Convocatoria de ayuda a proyectos de investigación liderados por jóvenes investigadores (9ª ed., 2019)

1. Datos de identificación.

Título de la propuesta	Ecología reproductiva de la orquídea amenazada *Anacamptis robusta*: alteraciones de su hábitat, mutualismos e hibridaciones. (ECOPRAT)
Categoría	Tomando la iniciativa
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2. Memoria Técnica. Actividades y resultados de investigación

2.1. Introducción (Planteamiento, objetivos y justificación)

Deceptive pollination, i.e. offering no reward in return for the pollination service, has been studied for decades in orchids to try to understand their evolutionary patterns. Approximately one-third of all the orchid species are reported to be deceptive (Ackerman 1986), which suggest the crucial role of floral deception in species diversification (Cozzolino and Widmer 2005). The most common non-rewarding pollination systems are based on sexual deception (e.g., *Ophrys* species) and food deception (e.g., *Orchis* species) (see, e.g. Jersàkovà et al. 2006; Martos et al. 2015; Wong et al. 2017). Sexual deception seems to be a highly specialised mechanism, whereas food deception is clearly a less-specific pollination mechanism (Cozzolino and Scopece 2008). Generalised food deception is largely mediated by visual signals such as showy flowers, spurs or nectar guides, and emitting floral scents similar to those of nectariferous plants (Dafni 1984; Ackerman 1986; Jersàkovà et al. 2009). Generally, reproductive output is lower in non-rewarding species than in rewarding ones (Neiland and Wilcock 1995; Johnson et al. 2004; Jacquemyn et al. 2009). This fact has been related to pollination limitation, which can be caused by low pollinator visits (frequency), low pollen arrival to stigmas (quantity) and the source of pollen (pollen quality) (Tremblay 2005).

Several factors influence the reproductive output in deceptive species, including (i) individual parameters, such as the plant height, inflorescence size and flower number per inflorescence (Scopece et al. 2017; Capó et al. 2019); (ii) population parameters, such as population size, shape and density of flowering stalks or co-flowering species (Vandewoestijne et al. 2009; Henneresse et al. 2017; Borràs and Cursach 2018); and (iii) environmental parameters, such as meteorological variables (Jacquemyn et al. 2009) and human disturbance (Traveset et al. 2018). Generally, plant height, inflorescence size and flower number increase reproductive success (O'Connell and Johnston 1998, Aragón and Ackerman 2004; Capó et al. 2019). Vandewoestijne et al. (2009) reported for three orchid species that reproductive success generally increased when population density and patch elongation decreased. Despite this, plant density effects are reported to be in the opposite direction in other deceptive species because even though high plant density attract more pollinators, they might fly away after a few un-rewarding visits and thus resulting in lower overall fruit set (Tremblay et al. 2005). On the other hand, Johnson et al. (2003) demonstrated that pollination success in deceptive orchids is enhanced by co-occurring nectar-producing species. Because of the diverse factors that influence on the reproductive success, spatiotemporal variation in the reproductive success of orchid species has been widely reported (Tremblay and Ackerman 2007; Jacquemyn and Brys 2010; Capó et al. 2019).

Hybridisation is a natural phenomenon that may be a driver of plant evolution (Rieseberg 1995; Piett et al. 2015), and it is common among Mediterranean terrestrial orchids (Luca et al. 2012). However, retro-hybridisation events (that is, crosses between hybrids and parental species) can threat parental species especially for endangered species or with a narrow distribution area (Conesa et al. 2010, Rita et al. 2018). Generally, food-deceptive orchid species, which show weaker pollinator specificity than sexual-deceptive species, rely on post-mating isolation as reproductive barriers to avoid hybridisation. In particular, late post-zygotic barriers such as hybrid sterility (Scopece et al. 2007; Cozzolino and Scopece 2008). This hybrid sterility may be caused by changes in both specific genes and chromosomal rearrangements (Rieseberg and Willis 2007). Indeed, Cozzolino et al. (2005) demonstrated that sympatric food deceptive orchids can share pollinators. On the other hand, competition between conspecific and heterospecific pollen can operate as a post-pollination pre-zygotic barrier limiting the frequency of the formation of hybrids in nature (Luca et al. 2014).

Anacamptis robusta (T. Stephenson) R.M.Bateman is a rare marsh food-deceptive species with a highly fragmented distribution: it occurs in North Africa (Argelia and Morocco) and in Mallorca Island (western Mediterranean basin). Despite Cozzolino et al. (2003) suggested that the European population of *A. robusta* is not been well genetically separated from the wide-spread related species *A. palustris* (Jacq.) R.M.Bateman, Pridgeon & M.W.Chase, we used here the *A. robusta* nomenclature until more scientific evidence resolves its taxonomical position. The population of *A. robusta* in Mallorca consists of several subpopulations (up to 14) very close to each other at the North of the island, most of which are included in a protected area (Albufera de Mallorca Natural Park). The whole population of the species in Mallorca shows dramatic interannual oscillations in the number of reproductive individuals; annual censuses since 1991 showed an oscillation from 166 in 2000 to >8,000 in 2014 (Viada and Maestre 2019). Besides, the species hybridises with the co-flowering and rewarding *A. coriophora* (L.) R.M.Bateman, Pridgeon & M.W.Chase (Bateman and Hollingsworth 2004). *A. robusta* is catalogued as Critically Endangered on the Red List of Spanish Vascular Flora (Moreno et al. 2008) and Endangered on the Red Book of the Flora of the Balearic Islands (Sáez et al. 2017), and it is protected by law as Vulnerable species (Decree 75/2005, BOIB no. 106, 16/07/2005). Moreover, the area where the largest subpopulation occurs (Son Bosc) has been declared a Critical Biological Area in order to protect it from being disturbed according to the corresponding Conservation Plan (BOIB no. 123, 22/08/2009). Several interconnected factors imperil the conservation of the Majorcan population: the loss of natural habitat, the competition with the natural succession, and disturbances related to human activities (such as urbanisation and trampling) (Sáez et al. 2010). Indeed, the attempt to construct a golf course in the nearby area in 2010 of Son Bosc involved dramatic consequences to the pollination networks that became less robust and resilient to future perturbations (Traveset et al. 2018). Besides, a fungus (prob. *Botrytis* sp., E. Moralejo com. pers.) that hamper the flower development has been recently reported in the Son Bosc subpopulation (Sáez et al. 2017).

Reproductive ecology of the species relies on pollinators to set fruit as some trials to perform agamospermy and spontaneous selfing failed in the past (J. Rita, com. pers.). Despite some pollinator census were performed both during the day and night (J. Rita, unpubl. data), no pollination events have never been seen. This is not a surprise due to pollinator visits to flowers in deceptive species are rare events and therefore difficult to record (Claessens and Kleynen 2011). Interestingly, Capó et al. (2017) reported *Megascolia bidens* (Linnaeus 1767) as a floral visitor of *A. robusta* (but not pollinating), which is shared with *A. coriophora* in conspecific areas. Indeed, *Anacamptis* x *albuferensis* R.M. Bateman has been formally described as the hybrid between *A. robusta* and *A. coriophora*, and molecular evidence indicated that *A. coriophora* would act as an ovule-donor whereas *A. robusta* act as a pollen-donor (Bateman and Hollingsworth 2004). However, no experimental crosses between parental species and retro-hybridisation crosses have been performed to evaluate their reproductive success (fruit set and percentage of embryo+ seeds), and in turn the effect on the conservation of the endangered species.

The aims of the present study were threefold: (i) to compare the reproductive success of *A. robusta* in disturbed versus non-disturbed subpopulations in terms of fitness (both fruit set and percentage of embryo+ seeds); (ii) to assess the effect of the coexistence with the rewarding *A. coriophora* on the reproductive success of *A. robusta*; and (iii) to evaluate by hand pollination experiments the role of pollen and ovule donor of *A. robusta* and *A. coriophora* in the hybridisation process, as well as the viability of crosses between parental species and retro-hybridisation events. Our hypotheses are that non-disturbed subpopulations of *A. robusta* would exhibit higher reproductive success than disturbed subpopulations and that the presence of the rewarding *A. coriophora* would increase the reproductive success of the non-rewarding *A. robusta*. Finally, we expect that *A. robusta* would act as pollen-donor while *A. coriophora* would act as ovule-donor. Overall, this study provides valuable data on the effect of the perturbation, the ecological context and the hybridisation phenomenon into the reproductive success of *A. robusta* and therefore helping to the conservation of the endangered species.

2.2. Descripción de la ejecución - Metodología

Study system

The study was conducted in s'Albufera de Mallorca Natural Park located in the north-east of Mallorca (Balearic Islands, Spain). Overall, three subpopulations were sampled in different areas of the Natural Park: Son Bosc (39˚46'N, 03˚07'E), Sa Font (39˚46'N, 03˚05'E) and Ses Puntes (39˚47'N, 03˚06'E). The area mainly includes a marshland-type habitat with a wide range of associated flora and pollinators. Son Bosc landscape, its plant species composition and pollinator communities changed drastically after the attempt to construct a golf course. Especially, the pollinator network was found to be less robust and more nested (Traveset et al. 2018). This perturbation, combined with the introduction of building materials, which induced ruderal (ie. *Glebionis coronaria* (L.) Cass. ex Spach) or non-native species (ie. *Oxalis pes-caprae* L.) to colonize the area, affected the stability of the place. Despite that, the creation of the Critical Biologic Area allowed managers to guarantee the survival of *A. robusta*.

Effect of habitat loss on the reproductive success of A. robusta

The subpopulation located in the disturbed area of Son Bosc was compared to the subpopulations of Ses Puntes and Sa Font, which are included in non-perturbed areas in the Natural Park. Prior to the study, a wireless environmental datalogger (Kestrel® DROP D2) was installed in each subpopulation to obtain air temperature and humidity data during the whole flowering span. Data was collected every hour from 17/04/2019 to 30/05/2019; dataloggers were installed in areas protected against direct sunlight and from rain. After the sampling season, mean day temperature and humidity were calculated as well as minimum and maximum day values. In order to evaluate the impact of the habitat loss in the former subpopulation, 50 individuals of each subpopulation were randomly selected and data about features and reproductive success was obtained. Concretely, the parameters analyzed were the plant height (from the basal part to the highest flower of the inflorescence), inflorescence length (from the lowest flower to the apex), flower number and fruit set (dividing the number of developed fruits by the number of flowers). Besides, we selected 10 fruits per subpopulation at random and ten subsamples of approximately 100 seeds per fruit were observed under a stereomicroscope. The number of seeds with fully developed embryo was recorded from the total sampled seeds as a proxy of seed viability. Spearman correlation indexes between plant features and fitness traits (fruit set and seed viability percentage) were evaluated. Statistical procedures were performed using R 3.6.1 (R Core Team). Generalized linear models (GLM) with binomial distribution were performed using locality as fixed categorical factor and plant features and fitness traits as response variables. Ad-hoc analysis was performed using Tukey test.

Attempting to know the pollinators of *A. robusta* and to obtain information about its floral visit rate in the studied subpopulations, phototrapping cameras (Bushnell® NatureView CamHD) were installed following the method optimized for insects and reptiles explained by Delgado-Fernández & Escobar-Flores (2017). A total of ca. 1,000 pictures were obtained per week in each locality for two months, and the pictures were thoroughly revised to check the putative pollinators. Plants with 4-8 opened flowers with non-visited androecia were selected to record. Cameras were rotating among 5-8 individuals in each population during the flowering season.

Spatial distribution and both interspecific and fungal infection effects on the reproductive success of A. robusta

To test the influence of spatial distribution parameters in the reproductive success of *A. robusta*, all the flowering individuals of 2019 were georeferenced using a differential GPS corrected through *Servei d'Informació Territorial de les Illes Balears* (SITIBSA) network. All GPS points were obtained with a maximum deviation of 0.05 m using the Leica System RTK RX 1200 and the Leica TPS800. At the same time, the plant features of *A. robusta* (see above), as well as the fruit set and visual signals of fungal infection were collected and associated with each position. In order to test interspecific influence between *A. robusta* and the conspecific *A. coriophora*, the position of the all *A. coriophora* individuals was also recorded.

With the spatial information, we obtained a relative position for each individual of *A. robusta* within the subpopulation. The minimum bounding geometry was calculated with a convex hull enclosing, and the resulting polygon was divided into 3 concentric parts by tertiles. Then, there was assigned to each individual a qualitative value of its position (external, intermediate or interior) (Vandewoestijne *et al.* 2009). Moreover, we generated a density grid that enclosed the subpopulation of *A. robusta* by generating cells form 1 × 1 m to 10 × 10 m and the number of plants per cell was calculated. Finally, we calculated the nearest neighbour distance (NND) for each individual between *A. robusta* (hereafter $NND_{robusta}$) and the NND for each *A. robusta* to the nearest *A. coriophora* (hereafter $NND_{coriophora}$). All these calculations were associated to each individual of *A. robusta* and were performed with Quantum GIS version 3.10.1 software (QGIS Development Team 2009).

To test the effect of relative position in the population and both intraspecific and interspecific plant density on the fruit set of *A. robusta,* a GLM with a binomial error structure and a logit link function. First, we selected the most pertinent cell size for the subpopulation by selecting best-fitting models based on Akaike Information Criterion corrected (AICc) for small sample sizes (Zuur *et al.* 2009) and using the '*dredge'* function in the '*MuMln'* package (Barton 2019) for automatic model selection. Only the *"intercept"* was fixed during the procedure of model selection, and the maximum number of variables was limited to one, to select a unique cell size. The appropriate cell size was 5 × 5m with no other model with ΔAICc ≤ 2. Second, for the resulting GLM the explanatory variables were relative position ($n = 3$) and density intervals ($n = 5$), and the response variables were fruit set and fungal infection. Finally, we generated a GLM where the explanatory variables were $NND_{robusta}$ or $NND_{coriophora}$ along with their interaction with plant height, and the response variable was fruit set.

Hybridization processes and interspecific compatibility

We performed a hand-pollination experiment to evaluate the interference of *A. x albuferensis* in the reproduction of *A. robusta* and the hybrid origin of *A* x *albuferensis* (Figure 1). The following treatments were performed in both parental species: (i) cross-pollination by a single pair of pollinia (1xALO), to test the intraspecific reproductive efficiency; (ii) cross-pollination by three pairs of pollinia (3xALO), to test the effect of pollen quantity into the intraspecific reproduction; (iii) interspecific pollination (INTER), to test the pollen and ovule donors that develops the hybrid individuals; and (iv) retrohybridization (HYB), to test the interference of hybrids to the parental reproduction. After the fructification of the individuals, data about fruiting success or failure were recorded and developed fruits were sampled to assess the seed viability (as explained above). The data collected were analyzed using generalized linear models (GLM) following binomial distribution and using fruit set and seed viability percentage as response variables and treatments as a fixed factor. After that, ad-hoc analysis was performed using Tukey test. Besides, pollen limitation index was also calculated following Larson and Barrett (2000) equation as:

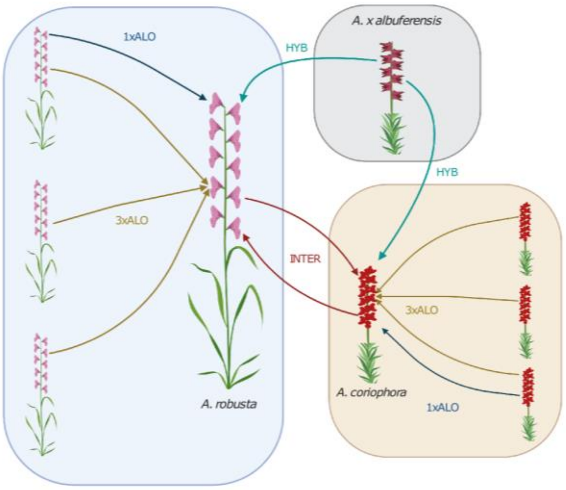

Figure 1. Experimental scheme of the hand-pollination experiment. Treatments performed were cross-pollination with one pair of pollinia (1xALO) and three pairs of pollinia (3xALO); interspecific crossing (INTER) and hybrid-parental pollination (HYB).

PL = 1 – (control fruit set / cross-pollination treatment fruit set).

2.3. Resultados obtenidos (cumplimiento de objetivos)

Fitness in disturbed vs. natural subpopulations

Differences among populations were found for *A. robusta* features as plant height (df = 2, F = 8.16, p < 0.001) in inflorescence size (df = 2, F = 11.05, p < 0.001), flower number (df = 2, deviance = 42.60, p < 0.001); as well as in plant fitness as fruit set (df = 2, deviance = 7.84, p = 0.019) but not in seed viability (df =2, deviance = 115.57, p = 0.897). Mean daily temperature and humidity showed similar patterns among subpopulations (Figure 2) including temperature variation from 7.5 – 27.0 ˚C and humidity variation from 45 – 100 %.

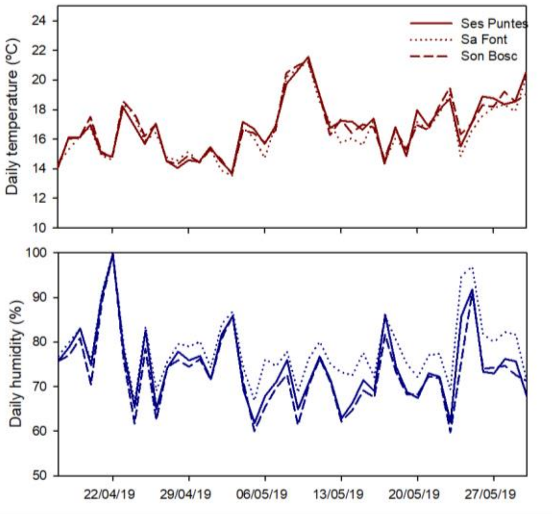

Figure 2. Daily temperature (red) and humidity (blue) in the studied populations during the flowering time. Data was collected from 13/04/2019 to 30/05/2019.

Concretely, plants in Sa Font were 45 cm (± 2.31) in height, while in Son Bosc and Ses Puntes, were 38 cm (± 1.77) and 34.13 cm (± 1.14) respectively (Figure 3). Same results were observed for the inflorescence size: 12.37 cm (± 0.63) compared to 8.93 cm (± 0.47) and 9.97 cm (±0.46); and in flower number: 17 (± 1.25) compared to 11 (± 0.72) and 13 (± 0.80) (Figure 3). Regarding the plant fitness, when comparing fruit set among subpopulations, Son Bosc showed the lower fruit set values [18.5 % (± 4.41)] against Ses Puntes and Sa Font [29.6 % (±3.56) and 27.1 % (± 3.68), respectively].

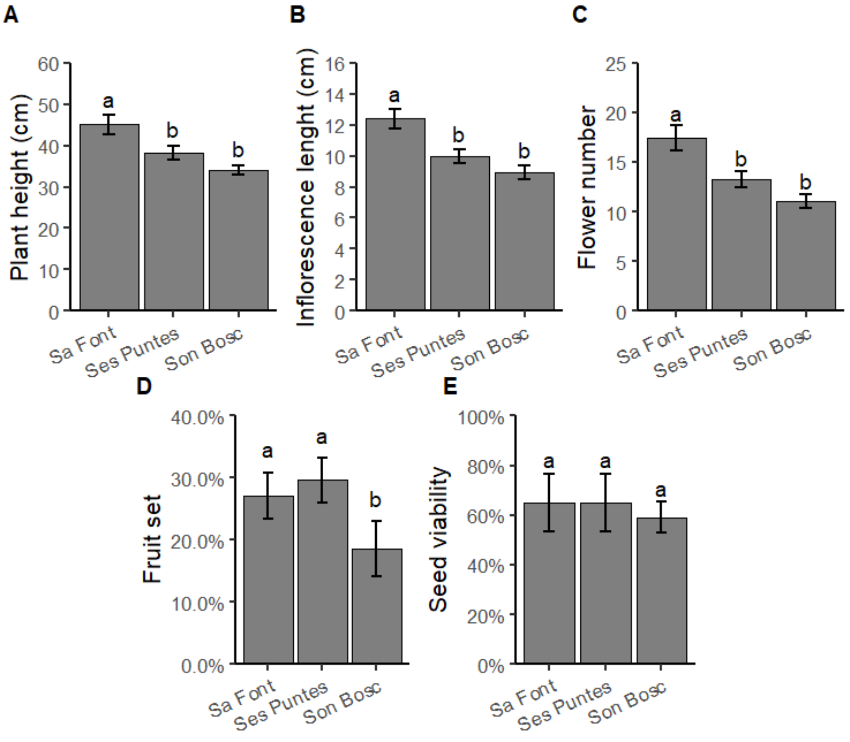

Figure 3. Mean of plant features analysed (A: plant height, B: inflorescence length, C: flower number) and reproductive success variables (D: fruit set and E: seed viability). Error bars indicate standard error. Letters indicate ad-hoc Tukey groups.

When analyzing differences in fruit set among subpopulations using plant features as a random variable in a mixed model, differences between subpopulations were still detected (df = 2, F = 3.17, p < 0.001). When correlating plant features, significant correlations were found for all the traits studied (height, inflorescence size and flower number), but in none of those variables correlated with the fruit set (Figure 4). No observation of pollinators has been reported in phototrapping cameras installed on each population studied. False positives were obtained mainly caused by movement of neighbor plants due to wind.

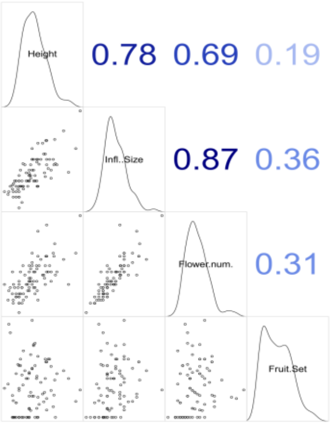

Figure 4. Correlation of plant features and fruit set values in the studied subpopulations. Upper panel indicate Tukey correlation indexes, diagonal panel indicate the distribution of variables analysed and lower panel indicate the correlation plot between pairs of parameters.

Spatial distribution and both interspecific and fungal infection effects on the reproductive success

Since all the models showed overdispersion, a quasibinomial distribution was applied instead. We found significant differences in the fruit set values for cells of 5 × 5m (df = 4; deviance = 30.40, p < 0.001) but *post-hoc* showed that only a single high-density cell had higher fruit set, therefore no significant effects of intraspecific density in reproductive success can be assumed. However, relative position within the subpopulation showed significant differences (df = 2, deviance = 76.52, p < 0.001). The external individuals showed higher fruit set than the rest of the subpopulation (Figure 5), although most of the subpopulation was found in this position.

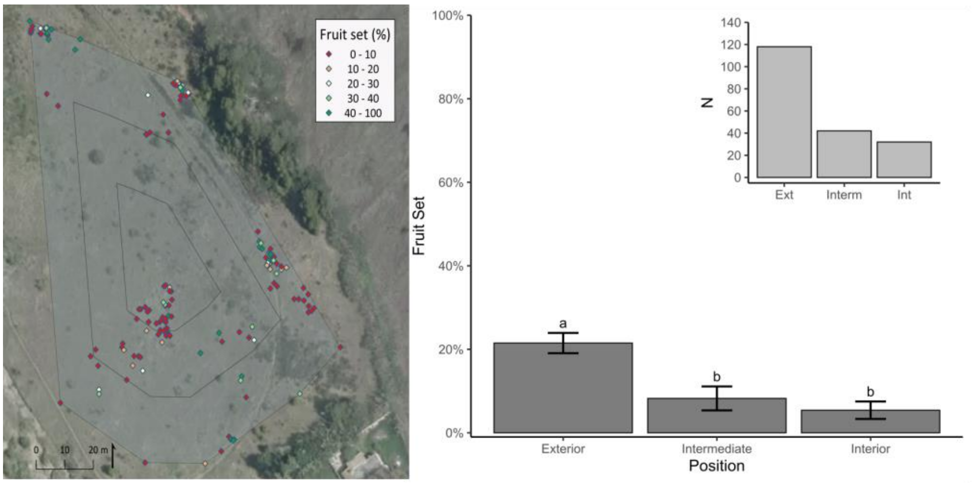

Figure 5. Spatial distribution of plants analysed (left) illustrating each fruit set value (heatmap) and fruit set means grouped by the position in the subpopulation (right). Number of individuals included in each group is indicated up to the right. Error bars indicate standard error and letters indicate ad-hoc Tukey groups. Heatmap scale has been corrected by the maximum values of the fruit set of the population.

No intraspecific effects for $NND_{robusta}$ were found (df = 1, deviance = 0.42, p = 0.725) and $NND_{robusta}$ did not show any interaction with plant features or $NND_{coriophora}$ (df = 1, deviance = 0.67, p = 0.658 and df = 1, deviance = 2.91, p = 0.352 respectively). On the contrary, we found significant interspecific effects for $NND_{coriophora}$ df = 1, deviance = 25.85, p = 0.005) with no interaction with the individual height (df = 1, deviance =0.83, p = 0.617). The equation of the model showed a negative effect of $NND_{coriophora}$ in the reproductive success of *A. robusta* [Fruit set = 0.0166 * $NND_{coriophora}$ – 2.0908]. Therefore, the proximity of *A. coriophora* to *A. robusta* decreased the fruit set of the latter.

Finally, regarding the fungal infection, the relative position also showed significative differences (df = 2, deviance = 13.87, p < 0.001) but the infection was more present in the internal portion of the subpopulation (Figure 6). Moreover, fungal infection had a negative effect on the reproductive success (df = 1, deviance = 227.81, p < 0.001).

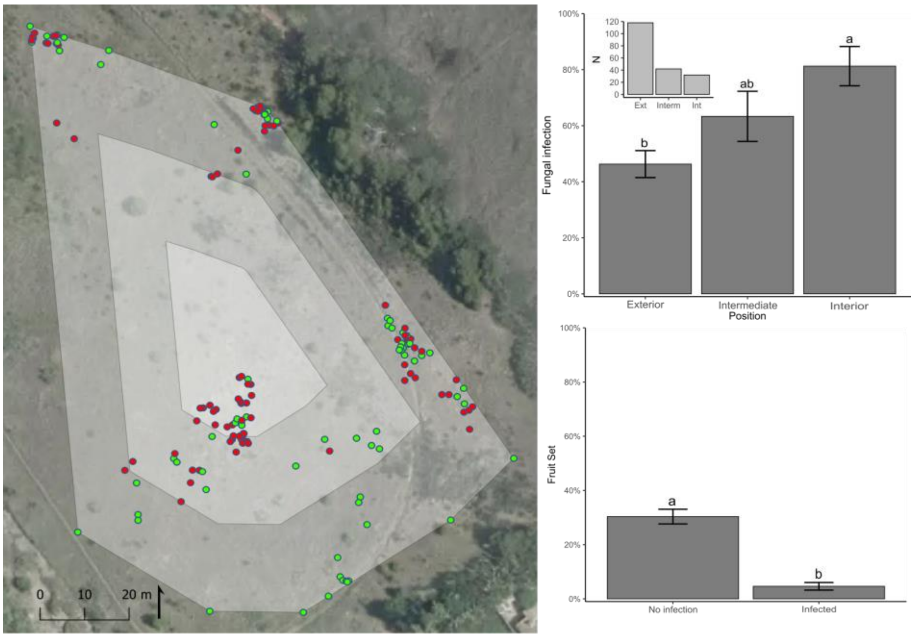

Figure 6. Fungal infection distribution in Son Bosc subpopulation. The map (up) shows the distribution of infected (red) and non-infected (green) individuals in the population area (green). Graphs shows the percentage of fungal infection in function of the spatial location in the population (left) and the fruit set differences between infected and non-infected individuals.

Hybridization process and reproductive limitations between species

Results obtained in the hand-pollination experiment are shown in Figure 7. Fructification was successful in cross-pollination treatments in both species. In *A. robusta*, differences in the fruit set between control and cross-pollination treatments indicated that the species suffers from pollen limitation (PL = 0.56), despite the opposite was obtained for *A. coriophora* (PL = 0.01). No differences were found for seed viability depending on the pollen quantity used in cross-pollination (one pair of pollinia, 1xALO, or three pairs of pollinia, 3xALO).

Interspecific outcrossing resulted in low levels (10 %) of fruit set when *A. robusta* acted as ovule donor, although the developed fruits did not produce any viable seeds. On the contrary, *A. coriophora* acting as ovule donor produced 45 % of viable fruits and from those, we obtained 17.33 % of viable seeds. Thus, the hybridization event was unidirectional, and *A. robusta* was the pollen donor and *A. coriophora* the ovule donor. In the case of retrohybridization events, fecundation using pollen from hybrids was only viable in *A. coriophora* ovules, which resulted in a fruit set of 40 % and produced 8.88 % of viable seeds.

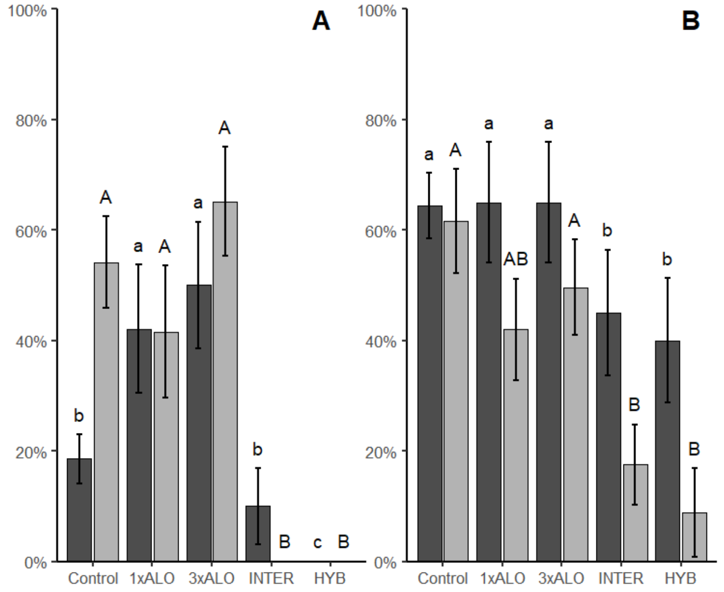

Figure 7. Mean of the fruit set (black bars) and seed viability (grey bars) resulted in each hand-pollination treatment performed in *A. robusta* (A) and *A. coriophora* (B). Error bars indicate standard error. Ad-hoc Tukey groups are indicated by lowercase letters (fruit set) and capital letters (seed viability).

2.4. Conclusiones y valoración de la ejecución

Implications of human disturbance for the conservation of A. robusta

As explained above, Son Bosc suffered from human disturbance and it is reported in the literature that plant community and pollinator network were affected. Our results showed that despite the subpopulation is maintained in the area and plant features such as height, inflorescence size and the number of flowers are similar to other natural subpopulations, there is a significant reduction in fruit set and seed viability.

Pollinator decline due to perturbation might be the main cause of the reduction in plant fitness (Cunningham 2000). Additionally, the disturbance may act as a driver for the introduction of non-native species, as observed in salt marshes in California (Martone & Wasson 2008), and thus they can result into new competitors for pollinators (Brown et al. 2002). Pollinator species of *A. robusta* are still unknown, despite we installed trapping cameras in the studied subpopulations, no floral visitors were detected in the studied subpopulations. Although the species probably receives a very low rate of visits, as has been widely reported for deceptive orchids (Neiland and Wilcock 1995, Tuomi et al. 2015), *A. robusta* reproduction relies on the action of pollinator is essential for its reproductive success (Rita and Vicens, unpubl. data). Under this scenario, to guarantee the conservation of this species in the Critical Biologic Area, the role of protecting pollinators is essential to ensure the plant fitness in a long-term period, as it has been seen for other non-rewarding orchids in the Balearic Islands (Capó et al. 2019).

Effect of biotic factors in the reproductive success of A. robusta

Despite Son Bosc is affected by human disturbances, there are other natural factors affecting both positively and negatively to the reproductive output of the species. For instance, it is considered that deceptive species can use rewarding ones as magnet species (Johnson et al. 2003), or sometimes they can enter in competition for pollinators (Tuomi et al. 2015). In Son Bosc, *A. robusta* coexists with the rewarding *A. coriophora* and because of the hybridization events, it is obvious that some pollinators are shared. In this sense, our results show that *A. robusta* is affected negatively by the proximity to *A. coriophora*. The latter species can attract pollinators by scent and nectar reward, being its visitation rate higher than nectarless species (Smithson 2006). Contrary to our hypothesis that *A. coriophora* may act as magnet species to *A. robusta*, our results showed that the individuals with a higher fruit set were found in the farthest areas from *A. coriophora* indicating competition for pollinators between both species. These results supports the fact that there are few hybrids in the study site despite the high abundance of both parental species therein.

Additionally, the existence of a fungal infection (probably by *Botrytis* sp.) in Son Bosc affected negatively the floral development of a wide range of individuals. This fungus has been reported to be lethal in a broad-spectrum of angiosperms worldwide, especially in crops (Williamson et al. 2007). Results indicate that the fungal infection is mainly distributed in the core of the subpopulation, and the infection affects strongly plant fitness (the fruit set of the infected plants was 5% compared to 30% of the non-infected plants).

Overall, the habitat in Son Bosc results into a complex ecological system in which human perturbation, interspecific competition for pollinators and fungal diseases are interfering with the reproductive success of the threatened species. From the other subpopulations located in the natural park (ca. 13 sites), the coexistence with *A. coriophora* is absent and the fungal infection has never been reported. For this reason, the conservation of this threatened species in Son Bosc strongly requires the consideration of the ecological context, especially avoiding the propagation of the fungus into peripheric individuals or even into other non-affected subpopulations. Further studies would be necessary to reveal the taxonomic identify of the pathogenic fungus and assess the causes of its introduction in Son Bosc.

Ecological implications of the hybridization events between A. robusta and A. coriophora

Anacamptis x *albuferensis* is originated by the outcrossing of *A. robusta* and *A. coriophora* as Bateman & Hollingsworth (2004) reported for the first time. Coinciding with the results therein, *A. robusta* can act uniquely as pollen donor whilst *A. coriophora* acts as ovule donor. From the results obtained in this study, it is observed that the fruiting success of the hybridization is high (45 %) but the seed viability decreases (17.53 %). This data combined with the germinative difficulties in orchid seeds (Arditti 1967) suggests that the hybridsation phenomenon is rare in natural conditions and barely interferes with the population stability of *A. coriophora*. On the other hand, no data has been provided about the *A.* x *albuferensis* autocompatibility because all the hybrid individuals collapsed in the pre-flowering stage due to the fungal infection in the reproduction season of the study. Some preliminary tests done by Rita & Vicens (unpublsh. data) showed that hybrids can fructify when pollination is hand-assisted, but the corresponding seeds are completely sterile. Thus, the survival of hybrids is strongly dependent on the parental species intercrossing.

Additionally, it has been proved that *A.* x *albuferensis* can retrohybridize with *A. coriophora* successfully producing viable seeds, despite its percentage is very low (8.9 %). This phenomenon has been reported in other threatened species such as *Lotus fulgurans* (Porta) D.D.Sokoloff (Conesa et al. 2010) stating that hybridization can result in a remarkable threat when the narrow species is acting as ovule donor. In this case, *A. robusta* is not able to fructify when is pollinated with pollen of *A.* x *albuferensis*, nor able to develop viable seeds when is pollinated with pollen of *A. coriophora*, then we assume that the species does not present any threat related with the existence of the hybrid. Moreover, further studies must be done to asses if the retrohybridization of *A.* x *albuferensis* with *A. coriophora* could be affecting the genetic structure of *A. coriophora* population.

Ecological perspectives to face the threats of A. robusta

Food deceptive orchids are generally strong pollinator-dependent (Macdonald et al. 2015) and their reproductive success is low (Jacquemyn et al. 2007). In contrast with rewarding species, deception allows plants to avoid geitonogamy mediated by the behaviour of the pollinator (that is, low visit frequency and improbable repetition of flowers allow populations to avoid geitonogamy events) (Johnson & Nilsson, 1999; Capó et al. 2019). This strong pollinator dependence indicates that the stability of the orchid population is linked to pollinator interaction.

On the other hand, the arrival of alien species in the habitat affects ecological networks worldwide (Carvalheiro et al. 2008; Hui et al. 2016) including not only non-native animals or plants but also fungi and bacteria (Loo 2018). The infection of *A. robusta* by fungi, probably *Botrytis* spp., decreases drastically its fructification rate and sometimes, prior to flowering time, the inflorescence is unable to develop correctly. This infection is reducing the natural reproductive success of the species in Son Bosc, which predicts a potential risk for the species in other population if the fungus arrives. As is done in many crops (Morgan 1984; Fedele et al. 2020), avoiding the fungi propagation and preventing from the internal expansion inside the affected subpopulation, can avoid future infections into other subpopulations and improve plant fitness in the affected subpopulation.

Conclusions

Anacamptis robusta showed a lower fruit set in disturbed populations compared to natural populations, which could be explained by a reduction of pollinators and the infection of a fungal pathogen, probably *Botrys* sp. The coexistence with the rewarding *A. coriophora* also affects negatively to *A. robusta* reproductive output, as there is a negative correlation between plant fitness and plant distance. Thus, this refuses our hypothesis of *A. coriophora* acting as a magnet species for pollinators and demonstrate the competition for pollinators between both species. In hybridization events, *A. robusta* acts only as pollen donor and *A. coriophora* as ovule donor. Occasionally, the hybrid *A.* x *albuferensis* pollinia is functional when crossing with *A. coriophora* ovules providing a hybrid F2. Thus, the conservation of *A. robusta* is not jeopardised by the hybridization phenomenon.

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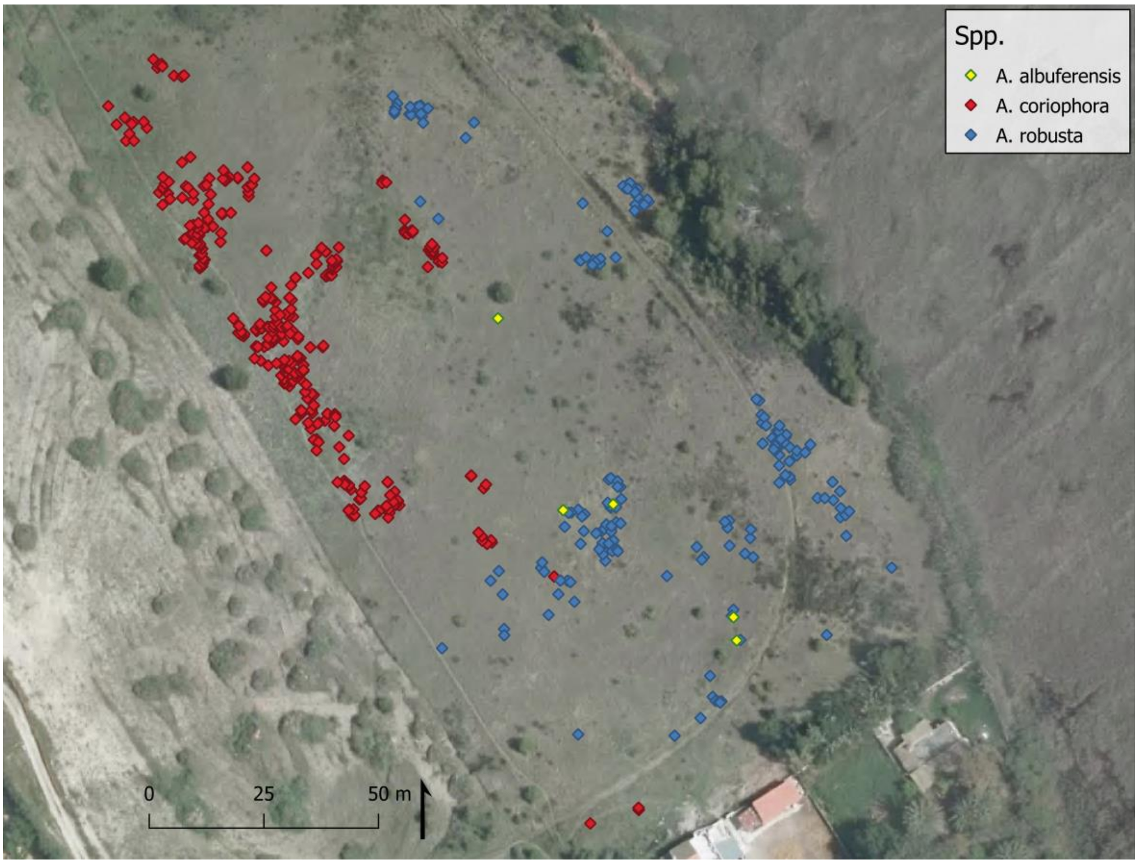
Supplementary information



S1. Distribution map of individuals of *A. robusta* (blue dots), *A. coriophora* (red dots) and *A.* x *albuferensis* (yellow dots) in Son Bosc Critical Biological Area.

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2.5. Publicaciones resultantes

2.5.1 Publicaciones en revistas científicas

Capó M, Borràs J, Perelló S, Rita J, Cursach J. 2020. *The reproductive ecology of the food deceptive Anacamptis robusta (Orchidaceae): Habitat disturbance, interactions with co-flowering rewarding orchids and hybridization events.* (In preparation)

2.5.2 Participaciones en congresos

Capó M, Borràs J, Perelló S, Cursach J, Rita J. 2019. *Sexo, mentiras y engaños: la extravagante reproducción de las orquídeas*. Oral communication. II Congreso y IV Jornadas de Jóvenes Investigadores en Formación: Fomentando la Interdisciplinariedad. Granada, Spain.

→ Premio a la mejor comunicación oral en el "*II Congreso y IV Jornadas de Jóvenes Investigadores en Formación: Fomentando la Interdisciplinariedad*"

Capó M. 2019. *ECOPRAT: Reproductive ecology of the threatened orchid Anacamptis robusta: Habitat alterations, mutualisms and hybridization*. Poster communication. XVI Reunión Anual de la Red Temática Ecoflor (Ecología y Evolución Floral). Granada, Spain.

2.5.3 Contribuciones técnicas y divulgativas

Presentación de los resultados al personal del Parque Natural de s'Albufera de Mallorca y recomendaciones de gestión de la especie a partir de los resultados obtenidos. (Fecha por definir, actividad confirmada por el director del PN).

Participación en el programa de radio Balears Fa Ciència (IB3 Radio) en la emisión del sábado 08/02/2020 para divulgar la ejecución y las conclusiones del proyecto. (Actividad confirmada por el programa).

3. **Informe de gastos del proyecto.** Relación de partidas de gastos y sus importes. Se deberán aportar justificantes originales de los pagos realizados (tickets, recibos o facturas).

Núm. factura	Concepto	Euros (€)
1	Sobres 60X110mm (1000 ud).	42.73
2	Pack de contadores manuales (6 ud)	15.09
3	3 X Kestrel Drop Datalogger	372.67
4	Desplazamiento y Alojamiento ECOFLOR2019	252.27
5	Impresión poster ECOFLOR2019	48.79
6	3 X Bushnell® NatureView CamHD	627.90
7	Etanol + Portaobjetos	64.65
8	Tela exclusión	39.02
9	3 X Tarjetas fototrampa	19.98
10	Pilas recargables, cinta aislante y adhesivo	90.64
11	3 X Pack estaquillas (100 ud)	11.85
12	3 X Cintas de color y 1 X cinta métrica	42.80
13	Pistola encoladora y barras de cola	13.70
14	Gastos desplazamiento en vehículo	358
	TOTAL	**2000.00**

Fdo:

Miquel Capó Servera

en Palma, a 31 de enero de 2020