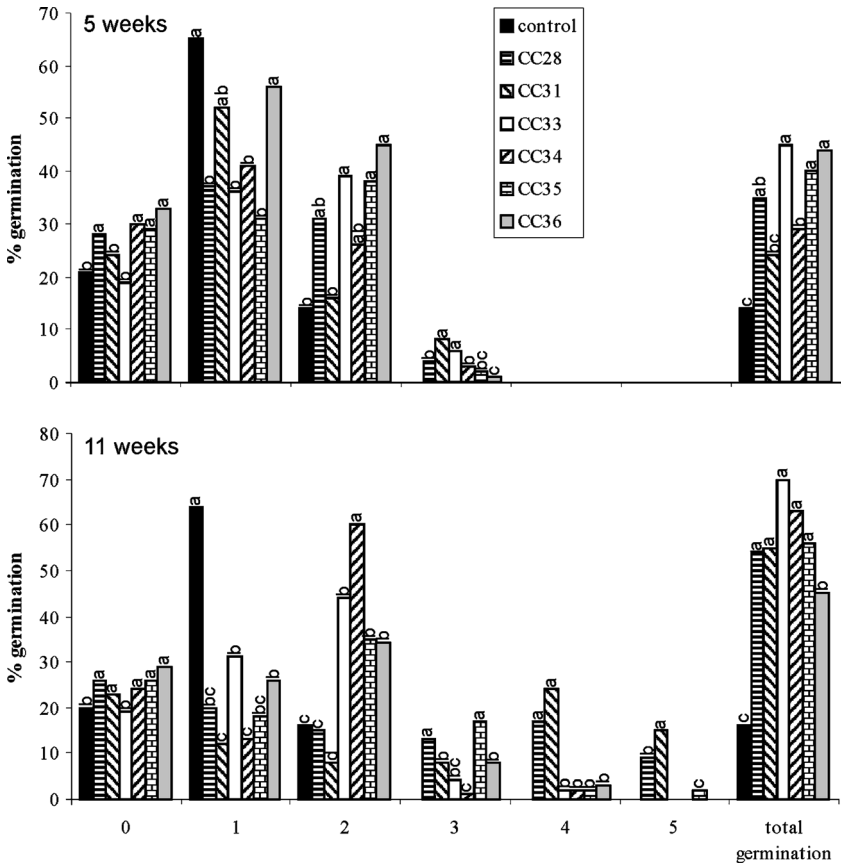

Fig. 4 Comparative effects of fungal strains on in vitro germination and protocorm development of *G. australis* after 5 and 11 weeks of symbiotic culture. *Histobars with the same letter in each graph* are not significantly different ($p = 0.05$)

other non-related species (Bonnardeaux et al. 2007; Stewart and Zettler 2002). It is a general view that there is a low fungal specificity in green terrestrial orchids like *G. australis* (Taylor et al. 2003). Nevertheless, high fungal specificity was also observed in some North American and Australian terrestrial species (McCormick and Jacquemyn 2013; Bonnardeaux et al. 2007).

We observed seed germination even in the control treatment without fungus, suggesting that at least in in vitro assays, *G. australis* has the ability to germinate asymbiotically without specific nutrients, although is unable to grow until advanced protocorm stages (>stage2). Other orchid species also demonstrated this behavior in water–agar medium (Rasmussen 1995; Øien et al. 2008), meanwhile others did not germinate at all under the same conditions (Fracchia et al. 2013; Steinfort et al. 2010). It would be interesting to further study this intrinsic characteristic of orchid seeds, and if there is a correlation between this trait and the mycorrhizal specificity of the species.

In our assays, the inoculation of single strain isolated exclusively from adult plants was enough to obtain seedlings with two to three leaves after 11 weeks of dual culture. These results indicate that fungal switch might not be a prerequisite for *G. australis* to be capable of growing until adult plants, nor specific fungal endophytes isolated from protocorm or from early seedling stages. The fungal switch mechanism was effectively demonstrated to occur in only one orchid species (McCormick et al. 2006), and proposed as the explanation for the failure of single mycorrhizal fungi to promote protocorm growth beyond the first developmental stages in other terrestrial orchids (Chutima et al. 2011).

As most temperate terrestrial orchids, *G. australis* has strategies to withstand the winter season underground fleshy roots and an apical bud that develops new leaves in late spring. In the in vitro assay, we obtained in a 3-month growing period seedlings with three to four leaves and fleshy roots of 1–2 cm length. In natural populations, we observed only in one population a low number of plants of similar size during the early summer, so these individuals could be 1-year plants that survived the harsh winter season. The relative fast growing rate of the species from seed to seedling could be an adaptive strategy to gain biomass during the short growing season (3 months), allowing the plants to overwinter. Nevertheless, we cannot exclude that lower developmental stages (protocorm stages 2–4) could also survive underground during the cold season, as it was demonstrated for other terrestrial orchids (Rasmussen 1995; Øien et al. 2008).

Our results demonstrate the ability of *G. australis* to germinate and grow until seedlings with two to three leaves with diverse endophytic fungi under laboratory conditions. In a

broad sense, these results suggest that the restricted distribution of *G. australis* is not related to its mycorrhizal specificity. However, as stated by Batty et al (2001), it is important to consider carefully the results that are obtained in in vitro symbiotic germination assays. Field variables (i.e., soil conditions, interaction with microorganisms) in natural habitats could interact with the establishment and efficiency of the symbiosis, thereby laboratory results might not be representative of the natural situation (Harley and Smith 1983; Masuhara and Katsuya 1994). In a recent review (McCormick and Jacquemyn 2013), the authors stated the importance of in situ assays, considering occupied vs non-occupied orchid habitats, in order to elucidate suitable microsite components for orchids establishment. This would probably reflect a combination of the distribution of abiotic conditions and the specificity of orchid fungus requirements, taking also into account that mycorrhizal fungi are not randomly distributed. In this context, a species that could associate with many different fungi, as happens for *G. australis*, would be less restricted than those species that associate with specific fungi.

As was stated before, other extrinsic or intrinsic plant factors can determine orchid species distribution and habitat occupancy (Swarts and Dixon 2009). It has been proposed that highly specific or scarce pollinators, as well as limited or failed fruiting success associated to nectar production could be drivers of rarity in orchid species (Neiland and Wilcock 1998). In the *Drakaea* genus, the highly specific pollination system of sexual deception may contribute to rarity in some species (Hopper and Brown 2007). *G. australis* has a particular trait not observed in other species from the genus: a trilobate and feathery stigma that coalesce at maturity in an amorphous viscous mass, a trait that could be associated to highly specific pollinators different from other *Gavilea* species with broader distributions (Chemisquy 2012).

Finally, the south Patagonian forest and surrounding steppe are exposed to several hazards that seriously place at risk of extinction the native orchid flora. Almost the whole distribution area of *G. australis* is exposed to high UV light irradiance due to the ozone depletion hole that affects the South Pole since more than 30 years (Thompson et al. 2011). This harmful effect occurs annually in spring and early summer, coinciding with the short plant growing and pollination season in southern Patagonia. Other risky factor is the extensive invasion of exotic herbivorous species such as beavers, feral boars, red deer, European rabbit and hare, muskrat, and American mink; all species that depredate native plants including orchids (Jaksic et al. 2002; Skewes et al. 2007). Also, climate change is affecting drastically the hydrological dynamics of the Andean region of south Patagonia, an effect that has worsened in the last years (Rabassa 2009). Additionally, both southern Argentinian provinces (Tierra del Fuego and Santa Cruz) are the country regions with the highest population growing rates in the last decade (25 and 39 %, respectively), thereby increasing pressure on the surrounding environment (INDEC 2010).

All this events highlight the importance and urgency to understand the biology of all Patagonian orchid species, and evaluate viable methods and strategies for their conservation. The distribution of the species and conservation status, mycorrhizal specificity, and methods to propagate symbiotically the species are highly valuable research that need urgent attention.

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