IN VITRO SEED PROPAGATION CONSTRAINTS OF MEXICAN LADY'S SLIPPER ORCHID, PICHOUAXTLE, *CYPRIPEDIUM IRAPEANUM*

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Abstract. *Cypripedium irapeanum* known in Mexico as "*Pichohuaxtle"* in Nahuatl, is the most emblematic Mexican lady's slipper orchid and one of the most studied cypripedioid species in Mesoamerica. Despite its protected status in Mexico, habitat loss driven by land-use change has severely reduced its populations. *Ex situ* conservation approaches are urgently needed to safeguard its genetic diversity, yet seed germination in the genus remains challenging due to innate dormancy and specific nutritional requirements. We collected seeds from two Mexican ecosystems where this species is distributed —*Quercus* and Tropical Dry Forests— and isolated mycorrhizal fungi from adult plants to examine both symbiotic and asymbiotic cultures. We found limitations in seed production due to flower ripening, ineffective pollination, or parasitism. Furthermore, germination was achieved after a cold treatment, and the best medium to obtain seedlings *in vitro* was Norstog medium. Two mycorrhizal fungi, isolated from adult plants belonging to the family Tulasnellaceae, formed a clade with previous isolates from American slipper orchids. One of these isolates promoted germination but later produced fungal incompatibility *in vitro*. Our study contributes to clarify previously undescribed processes in the biology of this highly appreciated and vulnerable species, supporting future efforts to propagate it from seed and obtain successful cultures.

Resumen. *Cypripedium irapeanum*, conocida en México como "Pichohuaxtle" en náhuatl, es la orquídea zapatilla de dama más emblemática del país y una de las especies cipripedioideas más estudiadas en Mesoamérica. A pesar de su estatus de protección en México, la pérdida de hábitat asociada al cambio de uso del suelo ha reducido severamente sus poblaciones. Las estrategias de conservación *ex situ* son urgentemente necesarias para salvaguardar su diversidad genética; sin embargo, la germinación de semillas en el género sigue siendo un reto debido a su dormancia innata y a sus requerimientos nutricionales específicos. Recolectamos semillas en dos ecosistemas mexicanos donde se distribuye la especie —bosques de *Quercus* y selva tropical seca— y aislamos hongos micorrízicos de plantas adultas para evaluar cultivos simbióticos y asimbióticos. Detectamos limitaciones en la producción de semillas debido a la maduración floral, la polinización ineficaz o el parasitismo. Asimismo, la germinación se logró únicamente tras un tratamiento de frío, y el medio más eficaz para obtener plántulas *in vitro* fue el medio Norstog. Dos hongos micorrízicos aislados de plantas adultas, pertenecientes a la familia Tulasnellaceae, formaron un clado con aislados previamente reportados en orquídeas zapatilla americanas. Uno de ellos promovió la germinación, aunque posteriormente produjo incompatibilidad fúngica en condiciones *in vitro*. Nuestro estudio contribuye a esclarecer procesos previamente no descritos en la biología de esta especie apreciada y vulnerable, y proporciona bases para futuros esfuerzos de propagación a partir de semillas orientados a obtener cultivos exitosos.

Keywords / Palabras clave: Ceratobasidiaceae, *Epulorhiza*, germinación simbiótica, incompatibilidad micorrícica, micorriza orquideoide, mycorrhizal incompatibility, orchid mycorrhiza, symbiotic germination, Tulasnellaceae

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Introduction. Many cypripedioid orchids are endangered due to anthropogenic activities and climate change. These factors affect ecological distribution and their associations, reducing seed production by decreasing pollinator populations or increasing parasites that prey on seed capsules (Faleiro *et al*., 2018; Liu *et al.,* 2021; Mcgough *et al.,* 2006; Rankou & Salazar, 2014). Mesoamerican *Cypripedium* L. is a group of interest because it is a sister clade to the rest of the *Cypripedium* (Guo *et al.*, 2012; Li *et al.*, 2011): a particular group formed by *C. irapeanum* Lex., *C. molle* Lindl. and *C. dickinsonianum* Hágsater, of which *C. irapeanum* is the most widely distributed in several habitats (Soto-Arenas & Solano-Gómez, 2007). All species have experienced a decline of almost 50% in population size as a result of habitat transformation, and propagation for reintroduction remains challenging due to persistent difficulties related to *in vitro* culture and mycorrhizal fungi associations (Moreno-Camarena & Ortega-Larrocea, 2022).

While habitat conservation has become a priority, understanding the life history of this elusive group is important as it may contribute to establishing protocols for propagation. In nature, protocorm and seedling recruitment (addition of new individuals to a population) in orchids are considered negligible contributors to population models because of the rarity of *in situ* germination observations (Nicolè *et al*., 2005). Therefore, a deeper understanding of the germination process and its relationships with orchid mycorrhizal fungi (OMF) is imperative to prevent further loss of genetic variability. Few seed-based propagation protocols have been developed, and most have been successfully applied only to European or North American species (Seaton & Ramsay, 2005; Shefferson *et al*., 2007, 2019; Shimura *et al*., 2009). In this study, we developed a set of methods for symbiotic and asymbiotic *in vitro* propagation based on previous protocols to test their effectiveness in *C. irapeanum*, the most valued of the Mexican lady's slippers.

Material and methods. *Collection of biological material.*— We sampled five populations of *Cypripedium irapeanum* (Fig. 1A), three in *Quercus* L. spp. forests (State of Mexico, Morelos and Puebla), and two in Tropical Deciduous Forests (TDF) (Veracruz). We also tried to isolate mycorrhizal fungi from *C. molle* in *Quercus* Forest in Oaxaca state (Fig. 1B**)**. Due to the endangered status of the genus, sampling authorization was granted by local institutions (SEMARNAT, Secretaría de Medio Ambiente y Recursos Naturales, SGPA/DGVS/07200/14 and SGPA/DGVS/5126/19).

Fungi: In each population, roots were collected from plantlets and adult non-flowering plants separated by at least one meter. One root per plant, including secondary roots, was collected, cutting it from the base of the rhizome (Fig. 1C–F). Roots were wrapped in aluminum foil with a small amount of soil and kept at 10 °C until isolation.

Seeds: Mature capsules were collected during the rainy season (August–September) and kept in paper bags then stored in a desiccator with silica gel at 24 °C and a relative humidity of 24%; once ripened, seeds from capsules belonging to the same plant were evaluated for their quality (amount of seeds without embryos, presence of fungi or other pests in dehiscent capsules) before being mixed. Semi-permanent slides were made (Fig. 2). Capsules collected from the State of Mexico and Veracruz were aborted due to wasp infestation.

Isolation, morphological and molecular characterization of orchid mycorrhizal fungi.— Roots were superficially washed to remove soil and organic matter. Colonization and digestion of hyphal coils was evaluated through semi-permanent slides of transverse root sections (*ca.* 0.5–1.0 cm) stained with acid fuchsin (0.1% in lactic acid and glycerol) and mounted in polyvinyllactoglycerol alcohol (PVLG) (Fig 1I–J). The velamen was removed from colonized segments, which were then rinsed in sterile distilled water and disinfected in 10% NaOCl (Brand Puro Sol®, 5% active Cl) for 1–2 min, followed by three additional rinses in sterile distilled water.

Under sterile conditions and using a microscope, root segments were cut in a Petri dish with a drop of distilled water; hyphal coils were extracted with a pipette and incubated onto three culture media: GPA (supernatant of green pea; Shimura *et al*.,2009), FIM (Fungal Isolation Medium; Clements *et al*., 1986) and AWA (Acidic Water Agar; Stewart, 2004). Media pH was adjusted to 5.8 (HCl 0.1 N and NaOH 0.1 N). All cultures were kept in the dark at 25 ± 1 °C.

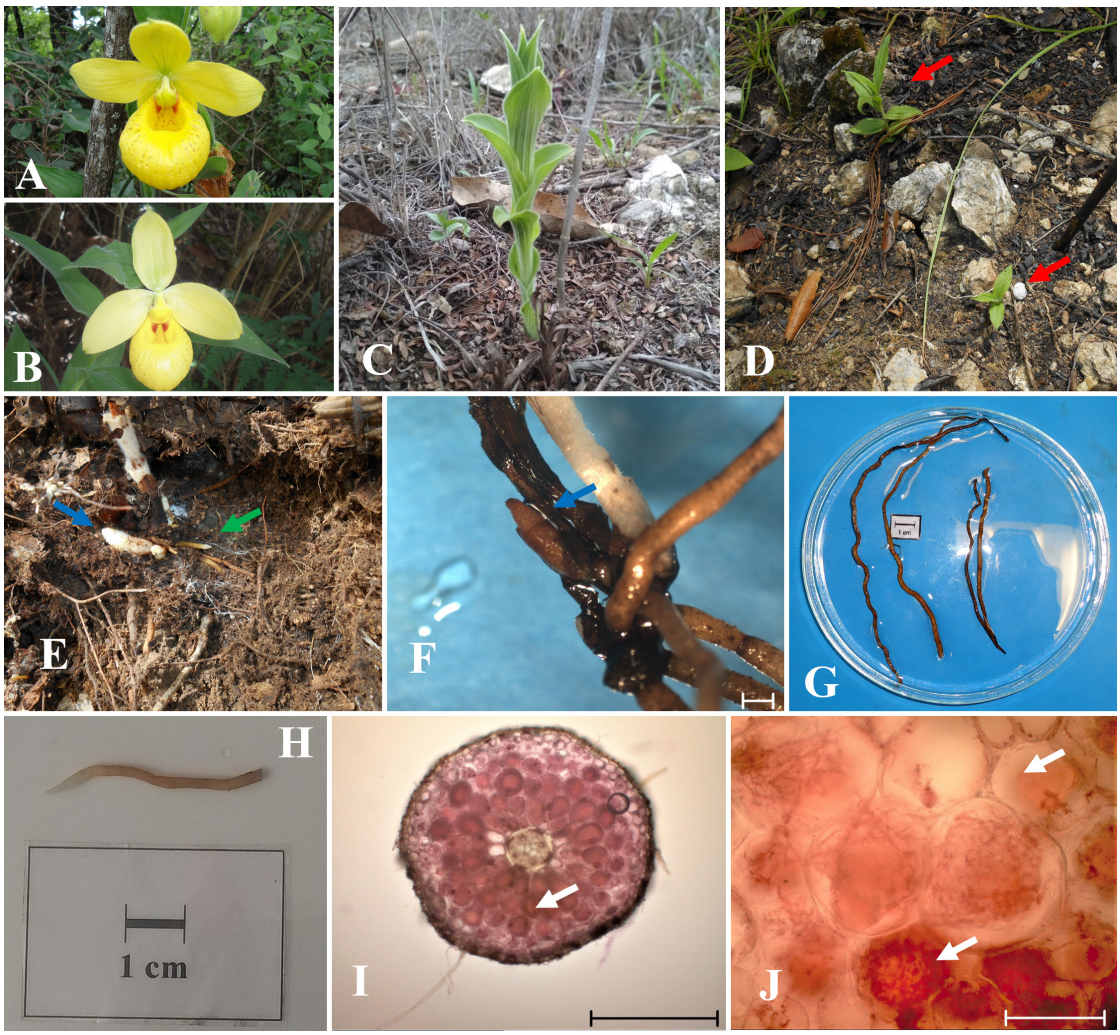

Figure 1. Flowers, collection, and root colonization diagnosis. **A, C**. *Cypripedium irapeanum* from *Quercus* forest at Puebla state, Mexico. **B, D**. *Cypripedium molle* from *Quercus* forest in Oaxaca state, Mexico. **E**. Underground shoot and root of *C. irapeanum* in Tropical Dry Forest. **F**. Lateral shoot in a 5-year plantlet. **G**. Aspect of long reddish adult roots. **H**. Aspect of lateral root. **I**. Mycorrhizal colonized cortex of transverse segment of lateral root. **J**. Hyphal coils in different degradation stages. Photographs by M. Moreno-Camarena.

After one or two days, hyphal tips growing from coils were excised and incubated on PDA medium (potato dextrose agar) or concentrated GPA (mashed peas without filtering it) and cultivated under the same conditions to obtain pure strains. From these colonies, hyphae were taken and stained with acid fuchsin and mounted on semi-permanent slides with PVLG to characterize micromorphology. *Rhizoctonia*-like fungi were selected considering branching of hyphae (*ca.* 90°), presence of basal septum and monilioid cells as well as color, appearance, and growth rate of colonies.

PCR amplification, sequencing, and phylogenetic analysis.— DNA was extracted from colonies with characteristics of *Rhizoctonia*. First, they were cultured in liquid PDB medium (potato dextrose broth) or GPE (green pea extract, boiled, non-crushed, and filtered pea) (pH 5.8) and incubated under agitation at 25 ± 1 °C. Once a colony had formed, the hyphae

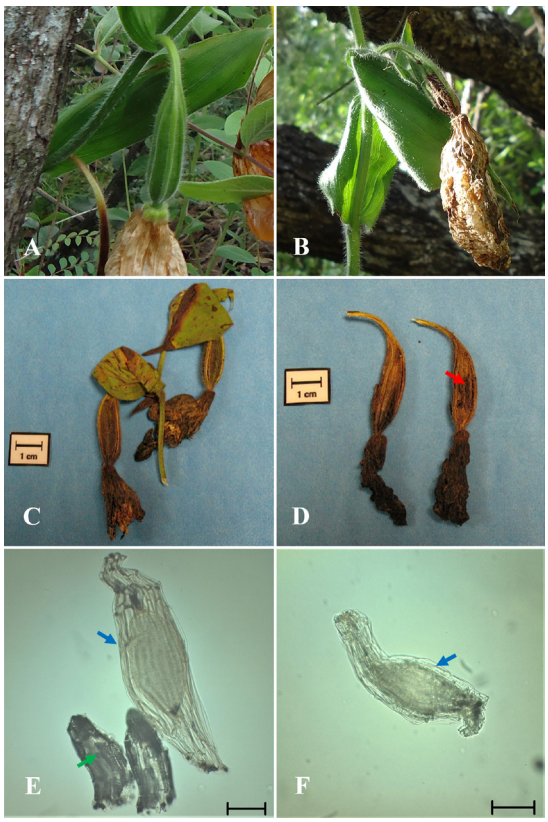

FIGURE 2. Aspect of capsules and seeds from *Cypripedium irapeanum*: **A**. from plants growing in Puebla state. **B**. Veracruz state aborted capsule. **C**. Dried capsules of *C. irapeanum*. **D**. Capsule of *C. irapeanum* damaged by parasites. **E**. Viable (blue arrow) and without embryo seeds (green arrow) from a capsule of *C. irapeanum* from Puebla state. **F**. Aspect of a single unviable seed due to the non-turgid appearance of the embryo cells that have adhered to those of the testa. Bar = 100 µm. Photographs by M. Moreno-Camarena.

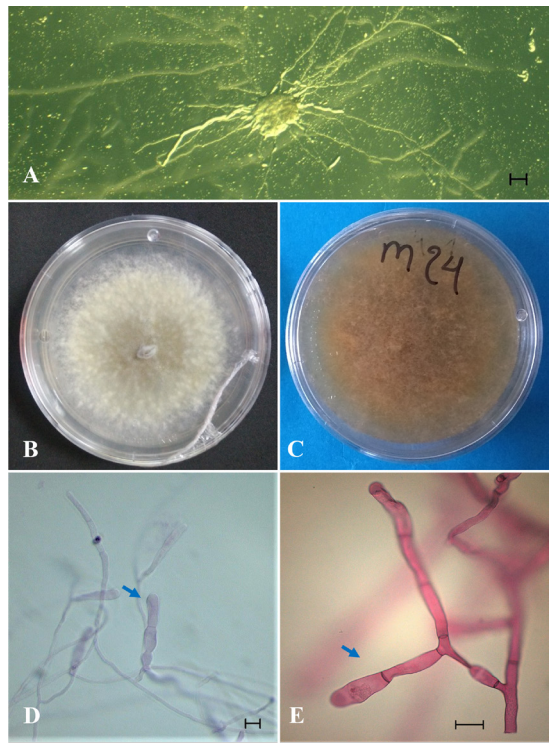

FIGURE 3. Mycorrhizal fungal isolates from *Cypripedium irapeanum*. **A**. Hyphal coil regrowing on green pea agar (GPA) medium (Bar = 100 µm). **B**. Aspect of a *Epulorhiza* colony on GPA. **C**. Aspect of a colony of *Ceratorhiza* isolated from *C. molle*. **D**. Monilioid cells (blue arrows) from *Epulorhiza* **E**. Monilioid cells from *Ceratorhiza* (Bar = 10 µm). Photographs by M. Moreno-Camarena.

were washed three times in sterile distilled water using a vacuum system to remove any residual medium. Extraction was performed with commercial kit RED Extract-N-Amp Plant PCR-(SIGMA®), following the manufacturer's recommendations.

PCR was performed in a thermocycler T100™ Thermal Cycler (BIORAD) with the primer combination ITS 1/ITS 4 and ITS1-ITS4 Tul (Jacquemyn *et al*., 2012; Shefferson *et al*., 2005; Shimura *et al*., 2009; Valdés *et al*., 2011). PCR mix contained 5.0 µL of Taq polymerase (Taq-&GO™ mastermix, MP Biomedicals), 0.25 µL of each primer, 3.0 µL of DNA (1:10 v:v), and 16.5 µL of deionized water for a final volume of 25.0 µL. PCR conditions consisted of a pre denaturation at 95 °C for 120 s, 35 cycles of denaturation at 95 °C for 60 s, annealing at 55 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 600 s (Gardes & Bruns, 1993; Shefferson *et al*., 2005; 2007). PCR products were verified in an electrophoresis gel at 2% stained with Midori Green. Successfully amplified samples were sequenced in LANABIO (Laboratorio Nacional de Biodiversidad, Biology Institute, UNAM).

Sequences were assembled and edited in Geneious Prime and deposited in GenBank under accession numbers (ON620090- ON620092). BLAST search (Basic Local Alignment Search Tool, blast, Max E-Value:10, Word size: 11, Gap cost: 5 2) (Altschul *et al*., 1997), MAFFT alignment (Katoh *et al*., 2002; Ka-

toh & Standley, 2013) and phylogenetic reconstruction (maximum likelihood) were performed with PHYML (substitution model Tamura-Nei TN93, 1000 bootstrap for node support) (Guindon *et al*., 2010), using plugins included on Geneious. *Multiclavula corynoides* (Peck) R.H.Petersen and *Scleroderma* Pers. (Accession numbers MCU66440 and HM196776, respectively) were used as outgroups (Yuan *et al*., 2010). An additional analysis was performed using representative sequences of OMF from different species of *Cypripedium* including *C. irapeanum* from previous reports (Valdés *et al*., 2011) and *C. molle* (Moreno-Camarena & Ortega-Larrocea, 2022). The same parameters for phylogenetic reconstruction were applied, with MCU66440 used as the outgroup.

Asymbiotic and symbiotic germination.— For *in vitro* germination assays, 0.5 mg of mature seeds were stored in envelopes of filter paper Whatman No. 1 (Seaton & Ramsay, 2005) in darkness for four months at 4 °C. After this time, seeds were disinfected by agitation for 30 min in a solution of $NaOCl:C_2H_6O:H_2O$ (15:5:80 v:v:v) plus 2 drops of Tween 20; envelopes were washed three times in sterile distilled water and incubated on Phytamax medium (modified) (SIGMA 1990), MS (Murashige & Skoog, 1962) and Norstog (Norstog, 1970). Symbiotic germination was conducted using two OMF isolates from adult plants of *C. irapeanum* population growing in a TDF and *Quercus* forest; fungi and seeds were sown simultaneously on Oatmeal Agar (OMA) (Clements *et al.,* 1986). Fungi were inoculated by placing a circle of 0.5 cm^3 of each inoculum; a negative control of OMA without fungi was also prepared. All Petri dishes were incubated in a light/darkness photoperiod (16/8) at 25 ± 1 °C and light intensity of 50 μmol m^{-2} s^{-1}.

Photographic records were taken every 12 days after sowing (das) at the following time points: T1:12, T2:24, T3:36, T4:48, T5:60, T6:72, T7:84, T8:96 and T9:108 das. A final account was done at 256 das to evaluate development. Morphogenesis was recorded according to developmental stages proposed by Stewart and Zettler (2002), with modifications due germination behavior of *Cypripedium* (Curtis, 1943). The stages were defined as follows: Stage 1—mature seeds; Stage 2—imbibed seeds; Stage 3—protocorm polarization; Stage 4—protocorm development with one leaf primordium; Stage 5—development of radicular meristem (pre-seedling); and Stage 6—seedling with developed roots and leaves (Fig. 4Q). ANOVA was applied to evaluate differences in germination between treatments. For statistical analysis, Stage 1 was not considered. A Least Significance Difference (LSD) was calculated using Statistica software (Ver. 7.0) (Stat Soft, 2004). Developed plantlets were cultivated in flasks containing the same germination medium to stimulate growth.

Results. *Seed collection.*— Collection of good quality seeds of this species was difficult to obtain because of the asynchronous formation of mature capsules, which were successfuly collected from one site in Puebla. In the State of Mexico, flowering was reduced due to harvesting pressure by local villagers, while populations in Veracruz failed to produce capsules with viable seeds. In all cases, several indehiscent capsules were infested with parasites (Fig. 2D).

Root colonization.— Primary roots were longer than 50 cm, and mycorrhizal colonization was scarce and heterogeneous along the length; the hyphal coils that were observed were normally digested and surrounded by numerous starch granules. Secondary roots were short (*ca.* 2–3 cm), thin (*ca.* 1 mm), and many had high levels of mycorrhizal colonization along the length. Hyphal coils were mostly undigested. Plantlets had numerous roots, and their colonization pattern was similar to that of secondary roots (Fig. 1F, I).

Fungal isolation and identity.— We isolated 33 strains from *C. irapeanum* from *Quercus* forest and TDF (Table 1). All fungi recovered from *C. irapeanum* belonged to the anamorphic genus *Epulorhiza* R.T.Moore (hyphae less than 4 μm, pearly monilioid cells, creamy submerged colonies on PDA, slow growing rate *ca.* 0.2 mm per day) (Fig. 3A–C). In contrast, those recovered from *C. molle* roots belonged to the anamorph genus *Ceratorhiza* R.T.Moore (hyphae of more than 4 μm, barrel-shaped monilioid cells, brownish colonies on PDA, aerial mycelium, growing rate of 0.5 mm per day). In all cases, monilioid cells were long and irregular (Fig. 3D, E). The best media for isolation and conservation of these fungi were GPA and AWA. Colonies maintained on PDA, or OMA exhibited a scarce growth rate and failed to survive over the long term.

Table 1. Description of orchid mycorrhizal isolates obtained from *Cypripedium irapeanum* (Mexican lady's slipper) and incubated on PDA medium (Potato Dextrose Agar). Values are presented as mean ± standard error (SE).

Habitat	Growth by day (cm)	Basal septa of hyphae (μm)	Diameter of hyphae (μm)	Monilioid cells (μm)	
				width	length
TDF	1.14 ± 0.22 a	1.79 ± 1.28 a	3.3 ± 1.1 a	7.7 ± 2.0 a	16.1 ± 4.5 a
QF	0.44 ± 0.25 b	1.44 ± 0.38 a	2.7 ± 0.4 a	7.8 ± 1.2 a	18.5 ± 4.2 a

Habitat: TDF-Tropical deciduous forest, QF-*Quercus forest.* Different letters represent statistical significance within a column.

Phylogenetic analysis (Fig. 4, green clade) revealed that isolates from *C. irapeanum* grouped into a clade comprising two sequences that belong to a clade of Tulasnellaceae associated with sequences recovered from *Vanilla planifolia* Andrews from Veracruz, Mexico (PQ423669) (Alejo-Viderique *et al*., 2025); *V. poitaei* Rchb.f. from Puerto Rico (DQ834391) (Porras-Alfaro & Bayman, 2007) and *Spathoglottis plicata* Ridl. from Singapore (AJ313456) (Ma *et al*., 2003). All reference sequences were obtained from roots growing in soil. The closest related fungi matched with uncultured endophytes from *Cypripedium parviflorum* Salisb. (DQ925544) (Shefferson *et al.*, 2007) and a sister OMF clade from *Paphiopedilum armeniacum* S.C.Chen & F.Y.Liu (Yuan *et al*. 2010) followed by more distant clades in Tulasnellaceae. An analysis incorporating OMF from other *Cypripedium* species (Fig. S1) revealed that isolates of this study belong to one of two distinct clades, associated with *C. candidum* Muhl. ex Willd. and *C. parviflorum* from Illinois, USA (DQ925539 and DQ925543). One OMF isolate from *C. irapeanum* in Puebla and State of Mexico (JF313324) (Valdés *et al*. 2011) is distantly related to the rest of OMF *Cypripedium* from Asiatic and North American species.

In the case of isolates from *C. molle* (Fig. 4, red clade), the sequences belonged to Ceratobasidiaceae, a group in which clades are less well supported. The closest related sequences where those from *Cephalanthera rubra* Rich. (Bell *et al*., 2020), *Thanatephorus* Donk associated with *V. aphylla* Blume from soil mycelium in Cuba (Porras-Alfaro & Bayman, 2007) and one isolate from *Cremastra variabilis* from Japan, all terrestrial species. When OMF from other slipper orchids were analyzed (Fig. S2), two well-supported clades were recovered: one formed by OMF associated with *Cypripedium tibeticum* King ex Rolfe, and *C. flavum* P.F.Hunt & Summerh and another within a clade including Asian species (*C. guttatum* Sw.*, C. subtropicum* S.C.Chen & K.Y.Lang*, C. plectrochilum* Franch) to which the *C. molle* isolate is related. The isolate recovered from *C. irapeanum* in Puebla by Valdés *et al.* (2011) also grouped separately.

Asymbiotic and symbiotic germination.— Successful germination occurred only on asymbiotic Norstog medium (Fig. 5) and was very low on MS and Commercial Phytamax™, reaching only protocorm polarization with no further development and eventual tissue oxidation (Fig. 5D). Conversely, polarization of protocorm and morphogenesis were observed only on Norstog medium and symbiotic-assisted germination. In general, seeds germinated 12–14 das, starting with embryo swelling and testa rupture, followed by protocorm formation and differentiation on a promeristematic zone (Fig. 5J). Morphogenesis continued until root and foliar meristems developed, with no differences in timing between symbiotic or asymbiotic treatments. Protocorms developed rhizoids only in symbiotic assays (Fig. 6D). Color varied from faintly green in symbiotic treatments to intense green on asymbiotic Norstog medium; additionally, protocorms on Norstog developed long roots and several foliar meristems (Fig. 5N–O). In symbiotic germination, although both isolates belong to the same molecular clade (Fig. 4), the isolate from oak-forest promoted 12% of germination, while the isolate from TDF reached only 0.6 % but promoted better development (Fig. 6C–E, Fig. 7). Unfortunately, neither promoted development beyond Stage 4 with a leaf primordium due to long-term incompatibility, expressed as invasion of the meristematic tissues of the protocorms (Fig. 5F–G). On asymbiotic Norstog medium, development continued until seedlings with multiple lateral shoots and long root development were obtained. Further culture in flasks with the same me-

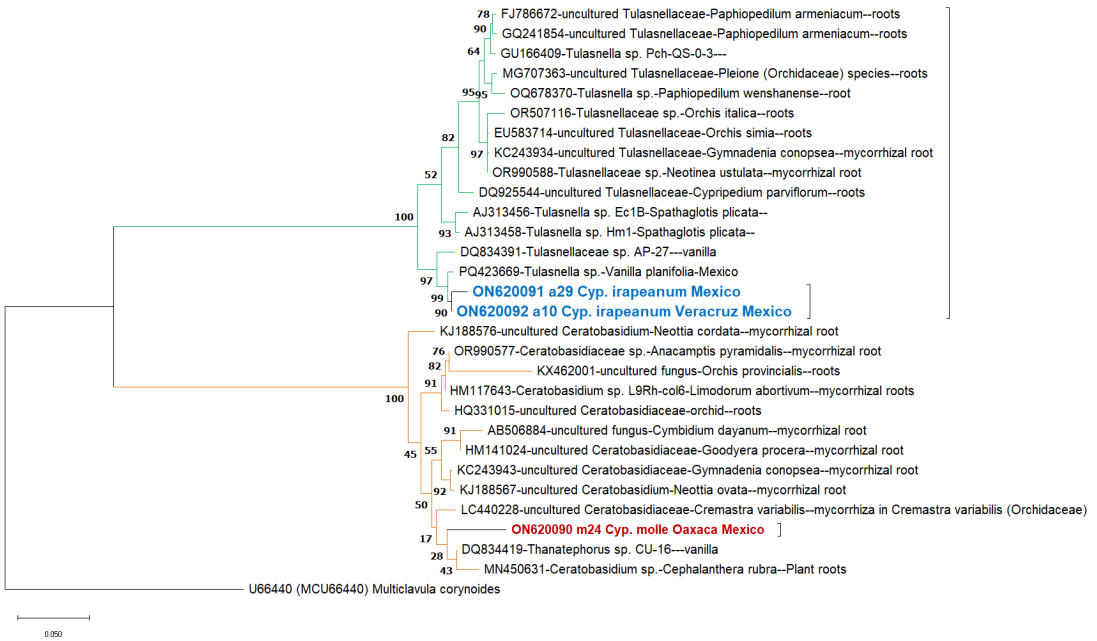

Figure 4. Consensus tree of phylogenetic relationships of *C. irapeanum* and *C. molle* (bold, italic, red labels) recovered by BLAST algorithm (Altschul *et al*. 1997). The green clade includes the isolates recovered in this study from *Quercus* forest and Tropical Dry Forest. Tree inferred using the Maximum Likelihood method and the Tamura-Nei 93 model. Branch lengths labels represent bootstrap support. Analyses and editions were performed in Geneious.

dium resulted in plantlets with several well-developed stems and leaves; however, plantlets died before *ex vitro* acclimatization (Fig. 5P–Q).

Discussion. Mycorrhizal colonization in *C. irapeanum* shows the same patterns previously described (Colin Rivera & Ortega-Larrocea, 2012; Moreno-Camarena & Ortega-Larrocea, 2022) and observed in other species of the genus, characterized as scarce, erratic, and sometimes rich in starch granules (Shefferson *et al*., 2007; 2019). Secondary roots—particularly in plantlets—are densely colonized by mostly undigested hyphal coils, likely indicating a strong mycorrhizal dependency at these stages (Rasmussen *et al*., 2015; Rasmussen & Pedersen, 2012).

However, the presence of mycorrhizal-compatible fungi in soil does not guarantee the successful recruitment of new individuals into a population, even though seed production is not low by orchid standards (*ca.* 100,000 seeds). *In situ* germination remains extremely low—estimated at around 0.001% (Hernández-Apolinar *et al*., 2012)— and is also remarkably slow. In a previous study, we reported a germination event after ten years of sowing seed baits (Moreno-Camarena & Ortega Larrocea, 2022). Low recruitment can be due to several seed-related factors, such as the presence of polyphenols and lignin in the coat—which make it highly hydrophobic —and the presence of abscisic acid— which inhibits seed development, increases as seeds reach maturity (Barsberg *et al*., 2013; Lee *et al*., 2005, Zeng *et al*., 2014). Additionally, compatibility issues with symbiotic partners also contribute to low recruitment, resulting in less than 1% of *in vitro* germination, as also observed by Shimura & Koda (2005).

This loss of symbiotic compatibility appears as browning and necrosis in protocorms and seedlings of *C. macranthos* var. *rebunense* (Kudô) Miyabe & Kudô (Shimura & Koda, 2005). In the case of *C. irapeanum,* incompatibility may arise because mycorrhizal fungi were isolated from adult plants that may not be responsible for germination, as the range of fungal associates changes throughout orchid's life cycle (Bidartondo & Read, 2008; Meng *et al*., 2019, Rasmussen *et al*., 2015). Shimura & Koda (2005) reported that protocorms develop numerous rhizoids, indicating that mycorrhizal colonization

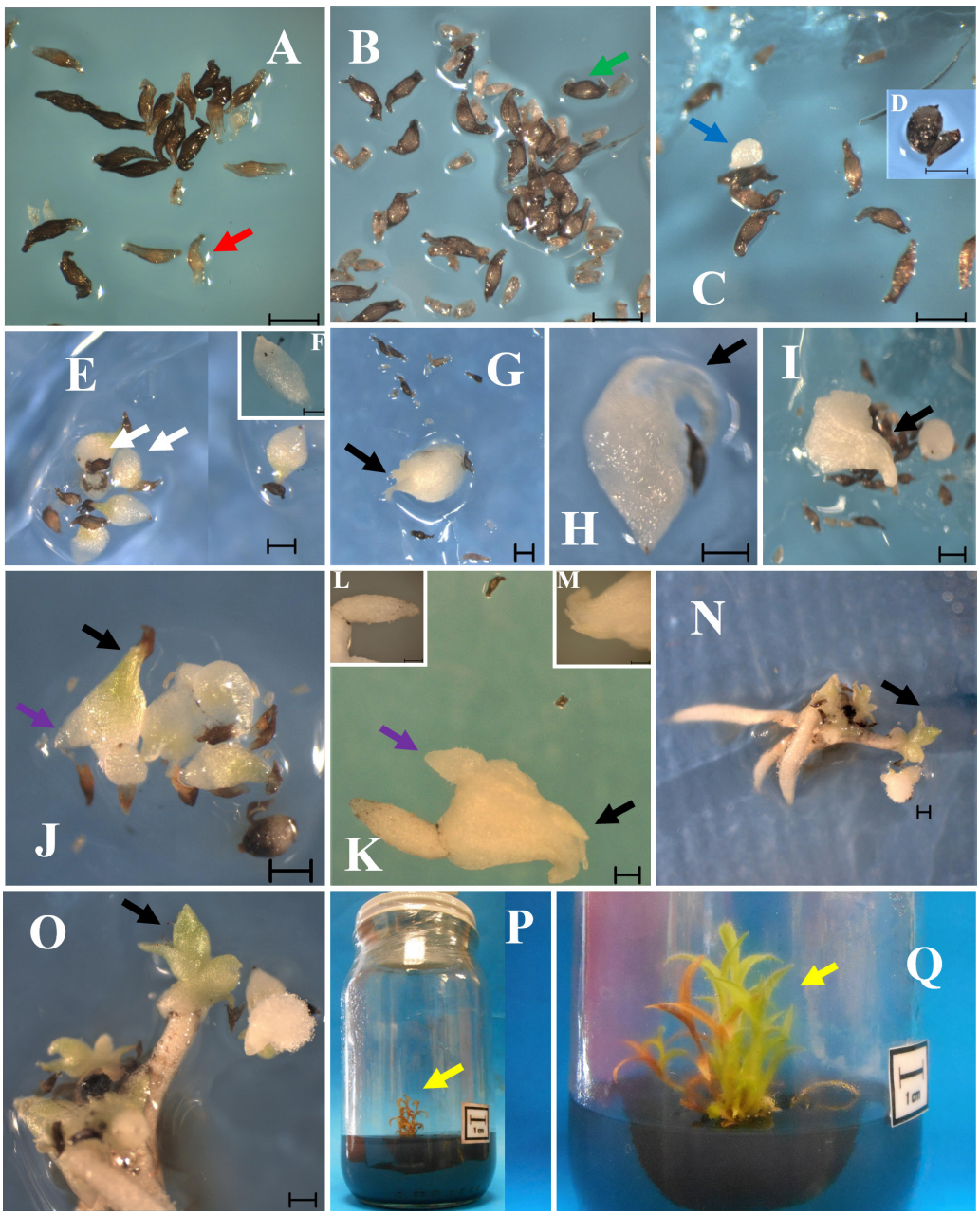

FIGURE 5. Asymbiotic germination on *C. irapeanum* in Norstog medium. **A**. Seeds at Stage 1, red arrow signals immature seeds, 0 days after sowing (das). **B**. Seed at stage 2 (imbibed) and **C** protocorms at stage 3 (polarization), 12 das. **D**. Protocorm oxidation at stage 3 in MS medium. **E**. Protocorms at stage 4 with leaf primordium at 54 das. **F**. Protocorm at stage 3 in Phytamax media 54 das with no photosynthetic activity. **G-I**. Protocorms at stage 4 at 96 das with different degree of development of leaf primordium (black arrow). **J**. Protocorm at stage 5 with leaf primordium and promeristematic zone (purple arrow) at 96 das. **K**. Seedling with developed leaf primordium (m, black arrow) and developed true roots (l, purple arrow), notice root hairs in **L**, and apical leaf in **M,** 122 das. **N-O**. Seedling with different degrees of development, 300 das. **P**. Seedlings after subculture on Norstog medium with added activated charcoal after 280 das. Bar from a-o represent 500 µm. **Q**. Same seedlings with first sprouting of leaves after 300 das. Photographs by M. Moreno-Camarena.

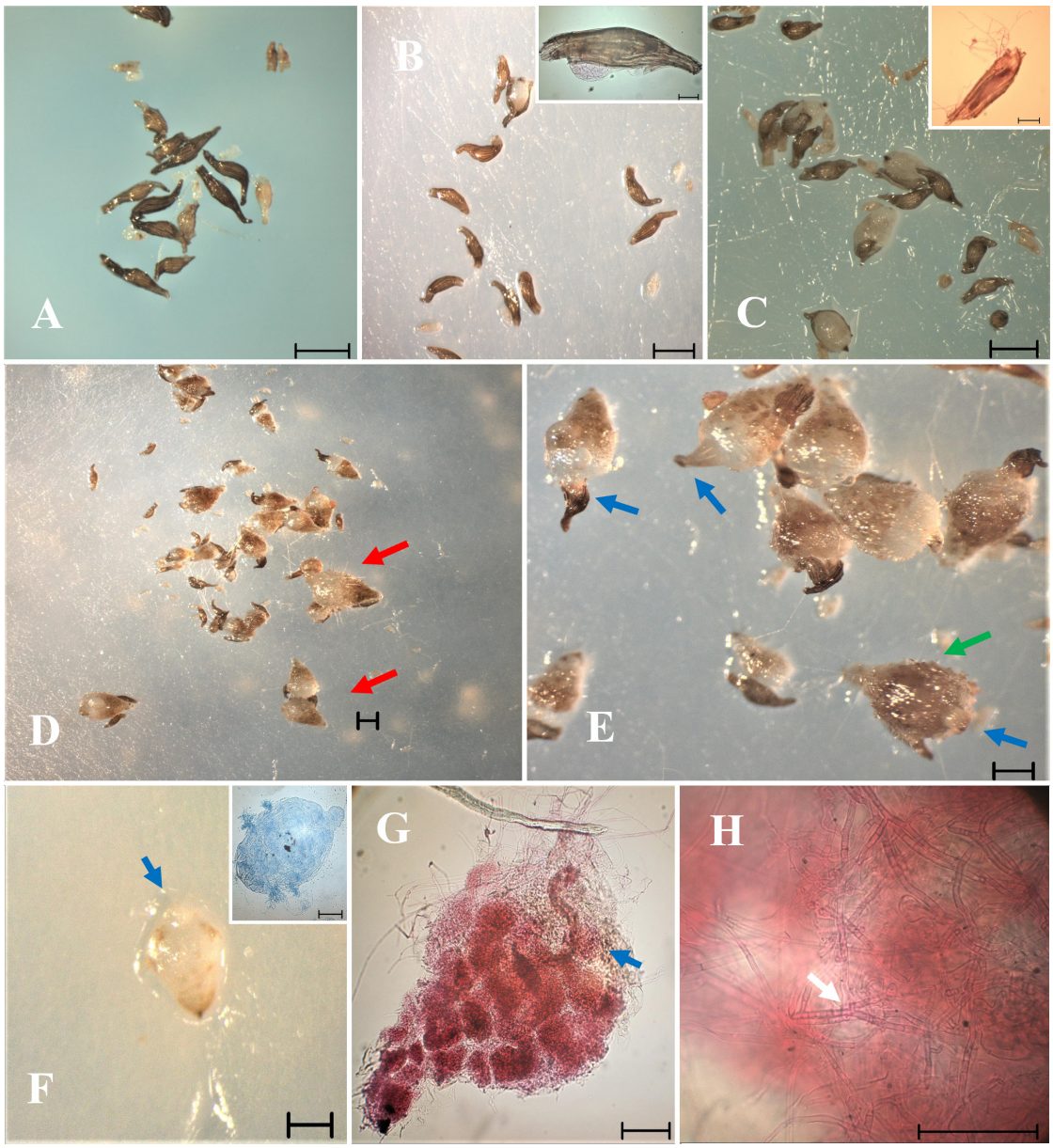

Figure 6. Symbiotic germination on *Cypripedium irapeanum*. **A**. Seeds at Stage 1, 0 days after sowing (das). **B**. Seed at stage 2 (imbibed) and 3, 12 das, detail (Bar = 100 µm). **C**. Protocorms at stage 3 (polarization), 22 das. **D-E**. Protocorms at stage 4 (red arrow) with leaf primordium (blue arrow) and colonized area (green arrow), 54 das. **F**. Protocorm at stage 4 at 109 das, detail to observe hyphal coils in base of protocorm. **G**. Protocorm at stage 4 at 187 das with leaf meristem (blue arrow) and hyphal coils colonizing it. Bar in A-G represents 500 µm. **H**. Detail of basal segment of protocorm, note hyphae branched *ca.* 90° (white arrow). Bar = 50 µm. Photographs by M. Moreno-Camarena.

begins via rhizoids; however, field observations and *in vitro* tests on *C. irapeanum* and *C. calceolus* L. (Rasmussen & Pedersen 2012, Moreno-Camarena & Ortega Larrocea, 2022) suggest that colonization starts from micropylar end cells before rhizoid formation. In fact, our findings illustrate that rhizoid development is a consequence of symbiotic colonization, occurring only after the association has been

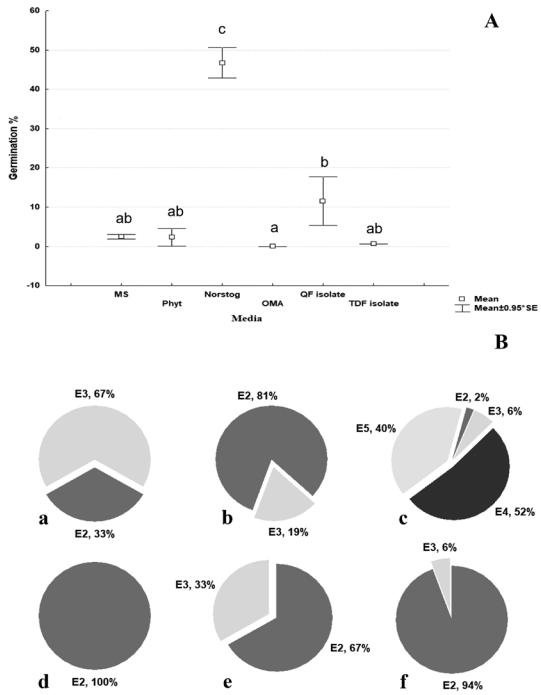

FIGURE 7. **A**. Germination percentage of *Cypripedium irapeanum* on different media. Letter indicates significative differences (LSD at 0.05). **B**. Developmental stages reached in different asymbiotic (a-d) and symbiotic (e-f) media: a) Murashige & Skoog; b) Phytamax; c) Norstog; d) Oat Meal Agar; e) *Quercus* Forest isolate; f) Tropical Dry Forest isolate.

established. Plantlets may possess the ability to control fungal growth within their tissues by phytoalexins in response to changing environmental conditions, such as nutrient and moisture availability, or other physical factors. For example, when *C. macranthos* var. *rebunense* was cultured on a medium with fungicide (Benomyl), it continued to grow and produced healthy plantlets (Shimura & Koda 2005). In further studies, two antifungal compounds—lusianthrin and chrysin— were found in the cytoplasm of orchid cells, both capable of inhibiting fungal growth (Shimura *et al*., 2007). Orchid mycorrhizal fungi appear to be a life-and-death struggle regulated by numerous factors, like seed composition and the fungi's degradation capacity (Barsberg *et al*. 2013; Zeng *et al.*, 2014), environmental conditions such as humidity and temperature (De Pauw & Remphrey, 1993, Shimura & Koda, 2005), the light/darkness regime, and nutrient availability. Loss of compatibility observed in our study may be due to the absence of specific stimuli needed to trigger plantlets to produce natural antifungal compounds, or to inherent *in vitro* conditions, as we did not evaluate complex fungal media or substrates that could help regulate fungal growth. *Epulorhiza* grow slowly and do not exhibit invasive growth over protocorms; instead, incompatibility occurring within the tissues. Further research is needed using different substrates and media, as well as with mycorrhizal isolates obtained from naturally occurring seedlings—even when recruitment is rare in the wild.

Undeniably, *in vitro* germination is consistently more successful than *in situ* germination, because many factors are stable. *In vitro* conditions provide seeds with continuous and regulated humidity for imbibition, readily available nutrients, and the possibility of scarifying or treat seeds to break dormancy. Dormancy-breaking in this genus has been linked to cold treatment when seeds come from temperate habitats (Rasmussen & Pedersen, 2012; Shimura & Koda, 2004; 2005), allowing for successful colonization by OMF. Methods to break dormancy can be physical, chemical or biological (*e.g.* sonication, solution of NaOCl or with mycorrhizal fungi, respectively), as long as they diminish the effect of inhibitors of germination (*e.g.* ABA, hydrophobic testa).

In situ germination requires several successive cold treatments, with germination rates increasing to 30% after the second period (Pedersen *et al*., 2012). This suggests that germination in this genus is not only extremely low but also likely spans several years in order to progress through each life stage (Curtis, 1943; Moreno-Camarena & Ortega-Larrocea, 2022, Pedersen *et al*., 2012; Rasmussen & Pedersen, 2012, Shefferson *et al*., Hutchings 2017). Optimum germination rates vary amongst species; and depend on the relationship between embryo development and barriers of the seed (formation of testa, chemical inhibitors, etc.); the days after pollination (40–105 days), and the environmental conditions of the species (with subtropical species requiring the longest periods) and may follow a possible phylogenetic gradient in germination requirements (Perner *et al*., 2022). Light or dark conditions requirements for incubation seem not constant in the genus, also suggesting an evolutionary tendency (Kaur, 2023;

Park *et al.,* 2023). Mesoamerican *C. irapeanum* follows this pattern: *in vitro* germination is accelerated by cold treatment (*ca.* 12 das), even though it grows in temperate natural habitats where prolonged cold dormancy periods in the soil are not assumed (Moreno-Camarena & Ortega Larrocea, 2022). On the other hand, nutrient medium preferences for this genus were described long ago by Harvais and Norstog (Oliva & Arditti, 1981) and confirmed recently (Kaur, 2023). Media with high phosphate and sugar contents such as MS and Phytamax (Murashige & Skoog, 1962; Seaton *et al*., 2005, Sigma-Aldrich, 1990), traditionally used for orchid germination, do not favor *C. irapeanum* germination nor *C. macranthos* Sw. (Huh *et al.,* 2016a, b). It remains unclear whether this effect may be dependent on seed origin, storage conditions, or seed maturity, since germination on MS medium has previously reached about 25 % (Hernández-Apolinar *et al*., 2012). However, in *C. acaule* Aiton, *C. formosanum* Hayata, and *C. macranthos* germination on media such as MS, Thomale, and VWD (Van Waes and Debergh) decreases as seed maturity increases, becoming null at full maturity (Zeng *et al*., 2012; 2014, Zhang *et al*., 2013). Conversely, germination of mature *C. debile* Rchb.f. seeds reaches up to 80 % in liquid MS medium, as the liquid environment may physically reduce resistance of the seed coat. Several studies use immature seeds (40-90 days after pollination-dap) to avoid dormancy and prevent seed coat impermeability, achieving considerably high germination rates on MS and Harvais— 56 and 25 % respectively— (Taniguchi *et al*., 2008; Zhang *et al.,* 2013). Root system formation may predate shoot leaves, varying amongst species but appears to be the predominant pattern in the genus (Kaur, 2023). The effects are often not predictable among species, population and even the year of experimentation (Hsu & Lee, 2012; Jiang *et al*., 2017).

Although these protocols do not reflect natural conditions, they may serve as a strategy to obtain seedlings that are more susceptible to inoculation by OMF, since adult stages likely have a greater ability to regulate the orchid-fungi relationship effectively (Fay *et al.,* 2018; Shimura *et al.,* 2007; Shimura & Koda, 2004; Yuan *et al*., 2010). Additionally, conventional media do not support the maintenance and growth of fungal strains well. Preferences for OMF media seem quite specific; for example, pea starch appears to be a more suitable energy source. Nevertheless, further studies are needed to determine whether this represents a limitation for isolating and culturing Tulasnellaceae fungi (Shimura *et al.,* 2009). Although OMF from European, Asian, and North American *Cypripedium* have been extensively studied (Shefferson *et al*., 2005; 2007; Yuan *et al*., 2010), attempts at isolation have only been reported for *C. macranthos* var. *rebunense* (Shimura & Koda 2005) and *C. irapeanum* (Valdés *et al*. 2011). In this study, we obtained several strains from *C. irapeanum* belonging to *Epulorhiza* species, which supports Shefferson *et al*.'s (2019) proposition of dominance by *Tulasnella cystidiophora* Höhn. & Litsch. associates within the genus. We found that OMF from *C. irapeanum* (Tulasnellaceae) and *C. molle* (Ceratobasidiaceae) are not closely related to clades recovered from other lady's slipper orchids, with the exception of a clade from *C. parviflorum.* Moreover, previously reported isolates of *C. irapeanum* from another locality in the state of Puebla belong to a different Tulasnellaceae clade than those found in our study. Ceratobasidiaceae has also been reported in association with *C. californicum* A.Gray and *C. fasciculatum* Kellogg ex S.Watson (Shefferson *et al*., 2005; 2019; Whitridge, 2004; Whitridge & Southworth, 2005); but symbiotic germination tests have not yet been performed. Such tests could provide valuable insights, especially since isolates obtained from plantlets of *C. molle* clearly differ from those found in adult plants (Moreno-Camarena & Ortega Larrocea, 2022). Isolates obtained from both OMF families are also related to fungi associated with *Vanilla* Mill. species, whose symbiotic germination has also been difficult as their seeds show very similar properties in the waterproofing of the testa by pigments, both families are phylogenetically related as ancestral clades of orchids (Givnish *et al*., 2015).

Final remarks. Mesoamerican *Cypripedium irapeanum* may associate with other clades of Tulasnellaceae mycorrhizal fungi, differring from those previously documented for other species of the genus. As in many *Cypripedium* taxa, in vitro germination proved difficult, likely reflecting nutritional and physiological requirements that differ between subtropical and temperate species. Germination success is shaped by multiple interacting factors, including seed dormancy, culture

medium composition, and fungal compatibility. The challenges in implementing effective propagation and conservation strategies may stem from several constraints: the use of asymbiotic media formulated for other recalcitrant taxa (e.g., cycads), and the specific requirements of fungal growth media, particularly the quality and type of starch used as a carbon source. Our results contribute to the limited body of information on the germination and early development of a Mesoamerican lady's slipper orchids. Despite the loss of compatibility during the process, adult fungal isolates remained capable of colonizing and initiating seed germination. Future work should focus on optimizing the balance between orchid and mycorrhizal fungi to produce robust seedlings suitable for greenhouse cultivation or reintroduction into natural habitats. Increasing the number of isolates and sequenced strains will be essential to determine whether the apparent preference of the Irapeana clade for Tulasnellaceae represents an evolutionary pattern or reflects sampling limitations.

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Supporting Information: Additional material related to this article is available in the online Supporting Information section.

Figure S1. Consensus tree of phylogenetic relationships of *Cypripedium irapeanum* recovered by BLAST algorithm and orchid mycorrhizal fungi (OMF) from other *Cypripedium* species in Tulasnellaceae family. Tree inferred by using the Maximum Likelihood method and the Tamura-Nei 93 model. Branch lengths labels represent bootstrap support. Analyses and editing were done using Geneious.

Figure S2. Consensus tree of phylogenetic relationships of *Cypripedium molle* recovered by BLAST algorithm and Orchid Mycorrhizal Fungi (OMF) from other *Cypripedium* species in Ceratobasidiaceae family. The tree was inferred by using the Maximum Likelihood method and the Tamura-Nei 93 model. Branch labels' length represents bootstrap support. Analyses and editing were performed using Geneious.