

Seed Science Research

Orchid E:S (Embryo-to-Seed) ratio as an indicator of germination behaviour and its ecological implications --Manuscript Draft--

Abstract:	Although the seeds of various Orchidaceae members can be readily germinated on sterile nutrient media, numerous species exhibit complete asymbiotic germination failure. Also, while seed morphology in orchids has been previously linked to dispersal, associations with germinability have not been widely explored. We compiled seed morphometric and germination data for 203 orchid species globally, drawing from international literature and our lab's unpublished findings. Based on (a) final germination percentage and (b) pre-treatment duration, two distinct groups of asymbiotic germination behaviour emerged - termed compliant and defiant, respectively. Additionally, a statistically significant relationship between germinability and the embryo length to seed length ratio (E:S) was observed. E:S values tended to be lower in species with defiant germination and higher in those with compliant germination. We further correlated seed morphometric and germination data with phylogenetically and ecologically significant parameters (subfamily, growth form, mycoheterotrophy level, climatic zone and habitat shadiness). Notably, lower E:S values and defiant germination predominate in orchids of shaded habitats, while compliant germination is more prevalent in open habitats. Lastly, taking the mycoheterotrophy continuum into consideration, we propose that the reduction of the E:S ratio is linked to the adaptation of various orchid lineages to shaded habitats through both an increased dispersal ability and their greater reliance on fungal symbiosis.
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Dear Editor and Reviewer of the Seed Science and Research Journal,

First let us thank you for taking time to review our work entitled: “**Orchid E:S (Embryo-to-Seed) ratio as an indicator of germination behaviour and its ecological implications**”.

Based on your comments and proposed changes that we incorporated along with our minor adjustments across the manuscript, we hope the work will be now suitable for publication

Regarding the Reviewer comments please find our answers below (the initial comment is highlighted in yellow):

However, there is one more general observation or question that I was wondering about. It seems to me that the relation between E:S and ecology of the species that was observed is an indirect relationship rather than a direct one. The authors brought up morphological dormancy, but as far as I can understand from orchids, the airspace has little to do with germination cueing, but as the authors explain, is perhaps more related to limiting dispersal. So, it may happen that both a small embryo and a lot of airspace are adaptations to the forest habitat, but not necessarily a direct explanation for low germination. As is the case for seeds with copious endosperm and small embryo. Therefore, I do wonder to which extent relative embryo length reflects absolute embryo length, where the latter seems to be a more accurate representation of embryo development and nutrient storage at dispersal and perhaps more directly linked to final germination. I would be curious to read the authors' reflections on this consideration.

Regarding the observation/question of the reviewer we want to point out a few parts of the work and also add some information that it is not currently presented which may clarify our reasoning; we also added several sentences across the discussion to further clarify our work:

- Firstly, in our work, we observe a statistical correlation between the ratio E:S and the germination behaviour of orchid seeds under asymbiotic conditions. Further investigation of the life and habitat traits of the species studied are used in an attempt to propose a hypothesis regarding our observed correlation.
- On the basis of our results, we conclude in hypothesising, that the present form of orchid seeds (with a variable E:S ratio) is the result of the evolution of mycoheterotrophy or the coevolution of dispersal ability and mycoheterotrophy. As it was pointed out by the reviewer, smaller E:S (relatively bigger air space) is a potential adaptation to the forest habitat and increased dependence on fungi, or in other words, lower E:S increases the dispersal ability of the seeds and – at the same time - their chances to find a suitable fungal host.
- We do not suggest that the E:S ratio may constitute a direct explanation of the type (or level) of an orchid species seed germination, but it can be instead an indicator of higher or lower asymbiotic germination (depicted here as compliant or defiant, respectively) which in terms of field seed germination is the result of a lesser or heavier dependence on its fungal symbionts. More specifically, an increased dependence on a fungal symbiont results in significantly harder (lower) germination under asymbiotic conditions, because the requirements for germination are higher both nutritionally (more complex media) and physically (longer scarification durations). And vice versa.

- Taking into account the proposed reduction of size for seeds and, as a result, of embryos in mycoheterotrophic families proposed by Leake (1994), and based on our observations of the relative dimensions (E:S), we speculate that a secondary reduction of the E:S ratio (driven by both dispersability and mycoheterotrophy) came about in the family Orchidaceae during the transitions of various lineages towards the heterotrophic end across the autotrophy \leftrightarrow full mycoheterotrophy continuum.
- Regarding absolute dimensions (length of seed, length of embryo): we did initially investigate whether there is any correlation of either of these to germination behaviour but no such relationship was found (L395-400). Taking the peculiar development of orchid embryo (globular embryo of a few hundred cells) into consideration along with the lack of any, meaningful, stored nutrient for the support of the protocorm until the photosynthetic stage, the absolute dimensions of embryos seem to have indeed little value to explain the germination behaviour in orchids.

L59-60 It is an old plant family, but 'one of the oldest angiosperm families' is perhaps somewhat of an overstatement.

L61-63 changed to: Evolutionarily, it stands as one old angiosperm family with an origin estimated at around 100-120 mya.

L97-98 'has thus far proven unsuccessful' but the reference dates from 30 years ago. Can you find a more recent reference?

L100-102 Along with the work of Rasmussen we have added two more recent works supporting the observation, in compliance to the reviewer comment. The use of the work of Rasmussen here is made due to the sheer volume of data presented both in different germination tests and number of species regarding terrestrial orchid germination throughout the book. Although a reference from 30 years ago, it can still be considered state of the art for the germination of terrestrial orchids. For the specific example mentioned (e.g. *Cephalanthera* spp.) the asymbiotic germination is still considered, in most species, unsuccessful while a few, more recent, cases of successful germination highlight cases such as the germination of immature seeds while germination of mature seeds remain unsuccessful.

L131 It is not clear to me what 'relative' refers to here. I would have thought that the small absolute size is characteristic for mycoheterotrophs

L136-137 The term relative in this position is indeed misleading and has been removed. It was used having in mind cases of families (e.g. Campanulaceae) which may have tiny seeds but they are not mycoheterotrophic. The lines 132-133 have been adjusted appropriately.

L140-144 In my opinion, low E:S ratio has nothing to do with morphological dormancy in orchids, as morphological dormancy refers to the fact that the embryo grows inside the seed prior to germination (thereby consuming the endosperm). Of course, it depends on how you define morphological dormancy, but to me this part can be skipped to avoid confusion.

We understand that defining dormancy is, in general, a challenge. In our work we use the definitions as proposed in the seminal works of Baskin & Baskin (usually referring to orchids seeds as having morphological or morphophysiological dormancy); a similar use is also made

in the recent works regarding orchids of Prasongsom et al. (2016 and 2022) which have comparable observations to our work. However, and in order to avoid any misunderstanding, modifications were made throughout the text by including the adjectif *sensu lato* in the usage of these two dormancy classes.

L153 Why not formulate the hypotheses here, prior to the study...

The general hypothesis was added in the text L160-165: Based on the ecological preferences of the species we suggest that the decrease in embryo size, relative to the overall seed length, is associated with the adaptation of different orchid lineages to shaded environments and their increased dependence on fungal symbiosis.

L188-191 Can you add one or two lines on how embryo and seed length measurements were taken? For example, along the same plane? Were literature measurements from photos or from data given in the text? In terms of open data and repeatability, it would be nice if the raw measurements were made available.

L201-207 describe the data used for the analysis both the literature measurements and the measurements conducted by the author, while minor adjustments were also added to further clarify the data structure. Regarding our dataset, the full data table will be made available in the supplementary materials of the present work which is now submitted along the revised version of the manuscript.

L290-295 How did you decide to categorize species as shade or open habitat species? I can easily imagine that for many species data-points were situated in both open and shaded habitats.

AND

L305-307 Also here, what was the cut-off to decide that a species was either tropical or temperate?

The method for categorizing the species both for the shadiness and the climate zone is described in L277-307 and L309-317. In general, the species were categorized as shaded or open habitat species and also as tropical or temperate based on two factors: 1) firstly the descriptions of their distribution and habitat in the literature, from which the data for their germination and seed morphology was also gathered, and 2) by using data points for the species in the dataset from GBIF for extracting their occurrences in climate zones and also the level of shade in their habitats (values extracted for the canopy cover dataset for the year 2000), due to lack of occurrence points or points with low accuracy this method was primarily used to validate the references from the literature for 127/203 species with available occurrence data. Regarding the habitat shadiness, as mentioned by the reviewer, not all data-points were situated in shaded locations and thus we used as a threshold the 30% canopy coverage with species occupying habitats with shadiness above this 30% (medium shadiness to deep shade) being categorized as shaded habitat species. The 30% was chosen based on other works on shadiness of habitats.

L298 Brackets are missing.

L317 Brackets added.

L317 Why exactly did you select these trait combinations to determine TI? Some combinations will give the same TI. Can you explain the rationale in the manuscript?

Adjustments were made throughout L338-357 to further clarify the reasoning of the TI and its values. The TI is an attempt to place all different factors on a single linear axis.

L336 The Euclidean distance of what?

No change was implemented for the current comment. Fig 5 was removed from the main text based on a comment from the reviewer and will be submitted as supplementary material in its initial (less complicated) form.

L376 and throughout the results section, is it possible to provide confidence intervals around the means? Also, medians are preferred over means for non-parametric tests, as they are based on ranks.

Based on the reviewer suggestion, we change throughout the text the values mentioned from mean values to median values along with their confidence intervals. We do keep the mean values in figs. 1 and 2, as both mean and median hold significant informative value.

L377 I assume they have 'relatively' less free airspace

L399 the suggested change was added: Thus, the seeds of the latter have relatively less free air space

L422-426 This section can go in the methods or discussion section.

The section was transferred to Material and Methods

L427-431 Fig 5. This figure is quite confusing. Maybe some annotations can be made on the figure to explain. On the other hand, it contributes relatively little to the results that are already evident and can be left out.

Based on the reviewer suggestion we removed the Fig. 5 from the main manuscript and we submit the initial, a less confusing, version of it as supplementary material. Several minor changes were also implemented throughout the text to accommodate for this removal (change of fig. numbering, removal of relative text to the original Fig. 5).

L510-513 Also here I don't see why a reference is made to morphological or morphophysiological dormancy. These seeds critically rely on their symbiont for germination, as outlined in the next sentence, hence this mechanism is not at all related to the fact that embryo growth inside the seed prior to germination is required as in morphologically dormant seeds.

As mentioned in a previous answer, we follow the description of orchid seeds as seeds with MD or MPD based on Baskin & Baskin book and the works by Prasongsom. The embryo may not develop further inside the seed prior to germination but the morphological changes occurring during the germination process (stages from rhizoid appearance to a fully formed protocorm) are considered part of the necessary development until dormancy is broken. As mentioned in the work of Baskin & Baskin 2014 for the family Orchidaceae: “indicating that germination is delayed only long enough for morphological development to occur, i.e., seeds have a special kind of morphological dormancy (MD)”.

Lastly, several typos were corrected across the text and mostly in the Reference section. We also added a few more references in important works on orchid biology that further support our work.

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Dear Editor,

The present study reveals a relationship between orchid seed morphometrics and their respective germination behaviour, thus the former can be an indicator of the latter. Furthermore, in the ecological context, seed morphometrics are correlated to several parameters, i.e., growth form, mycoheterotrophy, climatic zone, habitat shadiness and phylogeny (at subfamily level).

While most of the previously published works are focused on the relation of orchid seed morphology and their dispersal, our work attempts to explore the relationship of seed structure to germination behaviour. The correlations identified shed some new light on the ecological and evolutionary history of orchids.

The present study applies a new morphometric index (based on data drawn from the entire international literature) which can effectively predict germination behaviour of orchid species. It also showcases fungal symbiosis as an additional possible driver of the evolution of orchid seed morphology, other than dispersal (reported in previous studies).

On behalf of the authors

Sincerely yours

Spyridon Oikonomidis

1 **Title: Orchid E:S (Embryo-to-Seed) ratio as an indicator of**
2 **germination behaviour and its ecological implications**

3

4 **Running head title: E:S ratio and germinability of orchid**
5 **seeds**

6

7

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23 Keywords: E:S ratio; Germination behaviour; Habitat;
24 Mycoheterotrophy; Orchidaceae; Shadiness

25

26 **Abstract**

27 Although the seeds of various Orchidaceae members can be
28 readily germinated on sterile nutrient media, numerous species
29 exhibit complete asymbiotic germination failure. Also, while
30 seed morphology in orchids has been previously linked to
31 dispersal, associations with germinability have not been widely
32 explored. We compiled seed morphometric and germination
33 data for 203 orchid species globally, drawing from international
34 literature and our lab's unpublished findings. Based on (a) final
35 germination percentage and (b) pre-treatment duration, two
36 distinct groups of asymbiotic germination behaviour ~~groups~~
37 emerged - termed compliant and defiant, respectively.
38 Additionally, a statistically significant relationship between
39 germinability and the embryo length to seed length ratio (E:S)
40 was observed. E:S values tended to be lower in species with
41 defiant germination and higher in those with compliant
42 germination. We further correlated seed morphometric and
43 germination data with phylogenetically and ecologically
44 significant parameters (subfamily, growth form,
45 mycoheterotrophy level, climatic zone and habitat shadiness).
46 Notably, lower E:S values and defiant germination predominate
47 in orchids of shaded habitats, while compliant germination is
48 more prevalent in open habitats. Lastly, ~~building-ontaking~~ the
49 mycoheterotrophy continuum into consideration, we propose
50 that the reduction of the E:S ratio in embryo size (as a
51 proportion of the entire seed length) is linked to the adaptation
52 of various orchid lineages to shaded habitats through both an
53 increased dispersal ability and their ~~increased~~greater reliance
54 on fungal symbiosis.

55

56

57 **Introduction**

58 Orchidaceae is one of the most diverse flowering plant families
59 globally, boasting approximately 750 genera and an estimated
60 26,470 to 28,237 species (Christenhusz et al., 2017; Govaerts et
61 al., 2017). Evolutionarily, it stands as one ~~of the old~~ est
62 angiosperm families—family with its appearance estimated
63 around 100-120 mya (Givnish et al., 2015). Orchids can be
64 found on almost every continent, with the exception of the
65 poles, and Sahara and Great Victoria deserts (Christenhusz et
66 al., 2017). The orchid family is classified into five subfamilies,
67 with Epidendroideae and Orchidoideae being the largest,
68 comprising 21,100 and 4,965 species, respectively.
69 Vanilloideae, Cyripedioideae, and Apostasioideae are
70 represented by 245, 169, and just 14 species, respectively
71 (Christenhusz et al., 2017).

72 Orchids have captured the interest of scientists and plant
73 enthusiasts for over four centuries, owing to their vast variety
74 in flower morphology and unique life history, such as deceptive
75 pollination and mandatory symbiosis with a fungus for
76 germination under natural conditions (Arditti, 1984; Micheneau
77 et al., 2009; Schiff, 2017).

78 The successful germination and development of orchids in
79 vitro were initially described in the 20th century. Noël Bernard
80 pioneered symbiotic methods in the first decade, followed by
81 Lewis Knudson's introduction of asymbiotic methods in the
82 second decade (Bernard, 1909; Knudson, 1921). Orchid species
83 remain among the most challenging to propagate successfully
84 from seeds. Knudson's experiments in the early 1920s revealed
85 that for the successful asymbiotic seed germination and further
86 development of most orchids, a minimum external carbon
87 source is required. This necessity is attributed to the initial

88 mandatory fully-mycoheterotrophic stage of, presumably, all
89 orchid species in field conditions (Bernard, 1899; Rasmussen,
90 1995; Merckx, 2013).

91 The asymbiotic germination process of orchid species
92 exhibits high heterogeneity in terms of final germination (FG)
93 success. Some species have been reported to readily germinate
94 in distilled water (or water agar) without any carbon source,
95 although their development is halted at the rhizoid stage before
96 reaching the protocorm stage (Rasmussen, 1995; Oikonomidis
97 et al., 2020; Diantina et al., 2020). The majority of orchid
98 species require complex media for successful asymbiotic
99 germination. However, for several, especially terrestrial species
100 (e.g., *Cephalanthera* spp.), asymbiotic germination of mature
101 seeds has been thus so far proven unsuccessful (Rasmussen,
102 1995; Yamazaki and Miyoshi, 2006; Zhang et al., 2020). The
103 intricacy and heterogeneity observed in orchid seed
104 germination in the lab may mirror the complexity of
105 requirements for successful orchid seed germination and
106 seedling establishment in nature, particularly under symbiotic
107 conditions (Rasmussen, 1995; Rasmussen et al., 2015).

108 Orchid seeds, characterised by their microscopic sizes,
109 were not observed until the 16th century. Official references to
110 orchid seeds emerged in the literature in the mid-18th century,
111 notably in the works of Conrad Gessner and Georg Everhard
112 Rumphius (Kull and Arditti, 2002). Orchid seeds typically
113 comprise a spindled-shaped, thin seed coat (testa) that
114 encompasses the underdeveloped embryo. The embryos lack
115 endosperm, cotyledons, and a radicle, although a few
116 exceptions, such as *Bletilla striata*, exhibit rudimentary
117 cotyledons (Arditti, 1967). In certain cases, as in the genera
118 *Cephalanthera* and *Epipactis*, another tight cell layer that
119 encloses the embryo, known as the carapace, is also present
120 (Veyret, 1969; Lee and Yeun 2023). Ranging in size (longest

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121 dimension) between 150 and 6000 μm , with the majority falling
122 between 300 and 800 μm , orchid seeds rank among the smallest
123 in the plant kingdom (Movray and Kores, 1995; Baskin and
124 Baskin, 2014).

125 Orchid seeds exhibit significant variation in terms of
126 relative seed and embryo dimensions, testa cell morphology,
127 and coloration. Numerous orchid seed characteristics have been
128 suggested to hold taxonomic importance for infrageneric
129 identifications (Akçin et al., 2009; Gamarra et al., 2010).
130 Concerning seed and embryo dimensions, it has been proposed
131 that the relative increase of air space inside the seed (with the
132 embryo occupying a relatively smaller volume) is positively
133 correlated with flotation time. This correlation suggests a
134 potential adaptation to anemochory in orchid species, supported
135 by both morphological and phylogenetic data (Diantina et al.,
136 2020; Fan et al., 2020). The smaller ~~relative~~-size of seeds and
137 embryos, ~~accompanied by an and the~~ increased ~~in~~ air space
138 within the seed - a trait common among all mycoheterotrophic
139 plants - ~~is~~ have been considered a 'consequence' (or a
140 'prerequisite') of the transition from an autotrophic to a
141 partially or fully mycoheterotrophic lifestyle (Leake, 1994;
142 Eriksson and Kainulainen, 2011). All these are also prevalent in
143 the Orchidaceae family which, notably, embraces a significant
144 proportion of the fully mycoheterotrophic plants known today,
145 accounting to 215 out of a total of 535 species (Merckx, 2013).

146 In a previous study on the genus *Dendrobium* (Prasongsom et
147 al., 2016, 2022), it was ~~emphasized~~ emphasised that the small
148 size of a seed alone is insufficient for ~~categorizing~~ categorising
149 a species into the sensu lato morphological or
150 morphophysiological dormancy classes, according to Baskin
151 and Baskin (2014) definition. Consistent with these findings,
152 our team has also observed variations in the germination
153 behaviour of certain orchid species in relation to their relative

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154 embryo-to-seed size (Oikonomidis and Thanos, 2020). The
155 present study explores the relationships among seed structure,
156 germination behaviour, phylogeny and four biotic and climatic
157 variables (mycoheterotrophy level, growth form, climatic zone,
158 habitat shadiness). It reveals the correlation between orchid
159 seed architecture and germination behaviour, ~~while also~~
160 ~~formulating a hypothesis grounded in the ecological~~
161 ~~preferences of the species.~~ Based on the ecological preferences
162 of the species we suggest that the decrease in embryo size,
163 relative to the overall seed length, is associated with the
164 adaptation of different orchid lineages to shaded environments
165 and their increased dependence on fungal symbiosis.

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166

167 **Materials and Methods**

168 *The dataset of E:S and FG values*

169 Among the most common data available for orchids are the
170 dimensions of the seeds. In this study the ratio of embryo
171 length and seed length (E:S) is used to investigate the
172 relationship between seed morphology and germination
173 behaviour of 203 orchid species across the globe. Although in
174 previous studies (Arditti and Ghani, 2000; Diantina et al., 2020;
175 Fan et al., 2020) researchers have employed air space (the free
176 space between the embryo and the outer testa of the seed) to
177 investigate seed trait evolution, we have chosen to focus
178 primarily on the study of the E:S. The calculation of air space
179 for orchid seeds is based on the ~~generalization-generalisation~~ of
180 the shape of orchid seeds, and thus, slight mistakes in the
181 values might be common.

182
183 Data for both morphology (primarily dimensions of seeds
184 and embryos) and seed germination (FG, final germination)
185 were collected for a total of 203 species belonging to 71 genera.
186 This information was gathered through a comprehensive

187 bibliographical search, including 174 species, and from the
188 unpublished data of our lab, covering 29 species. The limiting
189 factor for the ultimate size of the dataset was the concurrent
190 availability of morphological data on seeds and germination
191 behaviour for each orchid species. The final dataset, in
192 comparison with the family, exhibits a considerable
193 overrepresentation of Orchidoideae over Epidendroideae (see
194 Table 1). This can be attributed to the cumulative effect of the
195 predominant focus in orchid research on temperate species of
196 the Northern Hemisphere, which are overrepresented by the
197 family Orchidoideae (Wraith et al., 2020). Furthermore, our
198 own research adds to this bias by concentrating on orchids from
199 Greece, where species in the Orchidoideae subfamily
200 predominate. It should be noted here that the E:S ratio was
201 calculated using mean values—of embryo and seed length,
202 measured under a stereomicroscope or a microscope, gathered
203 from the bibliography (tabular data for 174 species) while for
204 each of the 29 species studied by our group, a 50-seed sample
205 was used to calculate the mean E:S ratio values using the
206 Lumenera Infinity-1 camera and software under a
207 stereomicroscope.

208

209 *Compliant and Defiant germination*

210 Based on the final percentage of asymbiotic germination
211 and the intensity of pre-sowing treatments, we propose a binary
212 classification system for orchid seed germination behaviour:
213 compliant and defiant germination. For assessing data, we used
214 the appearance of rhizoids as a criterion for germination,
215 following the developmental classification system of Zettler
216 and Hofer (1998). Owing to the heterogeneity in the literature
217 regarding the precise stage considered for germination in
218 various orchid species, only works reporting the appearance of
219 rhizoids were included in our database. In cases of multiple

220 reports on the same species, we used those with the highest
221 germination values. More specifically, we define the
222 germination behaviour of an orchid species as defiant when
223 either the final asymbiotic germination percentage does not
224 exceed 40% or when a prolonged duration (> 1 h) of chemical
225 treatment is required to achieve germination higher than 40%.
226 Thus, defiant asymbiotic germination is the case where orchid
227 seeds do not germinate under typical lab procedures (nutrient
228 media and pre-treatments). In summary, the species in the
229 entire dataset can be ~~categorized~~ categorised into two groups
230 based on germination behaviour: 1) Defiant (FG: 0-40%, mean
231 = 12.8%, n = 85), and 2) Compliant (FG: 45-100%, mean =
232 77.6%, n = 118). Regarding the second criterion (duration of
233 chemical treatment), it is important to note that only 4 species
234 with germination over 40% are classified in the defiant group,
235 with the most extreme case being *Cephalanthera rubra* which
236 achieved 60% FG after 15 h of chemical treatment (Rasmussen,
237 1995). The dividing line of 40% was selected as a close
238 approximation to half of the highest mode value (85.7%),
239 which nearly coincides with the highest valley (38.5%). This
240 choice is based on: a) a Hartigan's test of unimodality (p-value
241 < 0.001), and b) a test using the 'multimode' package in R with
242 the ACR method (p-value = 0.13). In this test, three modes
243 (1.8%, 19.7%, and 85.7%) and two antimodes (13.2% and
244 38.5%) were identified. According to our criteria, the 203
245 species in our dataset are divided almost equally into the two
246 groups: 118 species (58%) are classified as compliant, while 85
247 species (42%) are categorized as defiant. It is worth noting that
248 in a previous preliminary work (Oikonomidis and Thanos,
249 2020), in addition to the defiant and compliant groups, we
250 proposed a third category for even easier germination,
251 encompassing species that could readily germinate on water or
252 water agar without any chemical treatment. However, due to

253 the small size of this group (n = 11), we decided, in this study,
254 to incorporate them into the compliant germination category.

255 *Biotic and abiotic factors*

256 For each species, we recorded data concerning their growth
257 form, mycoheterotrophy level, climate zone, and habitat type in
258 relation to shadiness. Regarding growth form, the species fall
259 into two categories: terrestrial and epiphytic. The data
260 regarding growth form were gathered from three types of
261 resources: 1) the literature, from which germination and
262 morphological data were collected, and which, in most cases,
263 also reported the growth form of the studied orchid species, 2)
264 the online database of Plants of the World (POWO), and 3)
265 personal observations, specifically in the case of European
266 orchids. In summary, 150 species are terrestrial, while the
267 remaining 53 species are epiphytic.

268 In terms of mycoheterotrophy, Orchidaceae members can
269 be ~~characterized~~characterised by three levels: autotrophy,
270 partial mycoheterotrophy, and full mycoheterotrophy,
271 regarding their dependence on fungal carbon and nitrogen for
272 survival in nature. Nevertheless, mycoheterotrophy is better
273 described as a continuum between autotrophy and full
274 mycoheterotrophy (Mercx, 2013). Within the entire database
275 used, only a small number (20 species) are fully
276 mycoheterotrophic. The remaining species are considered
277 partially mycoheterotrophs, because unlike fully
278 mycoheterotrophic species, cases where autotrophic orchids
279 have been differentiated from partially mycoheterotrophic ones
280 are infrequent in the literature, where the majority of orchid
281 species tested falls into this broad category (Schiebold et al.,
282 2017). Therefore, the remaining species in the dataset are
283 categorized as partially mycoheterotrophic (183 out of 203)
284 either because they have been identified as such in previous

285 studies or because classification data for them are not available
286 in the literature.

287 A dichotomous approach was adopted for both the climate
288 zone and habitat shadiness, with the two classes of climate
289 zones being temperate and tropical, and for habitat shadiness
290 being open and shaded. Species were assigned to classes based
291 on the description of each species, along with distribution maps
292 or accurate coordinates of populations obtained from various
293 resources: 1) the online database Plants of the World (POWO,
294 2019), 2) the World Checklist of Selected Plant Families
295 (WCSP, 2020), 3) the literature acquired while gathering seed
296 morphological and germination data, and 4) the online database
297 GBIF (www.GBIF.org). Regarding shadiness data, more than
298 two million occurrences were initially extracted from GBIF for
299 the species of the study. From these occurrences, data reported
300 before 2000, with missing coordinates, ambiguous species
301 identification, or spatial resolution higher than 100 m were
302 discarded. This resulted in a dataset of about a hundred
303 thousand points, from which only occurrences of species with
304 more than 50 records were retained, amounting to about forty
305 thousand occurrences for 127 out of the 203 species (60%) in
306 the dataset. The characterisation of habitat shadiness for the
307 study species relied mainly on the description of each species
308 and its habitat from available literature. Additionally, a cross-
309 validation of shadiness was conducted for the 127 species with
310 available occurrences. In this cross-validation, the canopy
311 cover for the year 2000 (Hansen et al., 2013) was
312 ~~utilized~~utilised. The mean value of tree canopy cover for each
313 species was calculated, and a minimum threshold of 30% was
314 applied to distinguish species in open habitats ($\leq 30\%$) from
315 those in shaded habitats ($>30\%$). This threshold selection aligns
316 with previous works on canopy cover classification, ~~such as~~
317 (Torres et al., 2013).

318 For characterising the climate zone of each species, the
319 Köppen-Geiger climate classification (Kottek et al., 2006) was
320 employed. In determining the climate zone, distribution data
321 were extracted from Kew databases (Plants of the World
322 Online and World Checklist of Selected Plant Families), where
323 maps or information about the countries where the species can
324 be found were available. The species in the dataset were
325 distributed in both temperate (127/203) and tropical (76/203)
326 climate zones.

327 *Statistics*

328 The germination dataset was not normally distributed; thus,
329 non-parametric tests (e.g., Kruskal-Wallis and Mann-Whitney
330 U test) were applied to check the statistical significance of the
331 data exploration results concerning the effect of the four
332 different classification variables on the morphometric
333 characteristics of orchid seeds.

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334 A Factor Analysis for Mixed Data (FAMD), illustrated in
335 Figure 3, was employed to ~~analyze~~analyse the similarity
336 between species in the dataset, taking into account variables
337 such as E:S ratio, habitat shadiness, growth form, climate zone,
338 and germination type. In an additional approach, and based on
339 the results from the rest of the analysis, we ~~utilized~~utilised a
340 combined index comprising all the previously mentioned traits.
341 Each trait, based on its level, was assigned a binary value of 0
342 or 1 for each species. By summing these values, we calculated
343 a Trait Index (TI) ranging from a minimum of 0 to a maximum
344 of 4. For instance, and considering one ~~of of the two~~
345 ~~extremes~~the two extremes, if an orchid species exhibits defiant
346 germination, is typically found in shaded habitats, is terrestrial,
347 and has a distribution mainly in temperate climate regions, then
348 it is assigned a value of 4, and refers to the species in the
349 mycoheterotrophic end of the auto-heterotrophy continuum.
350 Conversely, if the germination is compliant, the orchid is found

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351 in open habitats, is epiphytic, and has a distribution mainly in
352 the tropics, it is assigned a value of 0, referring to the species
353 on the autotrophic end of the continuum. Values between 0 and
354 4 arise from various combinations of the parameters for each
355 species with variable placements across the mycoheterotrophy
356 continuum. We use the TI in order to place all different factors
357 on a single linear scale.

~~358 The germination dataset was not normally distributed; thus,~~
~~359 non-parametric tests (e.g., Kruskal Wallis and Mann Whitney~~
~~360 U test) were applied to check the statistical significance of the~~
~~361 data exploration results concerning the effect of the four~~
~~362 different classification variables on the morphometric~~
~~363 characteristics of orchid seeds.~~

~~364 An agglomerative hierarchical clustering (Fig. 5) was~~
~~365 performed. The embryo length to seed length (E:S) ratio was~~
~~366 used as the clustering factor, and the distance matrix created~~
~~367 was calculated based on the Euclidean distance, with linkage~~
~~368 performed using the complete linkage method.~~

369 A Generalized Linear Model (GLM) (Fig. 4) with a
370 binomial error structure and logit link, using E:S [second-
371 degree polynomial: $y = C + b_1(E:S) + b_2(E:S)^2$] as a predictor
372 and germination class as the response variable, was conducted
373 to identify the effect of seed architecture on germination
374 behaviour. However, because this analysis does not take into
375 consideration the chemical treatment duration, the second
376 factor of the germination type classification, the results might
377 be somewhat distorted due to species that achieve high
378 germination percentages after long chemical durations.

379 A variable tree (Fig. 65) was created to represent the
380 dataset. Following expert reasoning, the variables were ordered
381 in a postulated hierarchical scale, where the first variable might
382 have an effect on the subsequent ones: 1) climate zone, 2)
383 habitat shadiness, 3) growth form, and 4) germination.

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384 Additionally, as the majority of the data were from the
385 subfamilies Orchidoideae (91/203) and Epidendroideae
386 (89/203), data exploration was also performed at the subfamily
387 level for these two groups separately. For the three other
388 subfamilies (Cypripedioideae, Vanilloideae, Apostasioideae),
389 the small number of observations in each did not allow
390 exploration of the relationships between E:S and the four
391 investigated variables.

392 Finally, the same data analysis was conducted with the
393 calculated volume of the seeds based on the proposed formulas
394 by Arditti (Arditti et al. 1979), but no differences compared to
395 E:S ratio data were observed in the final results. We also
396 investigated potential correlations between germination
397 behaviour and absolute seed and embryo dimensions but no
398 statistically significant results were observed. For brevity, these
399 analyses are not discussed further. ~~For brevity, this analysis is~~
400 ~~not further discussed.~~

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401 For the complete family dataset, all statistical tests were
402 performed, and the diagrams were created using R version 3.6.3
403 (R Development Core Team, 2005).

404

405 **Results**

406 In Fig. 1, the E:S ratio is plotted against each of the 5 factors:
407 germination type (Fig. 1a), habitat shadiness (Fig. 1b), climate
408 zone (Fig. 1c), mycoheterotrophy level (Fig. 1d), and growth
409 form (Fig. 1e). The statistical results of the analysis are also
410 presented in the figure. The differences in E:S ratio between
411 orchids exhibiting compliant germination and those with
412 defiant germination were found to be highly statistically
413 significant. Species with germination that is more challenging
414 under asymbiotic conditions have a much smaller E:S ratio
415 (~~mean~~-~~median~~ value = 0.23 ± 0.013) than those that germinate
416 almost readily (~~mean~~-~~median~~ value = 0.4138 ± 0.01). Thus, the

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417 seeds of the latter have relatively less free air space, i.e. the
418 space between the outer (testa) and inner (embryo) integuments
419 of the seed.

420 Orchid species typically growing in shaded habitats also
421 exhibit a smaller E:S ratio (mean-median value = 0.2324±0.01)
422 compared to those in open habitats (mean-median value =
423 0.3734±0.01). These differences are also found to be highly
424 statistically significant. Similarly, the differences in the E:S for
425 orchids found in temperate (mean-median value = 0.329±0.01)
426 and tropical (mean-median value = 0.3938±0.03) climates are
427 also highly statistically significant. It is clear that the smaller
428 E:S, and thus, the larger free air space, can be found in
429 temperate orchid species. Additionally, a significant difference
430 was observed between orchids with terrestrial growth form
431 (mean-median value = 0.2929±0.01) and those with epiphytic
432 habit (mean-median value = 0.46±0.02). Interestingly, the E:S
433 of the fully mycoheterotrophic orchids (mean-median value =
434 0.2219±0.02) ~~orchids~~ were also found much smaller than those
435 that were either partially mycoheterotrophic or autotrophic
436 (mean-median value = 0.3534±0.01).

437 The relationship between E:S and germination type, as
438 well as between E:S and habitat shadiness for the
439 Epidendroideae (Fig. 2a-b, respectively) and Orchidoideae
440 (Fig. 2c-d, respectively) subfamilies, is illustrated in Fig. 2.
441 These relationships follow the same pattern as the rest of the
442 Orchidaceae family. In both Orchidoideae and Epidendroideae,
443 the E:S differences between germination types exhibit highly
444 statistically significant differences, with mean-median values of
445 0.20—21±0.01 (defiant germination) and 0.48—47±0.02
446 (compliant germination) for Epidendroideae and 0.28—29±0.01
447 (defiant germination) and 0.36—33±0.01 (compliant
448 germination) for Orchidoideae. Regarding habitat shadiness,
449 the differences in statistical significance for Epidendroideae are

450 higher than those for Orchidoideae, with mean-median values
451 of 0.30-24±0.02 (shaded) and 0.43-42±0.03 (open) for
452 Epidendroideae and 0.28-29±0.03 (shaded) and 0.34±0.01
453 (open) for Orchidoideae.

454 The factor maps of the FAMD analysis (Fig. 3) reveal two
455 expected clusters, those of climate zone and growth form.
456 Additionally, there is a clustering between species found in
457 shaded or open habitats and those exhibiting defiant or
458 compliant germination, respectively. In the latter case, small
459 overlaps of the clusters are present, presumably due to other
460 factors affecting germination behaviour not considered in the
461 present study. The first and second dimensions of the FAMD
462 factor maps explain 75.1% of the variance in the dataset.

463 The GLM analysis reveals a relationship between E:S and
464 FG percentage (Fig. 4) with a range of pseudo-R² = 0.36-0.53.

465 ~~However, because this analysis does not take into consideration~~
466 ~~the chemical treatment duration, the second factor of the~~
467 ~~germination type classification, the results might be somewhat~~
468 ~~distorted due to species that achieve high germination~~
469 ~~percentages after long chemical durations.~~

470 ~~The clustering analysis (Fig. 5) demonstrates a pattern~~
471 ~~where species with higher TI values (3 or 4) group together,~~
472 ~~and their seeds have the smallest E:S. As we move towards a TI~~
473 ~~value of 0, E:S increases, presumably as a result of the~~
474 ~~correlation of the 4 variables with the morphology of the seed.~~

475 Finally, from the structured variable tree (Fig. 65), we can
476 observe four main patterns, encompassing 72% of the total
477 observations. These patterns include orchids that can be found:
478 1) in the tropics, in relatively open habitats as epiphytes and
479 exhibit compliant germination (13% of the observations), 2) in
480 temperate climates, in open habitats, are terrestrial, and exhibit
481 defiant germination (15% of the observations), 3) in temperate
482 climates, in shaded habitats, are terrestrial, and exhibit defiant

483 germination (18% of the observations), and 4) in temperate
484 climates, in open habitats, are terrestrial, and exhibit compliant
485 germination (26% of the observations).
|486

487 **Discussion**

488 The major finding of this study is a statistically robust
489 correlation between the embryo-to-seed size ratio (E:S) and
490 germination behaviour within the Orchidaceae family. This
491 correlation holds true not only for the entire family but also
492 when considering the two largest subfamilies, Orchidoideae
493 and Epidendroideae, analysed as independent datasets. In
494 simpler terms, species exhibiting larger embryos (relative to
495 seed size) tend to display comparatively facile (compliant)
496 germination, contrasting with those possessing larger air spaces
497 (exhibiting defiant germination).

498 Similarly, to previous assertions based on investigations of
499 *Dendrobium* species (Prasongsom et al., 2016, 2022), it is
500 becoming evident that not all Orchidaceae seeds should be
501 ranked (based on their minute size) within the sensu lato
502 morphological or morphophysiological dormancy classes
503 (Forbis et al., 2002; Baskin and Baskin, 2014). This study
504 elucidates that the orchids with smaller E:S ratios, consequently
505 featuring relatively larger air spaces within their seeds, are
506 more inclined to pertain to one of the aforementioned
507 dormancy classes. This inclination is attributed to their defiant
508 germination characteristics, thereby offering a nuanced
509 perspective on the relationship between E:S ratio and dormancy
510 type within the Orchidaceae family. On the other hand, species
511 with higher E:S ratios may exhibit non-dormant seeds. For
512 instance, *Anacamptis sancta* has been observed rapidly
513 producing protocorms within a few days of sowing
514 (Oikonomidis and Thanos, 2021).

515 In the Orchidaceae family, as well as in the Orchidoideae
516 and Epidendroideae subfamilies, the embryo-to-seed size ratio
517 (E:S) is observed to be smaller in species inhabiting temperate
518 regions worldwide, as well as in terrestrial life form, when
519 compared to their tropical and epiphytic counterparts, similar

520 observations were also made in previous works (Tsutsumi et al.
521 2007, Collier et al. 2023). Previous research has illustrated that
522 temperate terrestrial orchid species exhibit a greater need for
523 complex pre-treatments and germination media in contrast to
524 tropical epiphytic counterparts (Rasmussen 1995; Diantina et
525 al. 2020). This disparity is commonly interpreted on the basis
526 of the wider mycorrhizal compatibility and the simpler
527 nutritional requirements shown by epiphytic orchids (Teixeira
528 2013; Rasmussen et al. 2015). The reduction in embryo size, as
529 evidenced by general observations on mycoheterotrophic plants
530 by Leake (1994), and the substantial, secondary, decrease in the
531 embryo-to-seed size ratio (E:S) observed in the present study,
532 align with the prevailing concept of increased nutritional
533 demands in temperate terrestrial orchids.

534 It is noteworthy that the fully mycoheterotrophic species
535 exhibit the smallest E:S ratios among the species in the dataset.
536 The preponderance of these orchids is found within the
537 subfamily Epidendroideae, which is unsurprising given that out
538 of the 215 fully mycoheterotrophic species in the Orchidaceae
539 family, 155 belong to Epidendroideae. Notably, within this
540 subfamily, multiple instances of secondary returns to terrestrial
541 life forms have been documented (Chomicki et al., 2015; Fan et
542 al., 2020).

543 Considering both the extremely low values of E:S in fully
544 mycoheterotrophic orchids and the overall trend of lower E:S
545 values in shaded habitats compared to open ones, it is
546 postulated that a reduction in E:S during the family's evolution
547 accompanied the shift of several orchid lineages from open to
548 deeply shaded niches. This transition ~~led was the, potential,~~
549 ~~result of the shift of these species towards the fully~~ these species
550 ~~toward the fully~~ mycoheterotrophic end of the autotrophy-
551 mycoheterotrophy continuum. The seeds of these orchids fall
552 into the defiant germination category, posing significant

553 challenges for successful germination. In numerous cases for
554 such species, germination was not attainable under in vitro
555 culture (Rasmussen, 1995). Hence, these seeds are classified,
556 by many researchers, as deeply dormant, belonging to either the
557 morphological or the morphophysiological class (Rasmussen,
558 1995; Baskin and Baskin, 2014). In nature, the co-occurrence
559 of a compatible symbiont plays a crucial role in breaking seed
560 dormancy by scarifying the water-impermeable carapace and
561 providing nutrition for germination (Rasmussen, 1995).

562 In prior studies, it has been proposed that the larger air
563 space in seeds is an adaptation to anemochory (Arditti and
564 Ghani, 2000; Diantina et al., 2020; Fan et al., 2020).
565 Additionally, it has been suggested that the secondary return to
566 terrestrial habitats drove an increase in seed air space as a
567 counterbalance to seed dispersal capability, particularly from a
568 lower release height associated with the terrestrial habit. While
569 a larger air space does enhance floating capability (Arditti and
570 Ghani, 2000), it should not be considered an exclusive
571 adaptation to anemochory. The concept of convergence of
572 small E:S values absolute smaller seed size, as reported by
573 Leake in 1994, for the majority of mycoheterotrophic species,
574 further refined with the study of the relative seed to embryo
575 size along with the findings of in the present study, supports the
576 hypothesis of a decrease in E:S values (or increase of the
577 relative air space) co-occurring with an increase of the
578 dependence on fungal symbiont decreasing E:S values (while
579 simultaneously increasing the relative air space) as dependence
580 on symbionts increases. Moreover, the larger air space and the
581 corresponding longer flotation times, commonly observed in
582 orchids of shaded habitats (both terrestrial and epiphytic), may
583 play a crucial role in their dispersal within relatively encloused
584 obstructed environments such as forests, as they embark on a
585 'blind quest' for suitable fungal hosts. Furthermore, recent

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586 works support the anemochory as a derived state in the
587 Orchidaceae (Karremans et al. 2023) with zoochory being the
588 primary mode of dispersal for early diverging lineages. In
589 contrast, fungal relationships can be tracked even in the fossil
590 record (Poinar G. 2016), ~~even-although~~ such observations have
591 been challenged ~~from~~ ~~by~~ ~~some~~ ~~certain~~ researchers (Selosse et al.
592 2017).

593 While further investigations into the mycoheterotrophy
594 level of orchids and their seed morphology are essential for
595 elucidating the drivers of orchid seed trait evolution, we can
596 ~~speculate~~hypothesise that shifts across the mycoheterotrophy
597 continuum play a significant role as a driving force for orchid
598 seed morphology. The larger air space in terrestrial orchids and
599 those of shaded habitats should not be automatically accepted
600 as an adaptation to anemochory, especially considering the
601 existence of these traits in orchid groups where anemochory is
602 less common. The decrease in E:S could, in fact, be an
603 adaptation to the shift towards the fully mycoheterotrophic end
604 of the continuum, where the reduced relative size of the embryo
605 and, consequently, the resources expended per seed produced
606 might play a crucial role. This adaptation could contribute to
607 adult plant survival by allocating more resources to reflowering
608 in the next season or by increasing the potential number of
609 seeds produced per inflorescence.

610 In conclusion, orchid species inhabiting shaded habitats
611 produce seeds with smaller E:S ratios (and larger air spaces)
612 and deeper dormancy. Under in vitro asymbiotic conditions,
613 these seeds show ~~a defiant~~defiant germination behaviour, ~~i.e.~~
614 ~~either~~namely demonstrating poor final germination ~~levels~~
615 ~~and/or~~ requiring extended chemical pre-treatments for
616 successful germination. This pattern is, ~~also,~~ evident within the
617 Orchidoideae and Epidendroideae subfamilies; an
618 accumulation of ~~but with the expansion of available~~ data in the

619 ~~future might prove as well that this same pattern holds true, it~~
620 ~~may also be identified~~ in the subfamilies Apostasioideae,
621 Cyripedioideae, and Vanilloideae. ~~This,~~ secondary, decrease
622 ~~in of~~ E:S ~~towards in~~ shaded niches following transitions of
623 lineages from the autotrophic to the fully-mycoheterotrophic
624 edge of on the mycoheterotrophy continuum, potentially, might
625 signify the increasing dependence of orchids on their
626 symbionts, an extreme case in the reduction of seed and
627 embryo size observed in mycoheterotrophic plants (Leake,
628 1994). While this ~~speculation hypothesis~~ can explain both the
629 germination behaviour and morphometric traits of orchid seeds,
630 it requires further testing through the examination of the
631 morphology of seeds from additional orchid species and their
632 corresponding position on the autotrophy-mycoheterotrophy
633 continuum. It is crucial to approach the dominant drivers of
634 orchid seed morphology with caution, especially concerning the
635 shift from epiphytic to terrestrial habits. The E:S ratio (as a
636 proxy of the relative free-air space of an orchid seed) has been
637 considered an adaptation to anemochory in many previous
638 works, mentioned above. However, on the basis of our data, we
639 postulate that the evolutionary modification of seed
640 morphology in Orchidaceae (as depicted by the E:S ratio) could
641 be predominantly driven by the increased dependence on fungal
642 symbiosis at the germination stage. An alternative postulation
643 could implicate both anemochory and fungal symbiosis as
644 coevolving traits, driving the overall diversification of orchid
645 seed morphology and germination.~~s.~~ Further research is
646 necessary to elucidate these relationships.

647 Orchids rank among the most endangered plant families,
648 with 5 known extinct species and 986 out of 2013 species
649 assessed (48%) classified in a threat category (IUCN, 2024).
650 Orchids have nearly twice as many threatened species
651 compared to the totality of all organisms assessed thus far. In

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652 the forthcoming decades, ex-situ propagation will play a pivotal
653 role in the successful conservation of orchids. However, the
654 sheer number of orchid species, coupled with a significant
655 count of endangered ones and the limited resources available
656 for conservation, poses a difficult challenge in developing
657 protocols for the ex-situ propagation of orchid species for
658 conservation purposes.

659 The present study suggests the important predictive value
660 of the E:S ratio on germination behaviour of orchid seeds,
661 emphasising the value of relative embryo and seed dimensions
662 (compared to absolute ones) towards a better understanding of
663 orchid seed biology.~~The present study suggests the potential~~
664 ~~predictive value of the E:S ratio for the germination behaviour~~
665 ~~of orchid species. Recognizing Recognising~~ patterns in orchid
666 germination behaviour will be pivotal in successfully
667 conserving orchids, ultimately reducing the time required to
668 develop effective conservation protocols. In future germination
669 investigations, it is imperative to include data on orchid
670 germination in distilled water or water agar. Species that
671 germinate promptly under such conditions may exhibit even
672 higher E:S ratios compared to those in the compliant
673 germination category of our research. However, data on
674 germination under these conditions are scarce, particularly for
675 epiphytic orchids. These species appear to show ~~lower~~lesser
676 dependence on symbiotic relationships during their early life
677 stages, and information from these cases could contribute to a
678 better understanding of the evolution of seed traits in
679 Orchidaceae.

680

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685

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692 **Conflicts of interest**

693 The authors declare none.

694

695 **Data availability statement**

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697 The summarised dataset is available as supplementary material.

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699 **References**

700 **Akçin AT, Ozdener Y and Akçin A.** (2009) Taxonomic value
701 of seed characters in orchids from Turkey. *Belgian Journal*
702 *of Botany* **142**, 124–139.

703 **Arditti J.** (1967) Factors affecting the germination of orchid
704 seeds. *The Botanical Review* **33**, 1–97.

705 **Arditti J, Michaud JD and Healey PL.** (1979) Morphometry
706 of orchid seeds. I. *Paphiopedilum* and native California
707 and related species of *Cypripedium*. *American Journal of*
708 *Botany* **66**, 1128–1137.

709 **Arditti J.** (1984) A history of orchid hybridization, seed
710 germination and tissue culture. *Botanical Journal of the*
711 *Linnean Society* **89**, 359–381.

712 **Arditti J and Ghani AKA.** (2000) Tansley [review-Review](#) no.
713 110: Numerical and physical properties of orchid seeds and
714 their biological implications. *New Phytologist* **145**, 367–
715 421.

- 716 **Baskin CC** ~~and~~, **Baskin JM** (2014) *Seeds: Ecology,*
717 *Biogeography, and Evolution of Dormancy and*
718 *Germination, 2nd ed.* San Diego, USA, Academic Press.
- 719 **Bernard N.** (1899) Sur la germination du *Neottia nidus-avis*.
720 *Comptes Rendus Hebdomadaires des Séances de*
721 *l'Académie des Sciences* **128**, 1253–1255.
- 722 **Bernard N.** (1909) L'—évolution dans la symbiose, les
723 orchidées et leurs champignons commensaux. *Annales des*
724 *Sciences Naturelles* **9**, 1–196.
- 725 **Chomicki GL, Bidel PR, Ming F, Coiro M, Zhang X, Wang**
726 **Y, Baissac Y, Allemand CJ and Renner SS.** (2015) The
727 velamen protects photosynthetic orchid roots against UV-B
728 damage, and a large dated phylogeny implies multiple
729 gains and losses of this function during the Cenozoic. *New*
730 *Phytologist* **205**, 1330–1341.
- 731 **Christenhusz MJM, Fay MF and Chase MW.** (2017) *Plants*
732 *of the World: An Illustrated ~~encyclopedia~~ Encyclopedia*
733 *of ~~vascular~~ Vascular plantsPlants*. London, UK, Kew
734 Publishing.
- 735 **Collier MH, Fisher JS, Gribbins KM, Yoder JA and,**
736 **Zettler LW.** (2023) Differences in seed morphometrics of
737 representative orchids native to North America and Hawaii
738 using scanning electron microscopy. *South African Journal*
739 *of Botany* **152**, 222–229.
- 740 **Diantina S, McGill C, Millner J, Nadarajan J, Pritchard**
741 **HW and McCormick AC.** (2020) Comparative seed
742 morphology of tropical and temperate orchid species with
743 different growth habits. *Plants* **9**,
744 <https://doi.org/10.3390/plants9020161>.
- 745 **Diantina S, Kartikaningrum S, McCormick AC, Millner J,**
746 **McGill C, Pritchard HW and Mardarajan J.** (2023).
747 Comparative in vitro seed germination and seedling
748 development in tropical and temperate epiphytic and

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749 temperate terrestrial orchids. *Plant Cell, Tissue and Organ*
750 *Culture* **143**, 619-633.

751 **Eriksson O and Kainulainen K.** (2011) The evolutionary
752 ecology of dust seeds. *Perspectives in Plant Ecology,*
753 *Evolution and Systematics* **13**, 73–87.

754 **Fan XL, Chomicki G, Hao K, Liu Q, Xiong YZ, Renner SS,**
755 **Gao JY and Huang SQ.** (2020) Transitions between the
756 terrestrial and epiphytic habit drove the evolution of seed-
757 aerodynamic traits in orchids. *The American Naturalist*
758 **195**, 275–283.

759 **Forbis TA, Floyd SK and Queiroz A.** (2002). The evolution
760 of embryo size in Angiosperms and other seeds plants:
761 ~~implications~~ Implications for the evolution of seed
762 dormancy. *Evolution* **56**, 2112-2125.

763 **Gamarra R, Ortúñez E, Sanz E, Esparza I and Galán P.**
764 (2010) Seeds in subtribe Orchidinae (Orchidaceae): The
765 best morphological tool to support molecular analysis, pp.
766 323-326 in Nimis PL; Vignes R (Eds). *Tools for*
767 *identifying biodiversity: Progress and problems.* Trieste,
768 IT, Edizioni Università di Trieste.

769 **GBIF** (2020): *The Global Biodiversity Information Facility.*
770 Available from <https://www.gbif.org/> [accessed 8
771 September 2020].

772 **Govaerts R, Hind N, Lindon L, Chase M, Baker W, Lewis**
773 **G, Vorontsova M and Nicolson N.** (2017) Naming and
774 counting the world's plant families. pp. 4-9 in Willis KJ
775 (Eds). *State of the World's Plants.* London, UK, Kew
776 Publishing.

777 **Givnish TJ, Spalink D, Ames M, Lyon SP, Hunter SJ,**
778 **Zuluaga A, Iles WJD, Clements MA, Arroyo MTK,**
779 **Leebens-Mack J, Endara L, Kriebel R, Neubig KM,**
780 **Whitten WM, Williams NH and Cameron KM.** (2015)
781 Orchid phylogenomics and multiple drivers of their

- 782 extraordinary diversification. *Proceedings of the Royal*
783 *Society B* **282**, 20151553.
- 784 **Hansen MC, Potavov PV, Moore R, Hancher M,**
785 **Turubanova SA, Tyukavina A, Thau D, Stehman SV,**
786 **Goetz SJ, Loveland TR, Kommareddy A, Egorov A,**
787 **Chini L, Justice CO and Townshend JRG.** (2013) High-
788 resolution global maps of 21st century forest cover change.
789 *Science* **342**, 850–853.
- 790 IUCN (2024) *The IUCN Red List of Threatened Species.*
791 *Version 2023-1.* <https://www.iucnredlist.org> [accessed 8
792 January 2024].
- 793 **Karremans A, Wattevn C, Scaccabarozzi D, Escobar OAP**
794 **and, Bogarin D.** (2023) Evolution of seed dispersal modes
795 in the Orchidaceae: Has the *Vanilla* mystery been solved?
796 *Scientia Horticulturae* **9**, 1270.
- 797 **Kottek M, Grieser J, Beck C, Rudolf B and Bubel F.** (2006)
798 World map of the Köppen-Geiger climate classification
799 updated. *Meteorologische Zeitschrift* **15**, 259–263.
- 800 **Knudson L.** (1921) La germinación no simbiótica de las
801 semillas de orquídeas. *Bulletin—Boletín de la Real*
802 *Sociedad Española de Historia Natural* **21**, 250–260.
- 803 **Knudson L.** (1922) Nonsymbiotic germination of orchid seeds.
804 *Botanical Gazette* **73**, 1–25.
- 805 **Kull T, Arditti J.** (2002) *Orchid Biology: Reviews and*
806 *perspectives* *Perspectives*, VIII. Dordrecht, NT, Kluwer
807 Academic Publishers.
- 808 **Leake JR.** (1994) Tansley ~~review~~ *Review* no. 69: The biology
809 of myco-heterotrophic ('saprophytic') plants. *New*
810 *Phytologist* **127**, 171–216.
- 811 **Lee YI and Yeung EC.** (2023). The orchid seed coat: a
812 developmental and functional perspective. *Botanical*
813 *Studies*

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- 814 **Mercx VSFT.** (2013) *Mycoheterotrophy: The biology of plants*
815 *living on fungi*. New York, USA, Springer publishing.
- 816 **Micheneau C, Johnson SD and Fay MF.** (2009) Orchid
817 pollination: From Darwin to present day. *Botanical*
818 *Journal of the Linnean Society* **161**, 1–19.
- 819 **Movray M and Kores PJ.** (1995) Character analysis of the
820 seed coat in Spiranthoideae and Orchidoideae, with special
821 reference to the Diurideae (Orchidaceae). *American*
822 *Journal of Botany* **82**, 1443–1454.
- 823 **Oikonomidis S, Koutsovoulou K and Thanos CA.** (2020)
824 Germination of *Neotinea maculata* (Orchidaceae) in
825 nutrient media and water agar. *Flora Meditteranea* **30**,
826 394–399.
- 827 **Oikonomidis S and Thanos CA.** (2021). Germination of
828 *Anacamptis sancta* (Orchidaceae) in nutrient media, water
829 agar and various light regimes. *Flora Meditteranea* **31**, 271
830 – 276.
- 831 **Oikonomidis S and Thanos CA.** (2020) The good, the bad,
832 and the ugly: Morphometric traits of orchid seeds and their
833 implications in seed germination behaviour. in *1st*
834 *Panhellenic Scientific Meeting of Plant Physiologists*,
835 Athens, GR, Agricultural University of Athens, Greece.
- 836 **Poinar G.** (2016) Two new genera, *Mycophoris* gen. nov.,
837 (Orchidaceae) and *Synaptomitus* gen. nov.
838 (Basidiomycota) based on fossil seed with developing
839 embryo and associated fungus in Dominican amber.
840 *Botany* **95**, 1-8.
- 841 **POWO** (2019) *Plants of the World Online. Facilitated by the*
842 *Royal Botanic Gardens, Kew. Published on the Internet.*
843 URL: <https://powo.science.kew.org/> [accessed 11 October
844 2021].
- 845 **Prasongsom S, Thammasiri K and Pritchard HW.** (2016)
846 Seed micromorphology and ex vitro germination of

- 847 *Dendrobium* orchids. pp. 339-343, in *Acta Horticulturae –*
848 *Proceedings of the 1st International Symposium on*
849 *Tropical and Subtropical Ornamentals.*
- 850 **Prasongsom S, Thammasiri K and Pritchard HW.** (2022)
851 Seed dormancy concepts in orchids: *Dendrobium cruentum*
852 as a model species. *Seed Science and Research*, **32**, 175-
853 186.
- 854 **R Development Core Team** (2005) *R: A language and*
855 *environment for statistical computing.* R Foundation for
856 *Statistical Computing, Vienna, Austria.* ISBN 3-900051-
857 07-0, URL: <http://www.R-project.org>.
- 858 **Rasmussen NH.** (1995) *Terrestrial ~~O~~rchids: ~~F~~rom ~~S~~eeds to*
859 *~~mycotrophic~~–~~Mycotrophic~~ ~~plant~~Plant.* Cambridge, UK,
860 Cambridge University Press.
- 861 **Rasmussen NH and Rasmussen FN.** (2014) Seedling
862 mycorrhiza: a discussion of origin and evolution in
863 Orchidaceae. *Botanical Journal of the Linnean Society*
864 **175**, 313–327.
- 865 **Rasmussen NH, Dixon KW, Jersáková J and Těšitelová T.**
866 (2015) Germination and seedling establishment in orchids:
867 A complex of requirements. *Annals of Botany* **116**, 391–
868 402.
- 869 **Schiebold JMI, Bidartondo MI, Lenhard F, Makiola A and**
870 **Gebauer G.** (2017) Exploiting mycorrhizas in broad
871 daylight: Partial mycoheterotrophy is a common nutritional
872 strategy in meadow orchids. *Journal of Ecology* **106**, 168–
873 178.
- 874 **Schiff JL.** (2017) History of Orchids, in Schiff JL (Eds).
875 *Rare and Exotic Orchids.* New York, USA, Springer
876 publishing.
- 877 **Selosse MA, Brundrett M, Dearnaley J, Merckx VSFT,**
878 **Rasmussen F, Zettler LW and, Rasmussen HN.** (2017)
879 **Why Mycophoris is not an orchid seedling, and why**

- 880 *Synaptomitus* is not a fungal symbiont within this fossil.
881 *Botany* **95**, 865-868.
- 882 **Taylor DL, Bruns TD, Leake JR and Read DJ.** (2002)
883 Mycorrhiza specificity and function in mycoheterotrophic
884 plants. *Mycorrhizal Ecology* **157**, 375–413.
- 885 **Teixeira SJT.** (2013) Orchids: advances in tissue culture,
886 genetics, phytochemistry and transgenic biotechnology.
887 *Floriculture & Ornamental Biotechnology* **7**,1–52.
- 888 **Torres AB, Enriquez RO, Skutsch M and Lovett JC.**
889 (2013) Potential for climate change mitigation in
890 degraded forests: A study from La Primavera, Mexico.
891 *Forests* **4**, 1032–1054.
- 892 **Tsusumi C, Yukawa T, Lee NS, Lee CS and Kato M.**
893 (2007) Phylogeny and comparative seed morphology of
894 epiphytic and terrestrial species of *Liparis* (Orchidaceae)
895 in Japan. *Journal of Plant Research* **120**, 405–412.
- 896 **Veyret Y.** (1969) La structures des semences des
897 Orchidaceae et leur aptitude à la germination in vitro en
898 cultures pures. *Musée d'Histoire Naturelle de Paris,*
899 *Travaux du Laboratoire de la Jaysinia* **3**, 89-98.
- 900 **Vitt P, Taylor A, Rakosy D, Kreft H, Meyer A, Weigelt P**
901 **and Knight MT.** (2023) Global conservation
902 prioritization for the Orchidaceae. *Scientific reports*, **13**.
- 903 **WCSP** (2020) *World Checklist of Selected Plant Families.*
904 *Facilitated by the Royal Botanic Gardens, Kew. Published*
905 *on the Internet.* URL: <http://wmsp.science.kew.org/>.
906 [accessed 11 October 2021].
- 907 **Wraith J, Norman P and Pickering C.** (2020) Orchid
908 conservation and research: An analysis of gaps and
909 priorities for globally Red Listed species. *Ambio* **49**,
910 1601–1611.
- 911 **Yamazaki J and Mivoshi K.** (2006) In vitro asymbiotic
912 germination of immature seed and formation of

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913 protocorm by *Cephalanthera falcata* (Orchidaceae).
914 *Annals of Botany* **98**, 1197-1206.
915 **Zettler LW and Hofer CJ.** (1998) Propagation of the little
916 club-spur orchid (*Platanthera clavellata*) by symbiotic
917 seed germination and its ecological implications. –
918 *Environmental and Experimental Botany* **39**, 189-195.
919 **Zhang Y, Li YY, Chen XM, Guo SX and Lee YI.** (2020).
920 Effect of different mycobionts on symbiotic germination
921 and seedling growth of *Dendrobium officinale*, an
922 important medicinal orchid. *Botanical Studies* **61**.

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923 **Figures**

924

925 **Figure 1.** The relationship of E:S with: germination type (a),
926 habitat shadiness (b), climate zone (c), mycoheterotrophy
927 level (d), and growth form (e) for a dataset of 203 members
928 of the family Orchidaceae (see Table 1). The yellow dot
929 inside each viol-boxplot corresponds to the mean of the
930 group (m). The test statistics [$\log_e(W_{\text{mann-whitney}})$], p-value
931 (p), effect size (r), its confidence interval (CI_{95%}), and the
932 total number (n_{obs}) of observations are showed on top of each
933 diagram The number of observations in each category is
934 displayed beneath the group name on the x-axis.

935

936 **Figure 2.** The relationship of E:S with germination type and
937 habitat shadiness, depicted separately for the subfamilies
938 Epidendroideae (a, b) and Orchidoideae (c, d). The yellow
939 dot inside each viol-boxplot corresponds to the mean of the
940 group (m). The test statistics [$\log_e(W_{\text{mann-whitney}})$], p-value
941 (p), effect size (r), its confidence interval (CI_{95%}), and the
942 number (n_{obs}) of observations are showed on top of each
943 diagram.

944

945 **Figure 3.** FAMD factor maps, obtained through the Factor
946 Analysis for Mixed Data approach (FAMD analysis), depict the
947 clustering patterns of the 203 species in the dataset based on the
948 five variables studied (E:S, germination type, habitat shadiness,
949 growth form, and climate zone). Each factor map corresponds
950 to the clustering pattern of the species based on one of the four
951 categorical variables in the dataset. For each variable in the
952 corresponding factor map, the convex hull and the 95%
953 confidence interval ellipse are presented, with the group mean
954 at its centre.

955

956 **Figure 4.** The relationship between E:S and FG percentage,
957 accompanied by the best-fitted second-degree polynomial
958 curve. The colour of the dots represents the 'trait index' as
959 shown in the legend (for explanation of the TI values, see
960 Materials and Methods). The equation of the Generalized
961 Linear Model (GLM) regression is also shown (inset box),
962 along with the three pseudo-R² values correspond to: 1)
963 McFadden, 2) Cox and Snell, and 3) Nagelkerke, respectively.

964

~~965 **Figure 5.** A divisive hierarchical clustering dendrogram of the
966 studied orchids, split in two for better visualisation. The colour
967 of each species on the dendrogram corresponds to the 'trait
968 index' values: black for 4, red for 3, green for 2, blue for 1, and
969 turquoise for 0. The accompanying bar graph displays the E:S
970 value for each species, with the colours of the bars indicating
971 the subfamily of each species: dark green for Epidendroideae,
972 yellow for Orchidoideae, gray for Apostasioideae, dark gray for
973 Vanilloideae, and orange for Cypripedioideae. In addition, a
974 smaller version of the entire dendrogram is placed in the centre-
975 bottom, to the left of the split.~~

976

977 **Figure 65.** Variable tree representing the complete dataset of
978 the study. In each box the number of observations, the
979 percentage in the group and the level of the dividing factor
980 are presented. The root of the tree represents the number of
981 the observations in the entire dataset. The name of the
982 dividing factor for each step of the tree is also presented at
983 the base of the graph.

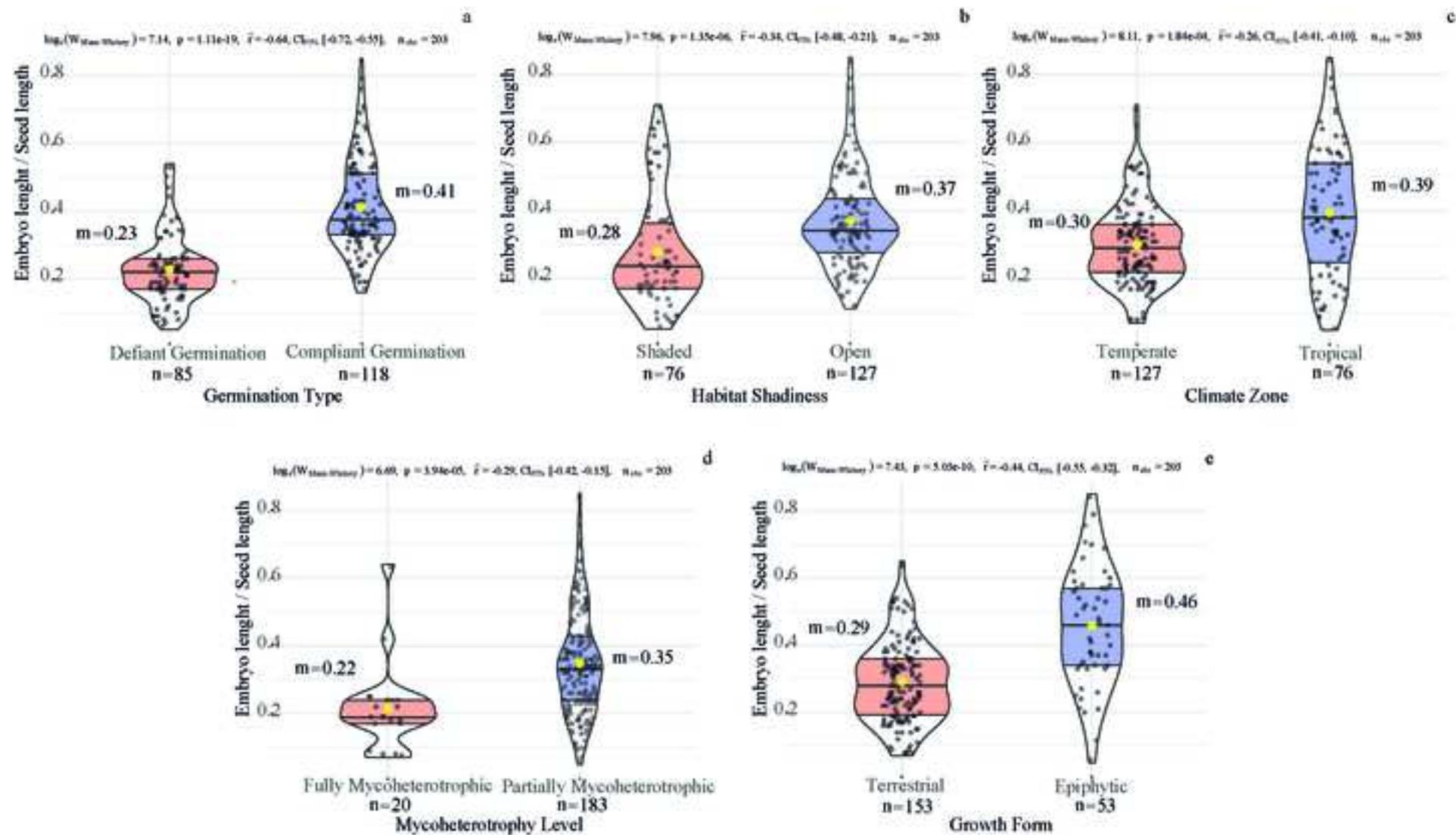
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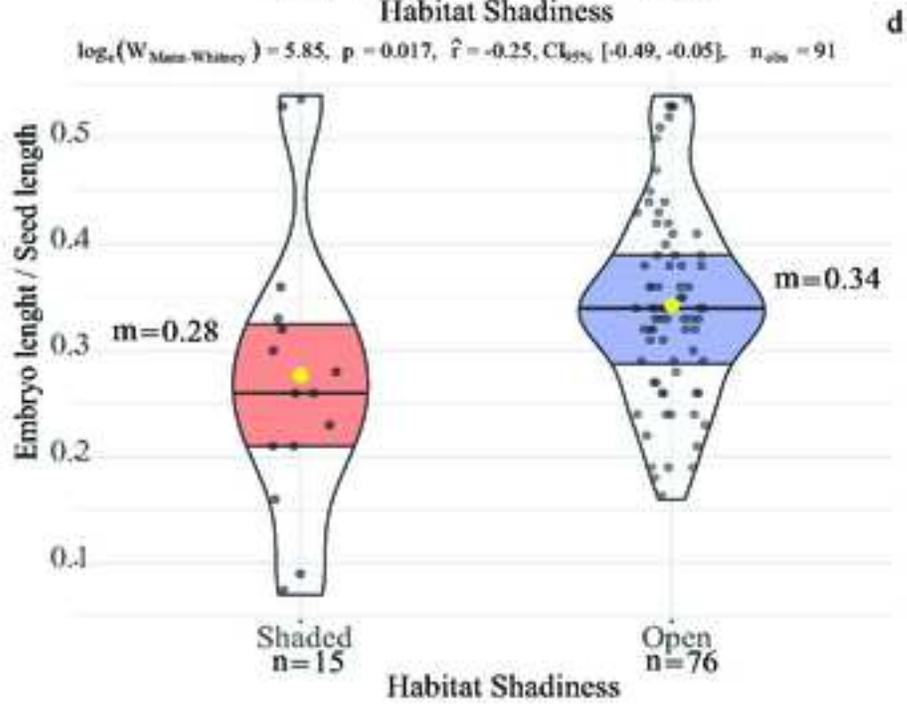
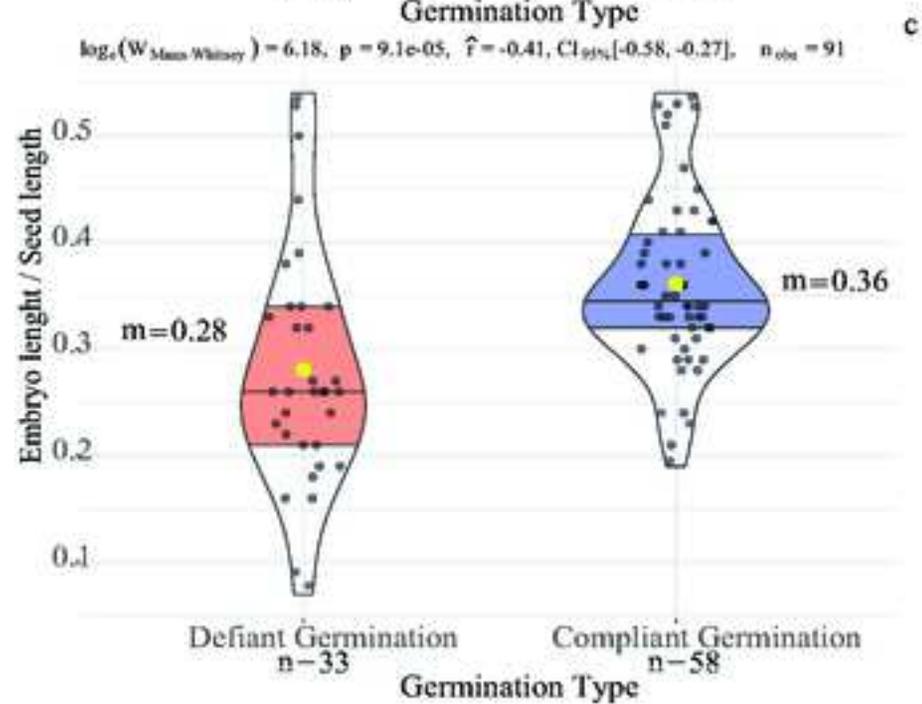
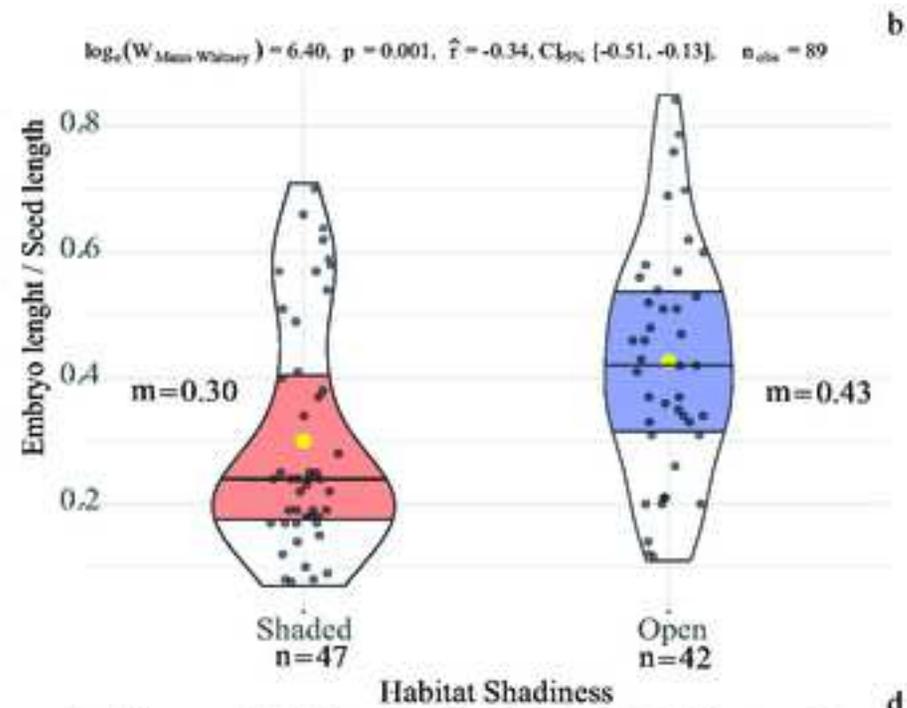
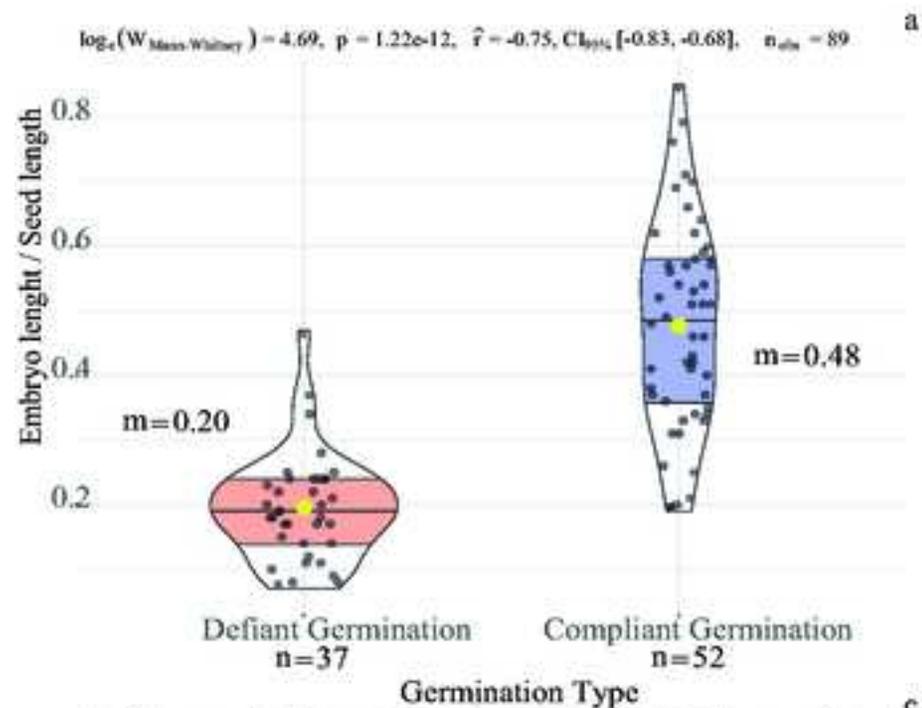
985 **Tables**

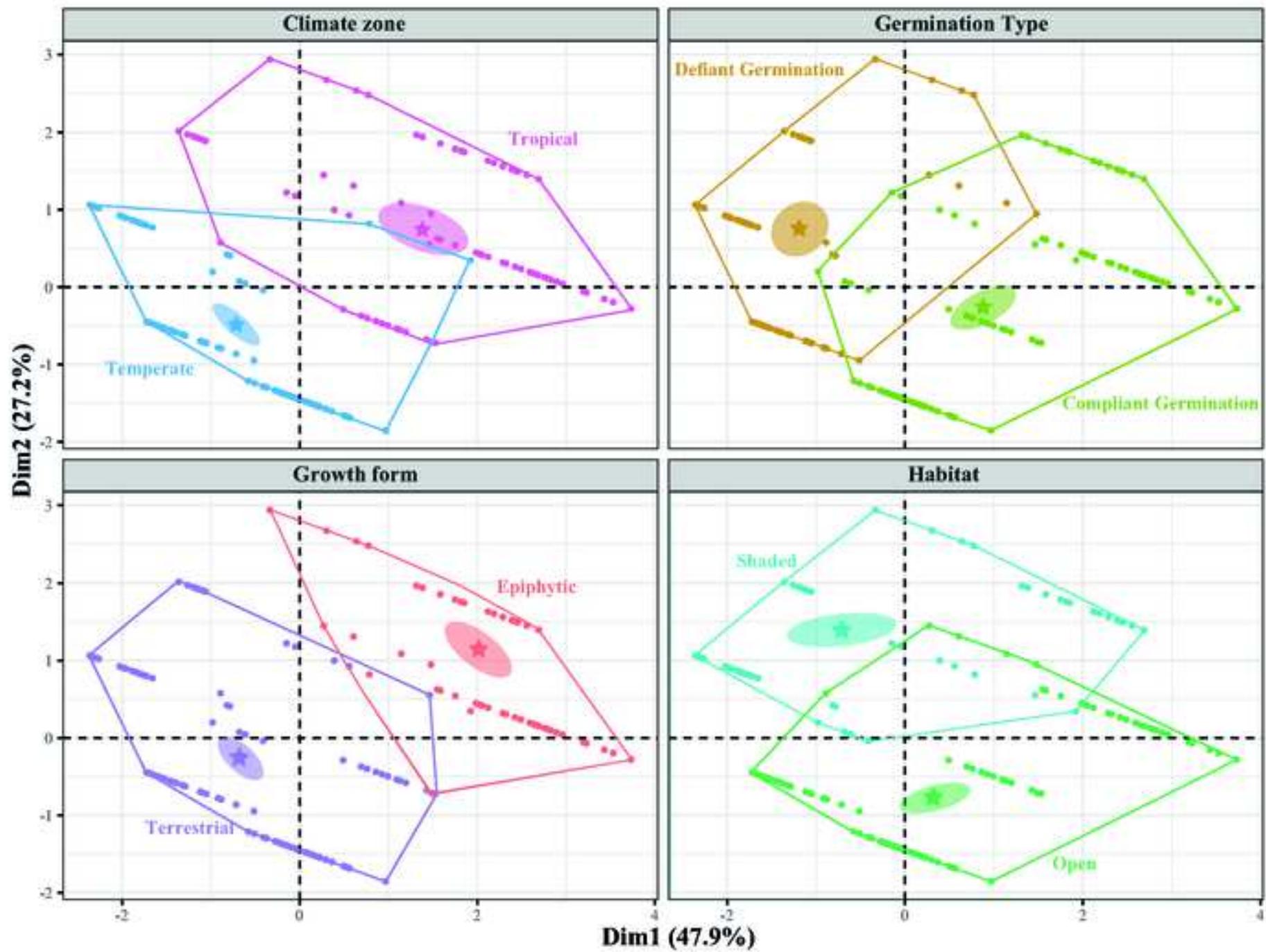
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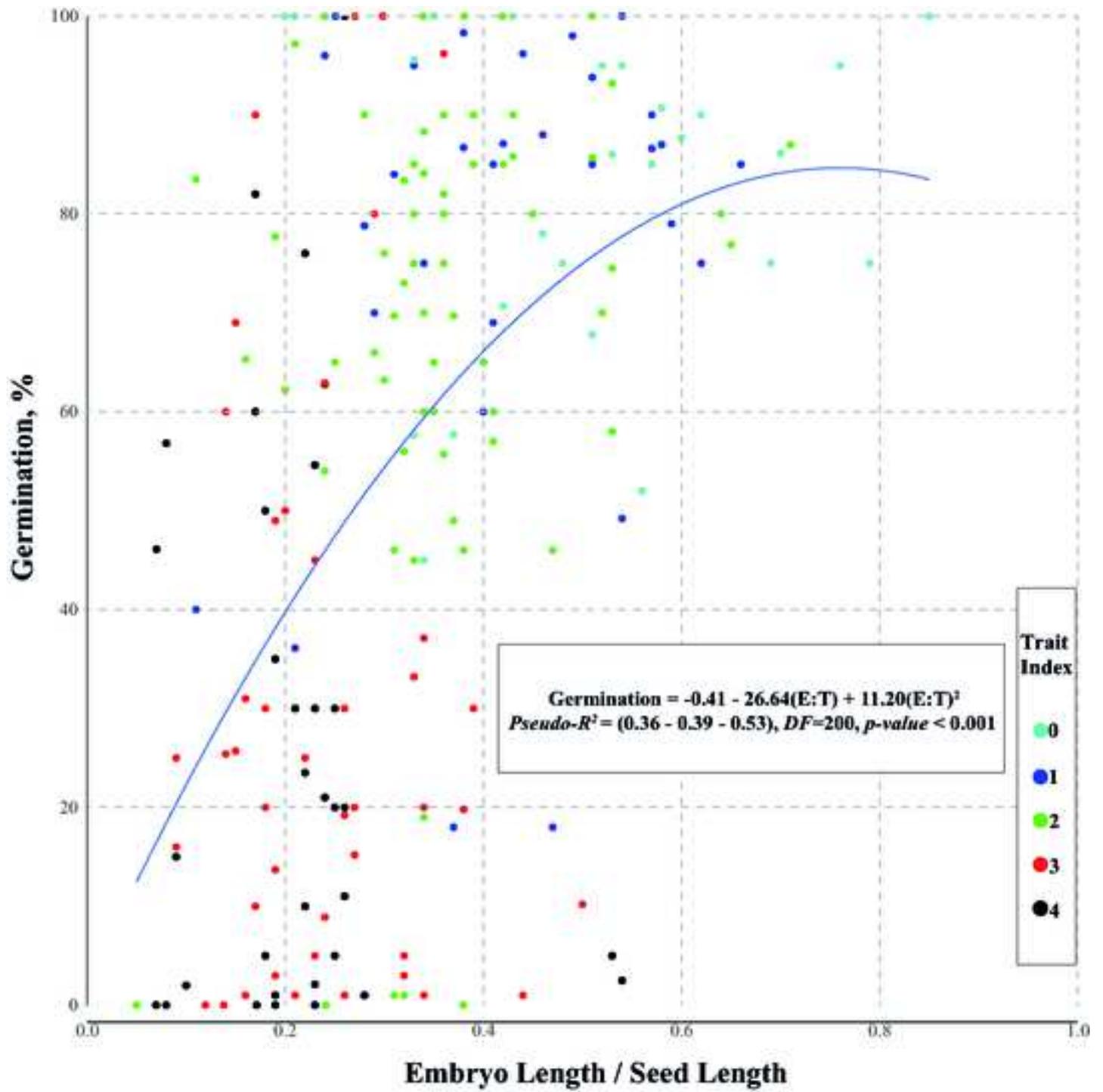
987 **Table 1.** Dataset breakdown by Orchidaceae subfamilies.

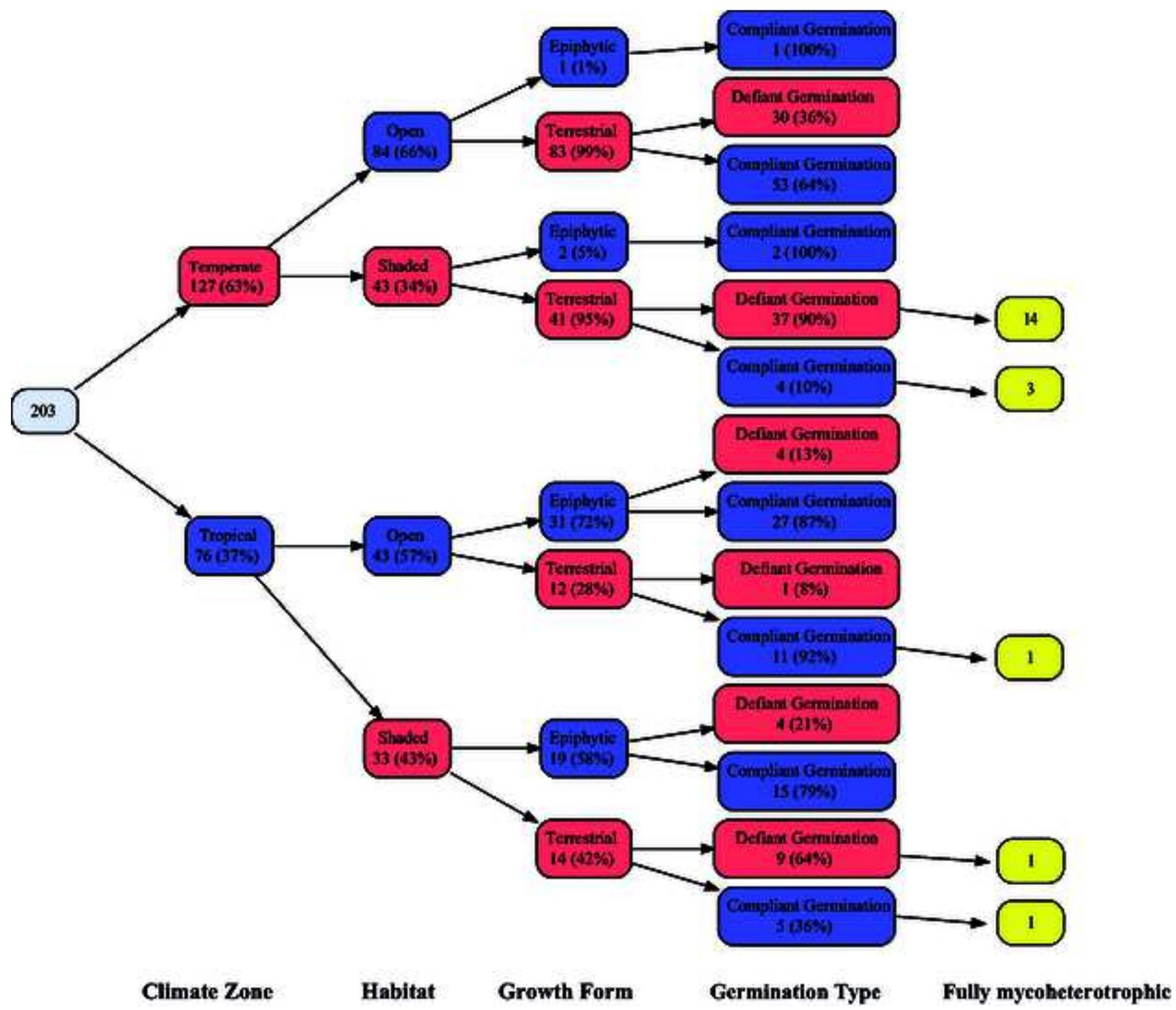
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Subfamily	D	D%	T	T%	±%
Apostasioideae	1	0.5	14	0.1	+0.4
Cypripedioideae	20	9.9	169	0.6	+9.2
Epidendroideae	89	44.0	21,100	79.6	-35.6
Orchidoideae	91	44.6	4,965	18.7	+25.9
Vanilloideae	2	1.0	245	0.9	+0.1
Total	203	100.0	26,493	100.0	

Note:

D: Total number of species in the dataset

D%: Percentage of species of the dataset in each subfamily

T: Total number of species in each subfamily (Christenhusz et al. 2017)

T%: Percentage of species of the family in each subfamily

±%: Percentage difference between species in the family and species in the dataset



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Supplementary Material
Supplementary_clustering_figure.tif



