



Università degli Studi di Cagliari

DOTTORATO DI RICERCA

IN SCIENZE E TECNOLOGIE DELLA TERRA E DELL'AMBIENTE

Ciclo XXXI

Patterns of reproductive isolation in Sardinian orchids of the subtribe Orchidinae

Settore scientifico disciplinare di afferenza
Botanica ambientale e applicata, BIO/03

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Esame finale anno accademico 2018 – 2019
Tesi discussa nella sessione d'esame Febbraio –Aprile 2019

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Chapter 1

Abstract

Orchids are globally well known for their highly specialized mechanisms of pollination as a result of their complex biology. Based on natural selection, mutation and genetic drift, speciation occurs simultaneously in organisms linking them in complex webs called ecosystems. Clarify what a species is, it is the first step to understand the biology of orchids and start protection actions especially in a fast changing world due to human impact such as habitats fragmentation and climate changes. I use the biological species concept (BSC) to investigate the presence and eventually the strength of mechanisms that limit the gene flow between close related taxa. Islands are considered *natural laboratories* due their unique evolutionary trends which have led to a flora whose biology remains, in many cases, unknown and controversial. The main outcomes of this thesis are: i) clarify the number of orchid species currently recorded in Sardinia and discuss the relevance of studying orchids on island conditions; ii) adopting the biological species concept, investigate zygotic barriers in two endemic close related *Ophrys* species and iii) discuss the zygotic barriers in the two most important functional groups of deception in mediterranean orchids.

Riassunto

Le orchidee sono globalmente riconosciute per i loro specializzati meccanismi di impollinazione come risultato della loro complessa biologia. Guidata dalla selezione e dalla deriva genetica, l'evoluzione avviene simultaneamente negli organismi legandoli in una rete complessa chiamata ecosistema. Il chiarimento di cosa sia una specie rappresenta il primo passo per la comprensione della biologia delle orchidee e quindi sviluppare azioni di protezione soprattutto in un mondo in rapido cambiamento a causa dell' impatto umano come il la frammentazione degli habitat e la gestione dell'ambiente. Ho adottato il concetto biologico di specie (BSC) per investigare la presenza ed eventualmente la forza dei meccanismi che limitano il flusso genico tra specie strettamente imparentate. Le isole sono considerate dei laboratori naturali a causa dei loro unici meccanismi di evoluzione che hanno portato ad una flora la cui biologia risulta essere, in molti casi, controversa o ancora sconosciuta. I principali obbiettivi di questa tesi sono: i) chiarire il numero di specie che sono attualmente presenti sull'isola di Sardegna e discutere l'importanza dello studio delle orchidee in situazioni di insularità; ii) adottare il concetto biologico di specie per investigare le barriere zigotiche in due specie sorelle ascritte al genere *Ophrys* e iii) discutere le barriere zigotiche nei due più importanti gruppi funzionali di inganno che si riscontrati nelle orchidee del Mediterraneo.

Preface

This thesis explores mechanisms of reproductive isolation in Sardinian orchids of the subtribe Orchidinae.

In the introduction, I briefly discussed the concept of species, reproductive isolation mechanisms and orchids as model system to investigate patterns of speciation.

In the first thesis chapter, I explored the relevance of orchids in continental island conditions for evolutionary and conservation research and why they should have more attention. Indeed, many studies carried out on island orchids are focused on oceanic islands species, less are on continental islands, in many cases the knowleges are extremely fragmented. In order to update the check-list of the orchids in Sardinia, I reviewed accademic and not accademic literature and implemented knowledge with data obtained from field activity. Due to their popularity, I also presented an artificial key which could be useful for field recognition. My final list comprises 63 species and 14 genera: thirteen species are recognized as exclusively of Sardinia. This richness reflects not only the interesting biogeographic history of Sardinia but also its diversity in habitats.

In the second chapter, I use the biological concept of species to investigate the existence of barriers that limit the gene flow between two endemic sister species within the sexually deceptive *Ophrys* genus: *Ophrys annae* and *Ophrys chestermanii*. Due to the hyper-specialized pollination syndrome, in *Ophrys* the role of postmating barriers has been traditionally thought to be weaker than premating. To evaluate the gene flow, I proceeded with intra and inter-specific manual crosses. The most relevant result was in manual crosses suggesting that postmating barriers might act as reinforcement of premating barriers; these crosses demonstrated an asymmetry in seed viability. Ecological analyses suggested that the two endemisms differ in habitat preferences underlying habitat preferences as a strong limit to gene flow.

The third chapter explores the “*lock and key hypothesis*” in orchids. This hypothesis is largely investigated in animals and it assumes that male and female genitalia match in a unique system to prevent interspecific crosses. As system model, I compared two functional groups of orchids on the base of their pollination strategy (food and sex deception). Here, I propose the Reproductive Standardization Index (RSI) to summarize the relationship among the stigmatic cavity length and the pollinarium one, calculated as the ratio between stigmatic cavity length and pollinarium length. I evaluated the variation of sterile and fertile traits and correlated male and female reproductive organs with the aim to define whether a specific morphology is promoted by evolution. Results showed that the functional morphology of fertile traits plays a pivotal role in limiting the gene flow in species that grow in sympatry. In particular it was observed that the Reproductive Standardization Index (RSI) is significantly different in the two pollination strategies and that the correlation between pollinium length and stigmatic cavity length is stronger in food deceptive species when compared to the sex deceptive ones.

The final chapter provides a general overview and synthesis on the key findings across the three case studies of the thesis, linking them to the current knowledge on orchids and their reproductive isolation and suggesting also potential conservation actions and directions for future research.

Chapter 2

Introduction

Concepts of species.

In biology no other concept has generated a vivid debate as the concept of species. The definition of this concept is crucial because it represents the starting unit of the process of evolution. Living forms vary from extremely simple as bacteria like *Mycoplasma pneumoniae* to extremely complex animals and plants such as *Homo sapiens* or orchids. In 1888, Darwin was aware that the variability of life could hardly have fallen into a single species concept so much that he wrote: “*Nor shall I here discuss the various definitions which have been given of the term species. No one definition has satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species*”. In evolutionary biology, this debate has remained vivid through the past two centuries. Mayr (1942) defined this debate as “*the species problem*” and more recently in 2001 Hey listed twenty-four potentially valid definitions of this concept.

In the 18th century, Linnaeus defined the species concept adopting morphological approach: organisms were organized in species on the base of sharing well defined morphological and anatomical features. In 1904, Poulton posed attention on mechanisms of reproductive isolation as a discriminating feature between species (Mallet 2004). This concept was then adopted by Dobzanky (1937) and Mayr (1942) who defined it including the role of the gene flow and its limits between different species as a “*groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups*”. This definition is generally called biological species concept (BSC). The “ecological species concept” was introduced by Van Valen in 1976. This definition takes into account the ecological niche concept as a relevant aspect of the concept of species so two groups of organisms could be defined separated species if they are ecologically different and even with ongoing gene flow. In 1989, Craftcraft implemented the debate defining a

species as “an irreducible (basal) cluster of organisms, diagnosable distinct from other such clusters, and within which there is a parental pattern of ancestry and descent”. Fundamental was the discovery of DNA and its adoption to explain evolutionary relationships between organisms (Craftcraft 1989).

Despite the twenty-four species concepts (Hey, 2001), currently the biological species concept (BSC) is the most accredited and the other definitions are in some way related to it.

Speciation.

Speciation is the process by which new species arise (Coyne & Orr 2004). Despite being of primary importance, our understanding of this process is still limited and it is strictly linked to the definition of species we apply. Using the biological concept of species, speciation can be viewed as the evolution of mechanisms of reproductive isolation between populations of the same species (Dobzhansky 1937). Ernst Mayer (1942, 1957) identified different ways of speciation in relation with geographic distance of the populations on which the evolution works.

Allopatric speciation occurs when one, or more, geographic barriers such as migration, local extinction, or geographic events interrupt gene flow among populations of the same species and so each population evolve separately (Coyne & Orr 2004). If the isolation persists for a sufficient period of time, they will have accumulated so many genetic differences that they will be reproductively isolated if they come into a second contact (Darwin 1859, Turelli *et al.* 2001).

Parapatric speciation occurs between populations that are not completely geographically isolated (Turelli *et al.* 2001). The distributions of these species overlap thus limiting gene flow and are often limited with abiotic gradients. Migrations between the populations involved are limited often because they are distributed along one or more abiotic gradients (Coyne & Orr 2004). This model might start in allopatry, but another scenario might start with the increasing of the distribution area. In each environment, each population is affected by directional selection and in overlapping area

hybrids are not favored, limiting the gene flow. Within the subpopulations, accumulated mutations might lead to reproductive isolation and finally to two different species, and thus, at the same time, in the overlapping area, hybridization zone will disappear.

The model called *sympatric speciation* occurs when two populations are not geographically isolated and they diverge as a consequence of a stable genetic polymorphism transmitted through generations (Dobzanky 1937, Mayr 1942). Initially, this model caused a theoretical debate because the answer to the question if incomplete reproductive isolation between subpopulations under divergent selection allow speciation was controversial (Via 2001). However, many recent studies show that this scenario is more likely to be plausible. Competitive speciation in lake fish, for instance, or parasites that shift hosts or plants with different anthesis or that shift pollinators are valid and can be considered as an easy exemplification of how this model is diffused (Wilson *et al.* 2000, Olsson *et al.* 2006, Breitkopf *et al.* 2015).

In plants, mechanisms such as hybridization and polyploidy play an important role which can lead in very short period of time to speciation (Stebbins 1950, Smocovitis 2001, Larson 2004,) Speciation by hybridization might arise when individuals of different species generate hybrids that are fertile, able to reach sexual maturity and they are in someway favored in some ecological niches. Two different models of speciation by hybridization are currently recognized: by homoploidy and polyploidy. The former model is when hybridization occurs between species with the same ploidy, the latter when hybridization occurs between two species with different chromomes numbers and the duplication of resulting chromosomes set (allopolyploidy) or after conspecific genome duplication (autopolyploidy) (Soltis & Soltis 2000).

The core of ecological speciation is the different ability of the individuals to adapt: in this model of speciation natural selection plays the main role (Van Valen 1976). Reproductive isolation is driven by the divergent selection of the enviroment and the interactions within the population. Due

to the fact that the evolutive processes that lead to reproductive isolation are different, ecological speciation might rise both in sympatry and allopatry.

Reproductive Isolation Mechanisms

The focus of biological species concept (BSC) consists in reproductive isolation mechanisms (RIMs), barriers that limit the gene flow (Dobzanky 1937, Mayr 1942). These barriers are defined according to whether they appear before or after zygotic's formation. Prezygotic barriers limit fertilization and they are:

-Habitat isolation, the two species do not meet.

-Temporal isolation, the two species have a different period of reproduction during the year.

-Mechanical isolation, male and female reproductive organs are morphologically different.

-Gametic isolation, biochemical interaction between male and female gametes prevent fertilization.

Postzygotic barriers keep isolation between two species after the formation of an hybrid zygote.

They are:

-Mortality of embryos, organisms do not exceed the embryonic stage.

-Incapacity of hybrids to reach sexual maturity (degeneration of hybrids).

-Reduced fitness of hybrids as they occupy an ecological niche intermediate between those of parental species. In plants, the probability of hybrids of being pollinated by another hybrid is very low.

All reproductive isolation mechanisms can vary greatly from species to species and often in the species studied such mechanisms are never present individually. It has been shown that these

mechanisms are not fixed and themselves are subject to temporal variation, depending on the evolutionary history of the species involved.

The model system

In 1859 Charles Darwin concluded his most revolutionary book “*On the Origin of Species*” with the sentence: “*There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.*” This amazing ending seems to be written specifically for orchids which are the subject of his following publication “*On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing*”. In this book Darwin explains the revolutionary theory of evolution previously expressed in the *On the Origin of Species*, free from ethical and theological implications related to the animal kingdom.

The family of Orchidaceae is probably the most recognizable family within the plant kingdom due to its fascinating and peculiar flowers (Ayasse *et al.*, 2000; Bateman & Rudall, 2006; Inda, Pimentel, & Chase, 2012; Chase *et al.*, 2015). Orchids have a cosmopolitan distribution, most of them live in tropical forests and over 80% of those are epiphytes, in contrast the orchids distributed in all the other ecosystems are terrestrial (Chase *et al.*, 2015). Orchids display an extraordinary floral diversity, with adaptations to different pollinators among close relatives, partitioning of individual pollinators by precise placement of pollinia on different parts of their bodies, and extreme convergence and divergence among crossable taxa. Floral displays vary in colors, patterns, shapes and scents which alone or in combination can act as signals for attraction of animal pollinators (Wilmer, 2011). Pollinator’s perception and its associated behavior plays a main role in selective environment for floral traits because it mediates the relationships between floral signals and pollen transport (Schiestl, 2013). In plants, the evolution of reproductive isolation is achieved typically by a combination of pre and postzygotic mechanisms that influences the gene flow

(Scopece, Widmer, & Cozzolino, 2008). The high specificity between plant and pollinator is considered a key mechanism for maintaining species boundaries in many orchids (Gill 1989; Tremblay *et al.*, 2005). On the base of the strategy adopted, orchids can be divided into those who give a reward, often sugar rich substance such as nectar, to pollinators and those who deceive pollinators promising a nest, sex or a food-rewards. One-third of all orchids offer no reward and thus attracts pollinators through deceptive strategies (Tremblay *et al.*, 2005). The most widespread systems of deception are food and sexual. For instance, the genera *Anacamptis*, *Dactylorhiza*, *Neotinea* and *Orchis s.s.* attract and deceive a high number of pollinators species miming other nectariferous plants (Dafni, 1983): they emphasize parts of flowers, such as spur, which contain the reward such a nectar so they are less species-specific (Xu, Schlüter, & Schiestl, 2012, Schiestl, 2002). In contrast, a highly specific pollination is typical of the genus *Ophrys*: to deceive its pollinator this strategy involves visual, tactile and even odor cues to imitate female so the male tries to mate with the flower (Ayasse *et al.* 2000) .

Given the biological species concept, the reproductive mechanisms of orchids and the insularity condition of Sardinia, orchids can be advantageously adopted as a system model to investigate trends of evolution.

Aim of the study

The aim of this PhD thesis is to discuss and analyze patterns of evolution in island condition in Orchidaceae. Initially a review of orchids of Sardinia is proposed to make the state of the art. The outcomes of this first paper is to give an updated check-list of orchids of the island. Here, I reviewed academic and not academic papers and implemented knowledge with new data. Due to the unclear systematic position of some taxa, I elaborated an artificial key to the orchids of Sardinia, which might be a useful tool to recognize orchids in the field. In addition, I discuss the relevance of continental islands as *natural laboratories* to investigate biodiversity patterns that are more likely to be established elsewhere.

The core of the other two papers are reproductive isolation mechanisms (RIMs) in the subtribe Orchidinae, known for their deceptive mechanisms of pollination. First, I analyzed RIMs to investigate the biological concept of species in two close related endemic species: *Ophrys annae* and *Ophrys chestermanii*. Given the scarce genetic difference between these two entities, I compared their morphology and conducted inter- and intra-specific crosses to quantify postmating barriers. The *lock and key theory* is the concept at the base of the last paper titled “Does sizes really matter?”. In animal kingdom, this theory is widely reported as a crucial tool to keep boundaries between species. Here, I investigated the relation between pollinia and stigma using four food deceptive species and four sexual deceptive species as models of the two functional pollination groups of orchids.

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Chapter 3

What we didn't know, we know and why it is important working on island's orchids. A synopsis of Sardinian studies.

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Abstract

Biological and ecological investigations of islands are crucial to explain ecosystems functioning. Many studies on biodiversity of islands are carried out in oceanic islands, in contrast, knowledge on continental islands such as those in the Mediterranean sea, are very often fragmented in space and time and often focused on single taxon. Here, a synopsis of the Orchidaceae of Sardinia is presented, based on literature research and recent botanical explorations. Our final list comprises 62 species and 14 genera: thirteen species are recognized as exclusively of Sardinia, four new species are recorded for the flora of the island, and one was no longer found. This orchid richness reflects the interesting geological history of the island that was linked to the mainland several times facing long periods of isolation. We also present an artificial key to field recognition. Our annotated synopsis shows the potential of continental islands to understand trends in ecology and evolution. Further studies are required in order to complete our knowledge of the orchid diversity on continental islands and specific conservation programs need to be developed to stop biodiversity loss.

Keywords: continental island, geographic isolation, Mediterranean basin, evolution, Sardinia, Orchidaceae.

Introduction

Geographic isolation is one of the factors that has most favored the rise of new species (Darwin, 1859) In fact, when populations of the same breeding group are separated, they face independent evolutionary histories defined by natural selection, genetic drift, adaptation and colonization to local conditions (Darwin 1859, Mayr, 1943). In biogeography, islands are the field to develop and test evolutionary and ecological theories (Darwin, 1859; Wallace, 1869; MacArthur, R.H. & Wilson, 1967; Losos & Schluter, 2000; Whittaker, Triantis, & Ladle, 2008). Here, plant richness is influenced by the distance from mainland and the age of the island (Traveset *et al.*, 2016) and diversification starts mainly via allopatry (Bramwell D., Caujapé-Castells J. 2011). However, the idea that islands should not be seen merely as target area for plant's colonization but also as 'halting places' has been proposed by Darwin (Darwin 1869) but surprisingly demonstrated recently (Harbaugh *et al.*, 2009). On the base of their origin, islands might be divided in two types: oceanic islands which have a volcanic origin and do not lay on the continental shelves and, on the contrary, continental islands which lay on the continental shelves and they had been linked with mainland at some point in their past. Biodiversity of oceanic island is determined by the ability of organisms to reach the island by another land, on the contrary on continental island biodiversity is defined by what remains on the island during the isolation process. Studies on island's biodiversity has been historically conducted on oceanic islands (Bramwell & Caujapé-Castells 2011, MacArthur & Wilson 1967, Losos & Schluter 2000; Whittaker et al. 2008) and recently on continental islands (Traveset *et al.*, 2016). With more than 12.000 islands, the Mediterranean basin is an amazing hotspot of biodiversity. Here, most of the islands are continental and originated during the Holocene, but few such as Stromboli, Vulcano and Santorini are volcanic (Rackham 2008). Plant biodiversity in the Mediterranean basin consists in more than 25.000 plant species (Myers *et al.*, 2000), hence each island or islet represents a small universe where ecological and evolutionary patterns have led to a unique ecosystem. However, studies on Mediterranean plants of islands are often run on species levels and rarely on higher taxonomic level or at community scale and are

generally conducted in the west-mediterranean islands (Traveset *et al.*, 2016). The cosmopolitan family of Orchidaceae is probably one of the richest in species in the Angiosperms and new species are described every year (Chase *et al.*, 2015). The extraordinary variability of their flowers and the peculiar uniqueness of their relationships with pollinators and fungi has always attracted humans from remote times to nowadays, allowing them to be adopted not only for their beauty but also considered as model to investigate biological trends (Darwin 1862; Bateman *et al.* 2011, and literature in them). Nevertheless, the concept of species in orchids still generates a vibrant debate and the literature about which character is taxonomically relevant is particularly rich (Devey *et al.*, 2008; Bateman *et al.*, 2011; Gögler *et al.*, 2015).

Sardinia is the second largest island in the Mediterranean basin and due to its strategic location, harbors a remarkable plant diversity; although it has been inhabited since ancient times, its size, ecosystem diversity and low human population density still favours the discovery of new orchids (Pavarese *et al.*, 2011).

These conditions determine a uncertainty on the number of orchids species: the most important published contribution on systematics and biology of orchids of Sardinia dates back to 1990 (Scrugli, 1990a) followed 20 years later by a PhD thesis (Lai, 2009) which represent the most updated document available specific for the island, including 60 species and 15 genera.

The aims of this paper are to i) update the check-list of Sardinian orchids presenting some notes about the observed orchid taxa, ii) propose an identification key, iii) discuss the relevance of orchids in island conditions for evolutionary and conservation research and why they should have more attention.

Materials and methods

Study area

Topography

Sardinia is located in the central western Mediterranean Sea between 38°51' and 41°15' latitude north and 8°80' and 9°50' east longitude with an area of 24,090 km² 270 km long, 145 km wide. The inhabitants are 1.66 million and the population density is 69 inhabitants per km². The nearest island is Corsica 11 km far and Italian peninsula is 187 km far.

Relevant mountain peaks are: Punta La Marmora (1,834 m), Monte Limbara (1,362 m), the Chain of Marghine and Goceano (1,259 m), the Sette Fratelli Range in the southeast, the Sulcis Mountains and the Monte Linas (1,236 m), the main wide alluvial valleys and flatlands are the Campidano in the southwest and the Nurra in the northwest. Seasonal rivers are relevant however the perennial rivers are the Tirso, 151 km, the Coghinas, 115 km, and the Flumendosa, 127 km. The only natural lake is Lago di Baratz, all the others are artificial. Large, shallow, salt-water lagoons are characteristic of the coast, particularly relevant is Molentargius lagoon in the city of Cagliari used for centuries for salt production.

Geology

The age of schistose sedimentary rocks varies from Lower Cambrian to Lower Carboniferous. These metamorphites results intruded by pleitonic granitoid rocks of Permo-Carboniferous age, belonging to the wide Sardinian-Corsican batholith (Carmignani *et al.*, 2016). Thick marine carbonatic sediments lie discordant on the Paleozoic Basement eroded during the Permo-Triassic period. A very thick Cenozoic succession constituted by continental and marine sediments, marles and limestones, of Eocene to Pliocene ages, rests on the Mesozoic sequence or more frequently, directly on the Paleozoic Basement. Acid to basic volcanites of Oligocene-Miocene and Pliocene-Pleistocene volcanic cycles are associated to the above-mentioned sediments. Finally, detritic, prevalently continental deposits of the ancient and recent Quaternary discontinuously cover all the

previous geological formations (Carmignani *et al.*, 2016). In the Early Oligocene Sardinia, Corsica and Balearic Islands were part of a Palaeozoic mountain chain present Iberia and migrated eastward in the south-eastern region of the basin (Mansion *et al.*, 2009). and together with other islands experienced a heterogeneous geological history characterized by successive formation of barriers and geographical corridors that either isolated or connected or separated it from to the mainland.

Climate

Sardinia has a mediterranean climate with annual temperature ranges from 11 to 17°C. Precipitations are typical of autumn and winter months and they vary from 400 to 1.100 mm per year: spring and summer are usually very hot and dry. Snowfalls are generally rare, but quite frequent in the highest mountain chains. The most relevant winds are the cold Mistral and the hot Scirocco from Sahara (Canu *et al.*, 2015).

Plant diversity

Sardinia is classified as one of the 34 most important biodiversity hotspot in the world (Mittermeier *et al.*, 2004): about 15% of its flora is endemic (2,295 species of which 347 are endemics, Bartolucci *et al.*, 2018). The dominant chorological element is the stenomediterranean (29%), followed by the Eurasian (17%) and the Eurimediterranean (16%).

Human Impact

It is still unclear when *Homo sapiens* colonized Sardinia; however, first evidences of a stable human presence are recorded during the Upper Paleolithic (Sporer, 1999) . During the Bronze Age, the Nuragic civilization arose from the 18th century BC to the 2nd century AD. Through the following centuries, due to its strategic location in the Mediterranean sea, coasts were colonized in succession from Phoenicians, Romans, Vandals, Goths and Byzantines and Saracens, leaving the inner part of the island particularly isolated causing, therefore, a strong genetic isolation in Sardinians. After five centuries of independence, the island was conquered by the Spanish Empire

till the rise of Sardinian Kingdom in 1720 under the House of Savoy precursor of Kingdom of Italy (1860) and the Unification of Italy in 1861.

The most ancient and relevant human activity on the island is sheep grazing (Bajocco *et al.*, 2012). In the last fifty years the island has suffered of land abandonment and an increasing human pressure on coasts caused mainly by new tourist settlements. A constant and inadequate landscape management is a direct cause of soil salinification and desertification (Zucca, Canu, & Previtali, 2010). Since 1956, sadly Sardinia hosts four North Atlantic Treaty Organization (NATO) military bases in strategic locations for a total of 213,6 sq km.

Three regional parks have been established: Regional natural park of Porto Conte (1999), Regional natural park of Molentargius-Saline (1999), Regional Natural park of Tepilora, Sant'Anna and Rio Posada (2014) and recognized as biosphere reserve by UNESCO in 2017. In addition, three National parks were created: the archipelago of La Maddalena National Park in 1994; Asinara National Park in 1997; Gulf of Orosei and Gennargentu National Park in 1998. National and Regional Parks cover an area of 1.141 sq km.

Check-list of Sardinian orchids

We carried out a literature search through ISI® Web of Science, Scopus and Google Scholar as well as through cross-referencing. The initial search terms for the query (August 17th, 2018) included "Orchids" AND "Sardinia"; we also performed a second query including each taxon recorded in Scrugli (1990a) and Lai (2009) AND "Sardinia". In addition, we implemented our investigation in the NIH genetic sequence database (GenBank) and the global archive of plant traits (TRY Plant Trait Database, Kattge et al. 2011) using the previous keywords. We included studies meeting the following criteria: i) performed in Sardinia, ii) involved taxa listed in Sardinia iii) published in peer-reviewed journals.

Because of its useful local relevance, we also took into account the grey-literature not published in peer-reviewed journals. In order to assess the conservation status of each taxa we consulted IUCN databases (<http://www.iucnredlist.org/>, last access 16/09/2018) and we also investigated their legal protection.

A specific search was conducted on the check-lists of Europe and Italy (Scrugli, 1990; Delforge, 2006, 2016; Lai, 2009; GIROS, 2009, 2016; Bartolucci *et al.*, 2018). Taxonomic identification was based upon The International Plant Name Index (www.ipni.org). We decided to base our work on the work of Lai (2009) updating it with new records and nomenclature changes.

In addition to the bibliographic research, we also included unpublished data collected in almost 10 years of field work carried out across the island during the decade (2009-2018) usually from October to July. We included phenology data taken from literature and integrated with our field observations. Descriptions of species and hybrids were prepared from both living specimens and herbarium material. New samples were deposited at the Herbarium CAG of Università degli Studi di Cagliari. Species identification was carried out adopting Lai (2009) and Delforge (2016).

Results

What we know

Our literature search resulted in a chronological list of 43 papers (Appendix I). Our review on literature show that despite the fact that the most investigated genus is *Ophrys* with a total of twelve papers, nine papers focused on cytogenetics, pollination biology and systematics with a particular emphasis on endemic entities. Thirteen papers on forty-three recorded are comparative studies between sardinian orchids and their close related taxa elsewhere mostly based on cytogenetic comparison (D'Emérico, Pignone, & Scrugli, 2000; D'Emérico *et al.*, 2002, 2005; Turco *et al.*, 2015) or phylogenetic analysis (Devey *et al.*, 2008, 2009; Breitkopf *et al.*, 2015; Bateman, Sramkó, & Paun, 2018) underlining the relevance of islands in understanding evolutionary processes.

Within the peer-reviewed literature (Appendix I), we identified the following topics: a) cytology 14%, b) taxonomy, 14%, c) pollination biology, 12%, d) cytogenetics, 12%, e) floristics, 16%, f) phylogeography, 16%, g) systematics and phylogenetics, 9%, h) systematics, 5%, i) climate change, 2%. The genus *Ophrys* is the most investigated genus with 15 papers, 191 citations. Eleven papers were related to cytology and cytogenetics. Their common aim is to define species karyotype to define evolutionary trends both in island conditions or within genera such as *Ophrys* or *Serapias* (D'Emérico *et al.*, 2000, 2005). Three papers were focused on mycorrhizae and endophytes (Cogoni; Riess; Scrugli, 1992) and they aim to understand the mutualistic relationship between plant and fungus especially in the subfamily Epidendroideae (D'Emérico, S., Grünanger, P., Scrugli, A., Pignone, 1999; D'Emérico *et al.*, 2000). Nine papers dealing with phylogeny and phylogeography underline the unique orchids richness of the island at species level as Zitari *et al.* (2011) or genus level where sardinian samples were used to reconstruct the phylogeny of *Serapias* (Bellusci *et al.*, 2008) or helpful to understand evolutionary trends in unresolved clade such as *Ophrys* (Devey *et al.* 2008, 2009, Breitkopf *et al.* 2015, Bateman *et al.* 2018). Six papers concerned floristics and they consist in national or local check-lists (Bacchetta, Pontecorvo, & Vacca, 2007; Bartolucci *et al.*, 2018). Five papers dealt with pollination biology and concerned on sex-deception in *Ophrys* endemisms to draw generalities on pollination of sympatric species (Cortis *et al.*, 2009; Gögler *et al.*, 2011, 2015) or to identify scent compounds and pollinators (Stöckl *et al.* 2008). A systematic and phylogenetic approach is characteristic of four papers. The aim of these publications is a comparison of close related species such as within the genus *Ophrys* or *Orchis* as in Pellegrino *et al.* (2005) continental islands (Cafasso *et al.*, 2001). Six papers deal with taxonomy mainly focused on local entities description with a morphometric approach. A phylogeographic approach is the topic of four papers: three at species level (Bullini *et al.*, 2001; Pavarese *et al.*, 2011; Zitari *et al.*, 2012) and one at genus level (Bellusci *et al.*, 2008). One paper dealt with climate change to predict species distribution (Ongaro *et al.*, 2018). In our database investigations, a total of 50 taxa out of 62 were found in GenBank (80.65%), 11.3% on IUCN while only ten species (16.12%) are currently

available in TRY Plant Trait Database. Regarding plant functional traits, in agreement with Chelli et al. (2018), Mediterranean orchids are poorly represented and our research on the global archive of plant traits (TRY database, Kattge et al. 2011) showed that mainly species with a European distribution are currently available (Table 1). We found general lack of available information on endemisms in GenBank, while non-endemic taxa are widely represented (Table 1).

What we did not know

Orchids have always fascinated even a non-academic audience so the grey literature is quite consistent (Appendix II). Often published in Italian or German language, these publications are focused mainly on single taxonomic groups or a single putative species. In addition, scientific disclosure includes several globally relevant books (Delforge, 2006; 2016) focused on European and Mediterranean orchids and very often cited in academic papers and papers and books edited by GIROS (Gruppo Italiano Ricerca Orchidee Spontanee) (GIROS 2008, 2016).

Our assessment produced a list of 62 species and 14 genera belonging to Orchidaceae (See List of Taxa), in contrast to the 60 species and 15 genera reported in the most recent check-list on Sardinian orchids (Lai 2009). Our list differs from Lai (2009) because three species are recent records: *Platanthera kuenkelei* var. *sardoa* in 2011 (Pavarese et al. 2011), *Anacamptis palustris* (2012) and *Orchis italica* 2017-2018 (unpublished data). 68 species were listed in Bartolucci et al. (2018) where we found species never recorded for the island (*Ophrys candica* (E.Nelson ex Soó) H.Baumann & Künkele, *Ophrys forestieri* (Rchb.f.) Lojac, *Ophrys parvimaclulata* (O.Danesch & E.Danesch) Paulus & Gack, *Ophrys scolopax* Cav. subsp. *apiformis* (Desf.) Maire & Weiller, *Ophrys panormitana* (Tod.) Soó, *Orchis mascula* (L.) L. subsp. *olbiensis* (Reut. ex Gren.) Asch. & Graebn, *Serapias neglecta* De Not). Hybrids currently recorded are twenty (Table 2). With 48 ssp. the subtribe Orchidinae represents the 75% of the family, while the genus *Ophrys* is the most species-rich genus (21 ssp.) followed by *Anacamptis* (8 ssp.), *Epipactis* and *Orchis* (6 ssp.) (Figure 1). As result of our field investigations, we did not find *Ophrys scolopax* subsp. *picta* (Link), but we

could not be sure of its extinction so we decided to include it in this checklist. West mediterranean species (29%) are the most common chorological element followed by Endemic (26%), Eurasian (15%), Mediterranean *s.s.* (13%), Eurimediterranean (12%), Stenomediterranean (7%) and East mediterranean (2%). Thirteen species are endemic of Sardinia, ten are *Ophrys*, two *Orchis* and one belongs to *Platanthera* (Table 3). Within endemisms, five species are exclusive of the island, six are Sardo-Corsican, one Sardo-Sicilian and one Sardo-Tunisian (Table 3). The first orchid to bloom is *Himantoglossum robertianum* starting in December or January (Table 4), the blooming peak is generally between April and May (Figure 2). The complexity of species concept is mainly related with the not always congruent results given by morphological and molecular analyses. Due the morphological complexity of some taxa such as *Epipactis* or *Ophrys* and the fact that this iconic family fascinated not only researchers but also amateurs, here an artificial key to the orchids on Sardinia is proposed as a helpful tool to field recognition.

In the last decade, our field investigations are mainly related on evolutionary trends of endemic taxa (Cortis *et al.*, 2009; Gögler *et al.*, 2009, 2011, 2015; Lussu *et al.*, 2018; Ongaro *et al.*, 2018) but also on geographic distribution as *Orchis italica* and phenological observations (Table 4, Figure 2). For instance, *Himantoglossum robertianum* was recorded to bloom in January and “rarely” in late December, we recorded earlier blooms more frequently. We monitored threatened species such as *Anacamptis palustris*, *Dactylorhiza elata* subsp. *sesquipedalis* and *Platanthera kuenkelei* var. *sardoa* because of the fragility of their environments. Unfortunately a general decline for all the three species was recorded. Supported by local municipalities, in the cases of *Dactylorhiza elata* subsp. *sesquipedalis* and *Platanthera kuenkelei* var. *sardoa*, we provided a fence on the only populations known.

Why it is important to work on island's orchids

The charming influence of orchids stimulates people more than any other plant family. In particular, island endemisms have received a special consideration. In Sardinia more than a quarter of the species are classified as endemic of the island. Studies on endemisms as *Ophrys normanii* (Cortis *et al.*, 2009; Gögler *et al.*, 2011, 2015), have contributed to clarify strategies of evolution within the Orchidaceae family such as hybridization as a speciation force or their studies might be helpful to understand the evolution of limited species groups such as the *Ophrys*. Indeed, orchids represent a perfect model to explain patterns of evolution because they have evolved complex interactions with other organisms such as animals for pollination and fungi to establish mycorrhizal symbiosis. On Mediterranean islands, in a long-term study conducted in Corsica, Vogt-Schilb *et al.* (2016) suggests that endemic species have lower colonization abilities than their widespread relatives, underlining the relevance of land abandonment in orchids dynamics. Hence, their study should not be independent with symbiotic organisms, in the case of orchids, for instance, pollinators.

The current biogeography of Sardinian plants is also affected by colonization through wind dispersal of seeds (Mansion *et al.*, 2008, 2009), a relevant process of species distribution (Nathan *et al.*, 2002; Nathan, 2006). This process is already recorded for other islands such as for instance Great Britain whose flora is one of the most detailed in the world (Braithwaite *et al.* 2006). Thanks to their dust-like seeds, orchids benefit from winds to migrate (Arditti & Ghani, 2000). Orchids migration northwards has been already recorded in Britain where *Ophrys balearica*, *Serapias parviflora* and *S. lingua* (Bateman *et al.* 2006) and recently *Ophrys tenthredinifera* (Fay 2015) have been found coming from the continent. In Mediterranean basin, predominant winds explain the genetic diversity and distribution of *Ophrys fuciflora* (Devey *et al.* 2009) and the role of Mistral might explain, in Sardinia, the recent record of *Orchis italica* in the north, an extremely common species on the continent.

In Sardinia, orchids don't seem affected by many factors that expose orchid species to the risk of extinctions (Hinsley *et al.*, 2018) but indirect human impact might represent the main cause of species decrease. Indeed, in Europe, where the human pressure has lasted for centuries, orchids are dramatically affected by urbanization and wrong land management which reduce or fragment habitat (Pellegrino & Bellusci, 2014), limiting orchid lifespan (Coates & Dixon, 2007; Swartz & Dixon, 2009; Parra-Tabla *et al.*, 2011) and affecting their reproductive success (Tremblay *et al.*, 2005; Huang *et al.*, 2009). Other human activities that might impact orchids in mediterranean are fire and wood cleaning. They drastically affect geophytes (Diadema, Médail, & Bretagnolle, 2007) and their symbiotic such as endophytes (Jasinge, Huynh, & Lawrie, 2018); however more knowledge about the effects of fire or the cleaning of woods in Mediterranean basin are needed especially in fragile systems such as islands. Nevertheless, coppicing could promote orchids because allow higher light intensities (Dorland & Willems, 2006), and so populations of orchids such as *Ophrys insectifera* drastically decreased due to the abandonment of the traditional methods of management of the grasslands, which facilitates the growth of taller species (Kull & Hutching 2005, Dorland & Willems, 2006). Coppicing could explain the orchid richness on roadside in Sardinia. In April and May, to prevent summer fires, local municipalities promote a haphazard mowing of grasses. During this time, many orchids species are fully in bloom and so a better scheduled mowing taking into account the life cycle of plant species -not only orchids- would certainly be important in the management of the landscape. A correct habitat management is crucial to protect biological diversity.

Climate changes towards overheating affect species in quantity and distribution (Sletvold *et al.*, 2013). Because of the diversity of Sardinian orchids, some species will benefit from a warmer climate, while others will succumb. Under this light, in Ongaro *et al.* 2018, three genera were analyzed: *Anacamptis*, *Ophrys* and *Serapias*. All the species investigated have a typical Mediterranean distribution and their common trend is to increase their distribution and migrate northwards and so they could be considered as potential winners (Ongaro *et al.* 2018). Not included

in that study, potentially losers might be all the eurasiatic genera which, in Sardinia, prosper in the inner and higher peaks such as, for instance, the rare red helleborine (*Cephalanthera rubra*).

Human impact might influence plants affecting pollinators communities (Smithson, 2005; Tremblay *et al.*, 2005). Globally the main causes of insects decreasing are landscape management, intense agricultural activities (Hegland *et al.*, 2009; Pellegrino & Bellusci, 2014; Bukovinszky *et al.*, 2017; Balfour *et al.*, 2018). These pressures are generally not intense in low populated islands such as Sardinia (Bajocco *et al.*, 2012) but, unfortunately, in Sardinia, studies focused on pollinators distribution are absent.

In mediterranean islands, anthropic pressure is directly expressed with turistic coastal settlements whose impact is particularly consistent. It is not a case that the two most endangered species in Sardinia, *Anacamptis palustris* and *Dactylorhiza elata* subsp. *sesquipedalis* are located on the coast in fragile habitats such as wet grassland.

Their peculiar life cycle exposes orchids to a greater danger of extinction when compared to other plants (Fay, 2012). All orchids are covered by CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and therefore require CITES permits for transport between countries. CITES permits are not required for actions within a country. Surprisingly, we found that only the 11% (seven species) of sardinian orchids are recorded on the IUCN red list; their population trends are listed in Table 1. However, since 1997 within the European Community there are no border controls due to the establishment of the EU single market. An important European tool to protect biodiversity is Habitat Directive (92/43/EEC in “Cod. Habitat Natura 2000 6210” which does not preserve orchids directly but “semi-natural dry grasslands and scrubland facies on calcareous substrates (*Festuco-Brometalia*) (important orchid sites), code 6210”. Currently Italy has not a more exhaustive national regulation and so it has not yet developed any law designed to directly protect orchids delegating the legislative provisions to the Regions and the Autonomous Provinces. The Autonomous Region of Sardinia has not issued any law yet. Although

at regional level the requests for protection of the flora have not yet been accepted, few small municipalities have shown, in spite of their economic constraints, particular interests in developing protection and conservation initiatives.

In the latest IUCN list, four of the five extinct orchids are island endemisms and this should be an alarm ring to focus more attention on the risk islands biodiversity currently face.

Investigations on species concept on islands are necessary not only to evaluate ecological and evolutionary patterns but also to better define continental species. In fact, more detailed molecular investigations are necessary to reveal species that differ more molecularly than morphologically, to identify those geographically isolated species that differ both molecularly and morphologically from close related species, to discover those unjustified entities not supported by both molecular and morphological analysis. Furthermore, studying orchids on the islands is essential because it raises dozens of evolutionary and ecological questions. What is their origin in islands that have had contacts with the continent or other islands at different stages of their geographic history? What species concept is most appropriate in the context of islands? Are there recurring evolutionary and ecological patterns on continental islands? Do these patterns vary from island to island and/or from islands to mainland? How relevant are orchids in ecosystems functioning? Which conservation approach should be more adequate in an area as complex as the Mediterranean basin? Which species or functional groups of species should have a conservation priority?

Hence, especially for islands where organisms have followed unique evolutionary trends, more integrated studies are necessary to fill existing gaps in knowledge in order to answer new biological questions and to develop adequate protection programs.

Conclusion

This synopsis is the most comprehensive analysis of studies on orchids in Sardinia and it could be helpful to reflect on how much we know about orchid biodiversity in continental island conditions: due to the complexity of the biology and evolution of orchids, we are aware that this list will face, in time, some thrilling changes reflecting the complexity of life on our planet. Given its peculiar location, geological history and biogeographic gradient, Sardinia represents an important source of orchid diversity and, accordingly, more investigations on these charismatic organisms are essential. The exclusive and often fragile ecosystems of islands stimulate the rise of new questions, the development and test of new theories at every ecosystem's level and the investigation on how species interact. Hence, comprehend these evolutionary and ecological patterns in unique and often basic biota such as islands might give us the time and the cultural tools to predict and evaluate ecosystem functioning.

List of taxa

Subfamily *Epidendroideae* Lindl., 1821

***Cephalanthera* Rich., Mém. Mus. Hist. Nat. 4: 51 (1818)**

Etymology: derives from greek *kephalé* (= head) and *antheros* referring to the globular shape of the anther. Rhizomatous species with numerous roots. *Cephalanthera* comprises 16 species. It is a mainly Eurasian genus with only one species in America (*Cephalanthera austiniiae* (A.Gray) Heller). Species are often cleistogamous. A single scape is normally departed from the rhizome. Inflorescence multiflora with slightly open and nectarless flowers. Three species are recorded for the island.

***Cephalanthera damasonium* (Mill.) Druce, Ann. Scott. Nat. Hist. 1906: 225 (1906).**

Distribution: Euri-Medit.

Phenology: V-VI

Habitat and ecology: Mid-shade, moist substrates from 200m up to 1000 m asl.

Pollinators: flowers usually not fully open, pollination is cleistogamous.

Discussion: It usually forms rich populations even it is not very common. It is mainly distributed on woody mountains of the center (Sarcidano) and center-east of the island. A single population is recorded in south-west of the island (Iglesiente).

$2n=36$

Synonyms: *Cephalanthera alba* (Crantz) Simonk.; *Cephalanthera pallens* Rich.; *Serapias damasonium* Mill.

***Cephalanthera longifolia* (L.) Fritsch, Oesterr. Bot. Z. 38: 81 (1888).**

Distribution: Eurasiat.

Phenology: IV-VI

Habitat and ecology. It prefers shady position in wet meadows in oak woods.

Pollinators: *Halictus* sp. or *Lasioglossum* sp.

Discussion: It is pollinated by pollinators of *Cistus salvifolius* L. Within the genus *Cephalanthera*, *C. longifolia* is the most common species especially in Sarcidano.

$2n=32$

Synonyms: *Cephalanthera ensifolia* Rich.

***Cephalanthera rubra* (L.) Rich., De Orchid. Eur.: 38 (1817).**

Distribution: Eurasiat.

Phenology: V-VII

Habitat and ecology: Oak woods.

Pollinators: *Chelostoma* sp., *Dufourea* sp., *Heriades* sp. or *Osmia* sp.

Discussion: Currently just three populations are recorded, two populations are known in Monte Arci and one in Monte Arcosu.

2n=36

Synonyms: *Cephalanthera maravignae* Tineo; *Serapias rubra* L.

***Epipactis* Zinn, Cat. Pl. Hort. Gott.: 85 (1757), nom. cons.**

Etymology: derived from greek *epipaktis* to define species of the genus *Helleborus* (autore), not known in referring of orchids.

***Epipactis exilis* P.Delforge, Naturalistes Belges 85: 246 (2004).**

Distribution: E-Medit.

Phenology: VI-VII

Habitat and ecology: shady and moist sites in oak woods.

Pollinators: wasps.

Discussion: Due its variation different nomenclatural issues are related to this species

Synonyms: *Epipactis baumanniorum* Ströhle; *Epipactis gracilis* (Hook.f.) A.A.Eaton, *Epipactis gracilis* B. et H. Baumann nom. illeg.; *Epipactis persica* (Soò) Nannfeldt; *Epipactis persica* (Soò) Nannfeldt subsp. *gracilis* W. Rossi nom inval.

***Epipactis helleborine* (L.) Crantz, Stirp. Austr. Fasc., ed. 2, 2: 467 (1769).**

Distribution: Eurasiat.

Phenology: IV-VII

Habitat and ecology: from moist to dry soil, from shady to sunny conditions from 400m up to 1200m asl.

Pollinators: wasps.

Discussion: Populations are usually rich and located on all the main mountains of the island. The wide range of tolerated habitats causes a great morphological variation that is at the core of nomenclatural problems.

$2n=38$

Synonyms: *Epipactis latifolia* (L.) All.; *Helleborine latifolia* (L.) Moench.

***Epipactis microphylla* (Ehrh.) Sw., Kongl. Vetensk. Acad. Nya Handl. 21: 232 (1800).**

Distribution: Medit.-Caucas.

Phenology: IV-VII

Habitat and ecology: mostly on shady site in deep and moist substrates of deciduous forests from 100 m to 1200 m asl.

Pollinators: wasps.

Discussion: It is a common species, however populations are always composed by isolated plants.

$2n=40$

Synonyms: *Helleborine microphylla* (Ehrh.) Schinz & Thell.; *Serapias microphylla* Ehrh.

***Epipactis muelleri* Godfery, J. Bot. 59: 106 (1921).**

Distribution: Euri-Medit.

Phenology: VI-VII

Habitat and ecology: Sun or mid-shade positions generally on dry substrates at 750m asl.

Pollinators: wasps.

Discussion: Sometimes some plants can be confused with *E. helleborine* which usually blooms later.

$2n=38-40$

Synonyms: unknow.

***Epipactis palustris* (L.) Crantz, Stirp. Austr. Fasc., ed. 2, 2: 463 (1769).**

Distribution: Eurasiat.

Phenology: VI-VII

Habitat and ecology: Sun or full-sun, on wet substrate in the center of the island (Sarcidano). In 2018 a fences has been placed around a population in the municipality of Laconi.

Pollinators: wasps.

Discussion: Currently the three populations known for the island are threatened by horse grazing.

$2n=40$

Synonyms: *Epipactis longifolia* All.; *Helleborine palustris* (L.) Schrank; *Serapias palustris* Mill.

***Epipactis tremolsii* Pau, Bol. Soc. Aragonesa Ci. Nat. 13: 43 (1914).**

Distribution: W-Medit.

Phenology: II-V

Habitat and ecology: sun or partial shade on dry calcareous substrates from 400m to 900m asl.

Pollinators: wasps.

Discussion: Sometimes confused with *E. helleborine*.

2n= unknown

Synonyms: *Epipactis helleborine* (L.) Crantz subsp. *tremolsii* (Pau) E. Klein.

***Gennaria* Parl., Fl. Ital. 3: 404 (1860).**

Etymology: Parlatores dedicated this genus or to Patrizio Gennari (1820-1897), a famous florist from the city of Cagliari (Pignatti, 1982), or to De Benedetto Gennari (1570-1610) (Landwehr, 1982). A genus formed by two species *G. diphylla* (Link) Parl. and *G. griffithii* (Hook.f.) X.H.Jin & D.Z.Li. Inflorescence dense and small and green flowers.

***Gennaria diphylla* (Link) Parl., Fl. Ital. 3: 405 (1860).**

Distribution: Medit.-Atl.

Phenology: II-V

Habitat and ecology: Pinewood and acidic garrigues up to 400m asl.

Pollinators: unknown.

Discussion: Extremely rare and mainly located in the north-east coast, but since 1995 new populations were discovered on the west coast (Giotta and Piccitto 1995, Orrù and Senis 2007), and in the south-east coast (Scrugli and Cogoni 1995).

2n=34

Synonyms: *Coeloglossum diphyllum* (Link) Fiori & Paol.

***Limodorum* Boehm., Defin. Gen. Pl.: 358 (1760), nom. cons.**

Etymology: incertain derivation indeed the greek word *limodes* means hangry probably referring to the parasitic behaviour of these plants, otherwise Teofrasto used *aimodoron* to define a non-orchid parasitic plant, successively the initial a change to λ. A mediterranean genus with three species are currently ascribed to this genus, two are recorded in Sardinia. Leaves small and purple ish due the abundance of anthocyanins. Lax inflorescence and very showy flowers when opened because cleistogamy is often the most relevant pollination system.

***Limodorum abortivum* (L.) Sw., Nova Acta Regiae Soc. Sci. Upsal. 6: 80 (1799).**

Distribution: Euri-Medit.

Phenology: IV-VI

Habitat and ecology: Mid-shade cool mediterranean forest dominated by *Quercus ilex* L. or *Pinus ssp.* Up to 1100m.

Pollinators: mostly cleistogamous.

Discussion: Flowers are rarely fully open more often completely closed. Populations can consist in more the 10 plants that can not emerges for years. This species is the most widespread of the two recorded in Sardinia.

2n=56

Synonyms: *Ionorchis abortiva* (L.) Beck; *Orchis abortiva* L.

***Limodorum trabutianum* Batt., Bull. Soc. Bot. France 33: 297 (1886).**

Distribution: W-Medit.

Phenology: V-VI

Habitat and ecology: Mid-shade cool mediterranean forest dominated by *Quercus ilex* L. up to 900m

Pollinators: mostly cleistogamous.

Discussion: Flowers rarely often completely closed. This species is recorded only in two sites with conspicuous populations.

$2n=60$

Synonyms: *Limodorum abortivum* (L.) Swartz subsp. *trabutianum* (Batt.) Rouy.

***Neottia* Guett., Hist. Acad. Roy. Sci. Mém. Math. Phys. (Paris, 4to) 1750: 374 (1754), nom. cons.**

Etymology: both words *Neottia* (from greek) and *nidus -avis* (from latin) refer to the peculiar roots morphology that looks like a bird nest. Eurasiatic genus of c. 50 species, known for its low or absent photosynthetic activity.

***Neottia nidus-avis* (L.) Rich., De Orchid. Eur.: 37 (1817).**

Distribution: Eurasiat.

Phenology: IV-V

Habitat and ecology: shady woodlands dominated by *Quercus ilex* L. from 400m to 1000 m

Pollinators: mostly cleistogamous.

Discussion: Not very common, but locally very well represented.

$2n=36$

Synonyms: *Ophrys nidus-avis* L.

***Neottia ovata* (L.) Bluff & Fingerh., Comp. Fl. German., ed. 2, 2: 435 (1838).**

Distribution: Eurasiat.

Phenology: V-VI

Habitat and ecology: Wood or wet meadows from 550m to 1100m asl.

Pollinators: quite generalistic. It is pollinated by Coleoptera, Diptera and Hymenoptera..

Discussion: Very limited distribution mainly in the center of the island, however populations are locally rich. In 2006 a new population was found in the Sette Fratelli Park (south east).

$2n=34$

Synonyms: *Listera ovata* (L.) R. Br., *Ophrys ovata* L..

***Spiranthes* Rich., De Orchid. Eur.: 28 (1817), nom. cons.**

Etymology: *speira e anthos* in referring to the spiral disposition of flower along the inflorescence. A temperate genus which includes 42 species pollinated by bees with a very selective mechanism.

***Spiranthes aestivalis* (Poir.) Rich., De Orchid. Eur.: 36 (1817).**

Distribution: Medit.-Atl.

Phenology: VI-VII

Habitat and ecology: Full sun on rocky banks of the torrents to 1000m asl.

Pollinators: Unknown.

Discussion: Solitary bees (Hymenoptera)

$2n=30$

Synonyms: *Ophrys aestivalis* Poir.

***Spiranthes spiralis* (L.) Chevall., Fl. Gén. Env. Paris 2: 330 (1827).**

Distribution: Medit.-Caucas.

Phenology: IX-XI

Habitat and ecology: Garrigues and meadows to 1060m asl.

Pollinators: Solitary bees (Hymenoptera).

Discussion: It is common on the island and populations usually are very rich.

2n=30

Synonyms: *Ophrys spiralis* L.; *Spiranthes autumnalis* Rich.

Subfamily Orchidoideae Eaton, 1836

***Anacamptis* Rich., De Orchid. Eur.: 25 (1817).**

Etymology: from greek *anakamptein* referred to pollinia or to the crest at based of the labellum or to lateral sepals. The epithet *pyramidalis* refers to the iconic inflorescence shape. A eurimediterranean genus originally included only *Anacamptis pyramidalis* (L.) Rich. but currently includes the species previously largely attributed to the genus *Orchis* (Bateman *et al.* 2003). Flowers usually big and very showy, sometimes nectarless and usually food deceptive species. Species are usually very common and don't have any soil preference.

***Anacamptis collina* (Banks & Sol. ex Russell) R.M.Bateman, Pridgeon & M.W.Chase, Lindleyana 12: 120 (1997).**

Distribution: Medit.

Phenology: II-IV

Habitat and ecology: Full sun, garrigues, dry meadows on a wide range of substrates.

Pollinators: *Apis mellifera*, *Andrena* sp., *Eucera* sp.

Discussion: Even if it is recorded in all the island populations are rare and not very dense.

2n=36

Synonyms: *Orchis saccata* Ten.; *Orchis collina* Banks & Sol. ex Russell

Anacamptis fragrans (Pollini) R.M.Bateman, Bot. J. Linn. Soc. 142: 12 (2003).

Distribution: Medit.

Phenology: IV-VI

Habitat and ecology: Grassland, garrigues, and meadows in full sun on dry substrates to 900m asl.

Pollinators: Uncertain. Discussion: Not very common and distributed especially in the central east part of the island. Few population are recorded in the north, but also on the south west-coast.

2n=36

Synonyms: *Orchis fragrans* Pollini; *Orchis coriophora* L. subsp. *fragrans*

***Anacamptis laxiflora* (Lam.) R.M.Bateman, Pridgeon & M.W.Chase, Lindleyana 12: 120 (1997).**

Distribution: Medit.-Atl.

Phenology: IV-VI

Habitat and ecology: Full sun on wet or damp substrates.

Pollinators: It adopts a food deception strategy so a wide range of species are adopted as pollinators.

Discussion: Its presence is recorded throughout the island but its distribution is threatened by the anthropic action on its habitat.

2n=36

Synonyms: *Orchis laxiflora* Lam.

Anacamptis longicornu (Poir.) R.M.Bateman, Pridgeon & M.W.Chase, Lindleyana 12: 120 (1997).

Distribution: W-Medit.

Phenology: II-V

Habitat and ecology: Full sun, garrigues, meadows on a wide range of substrates ti 1200m asl.

Pollinators: *Andrena albopunctata*, *A. ovatula*, *Apis mellifera*, *B. rupestris*, *B. sylvarum*, *B. sylvestris*, *B. terrestris*, *B.vestalis*, *Bombus lapidarius*, *Eucera hungarica*, *Halictus patellatus*, *Lasioglossum xanthopus*, *Osmia bicornis*.

Discussion: It is the most common *Anacamptis* in Sardinia and populations are recorded throughout the island. During its blooming season, it is very common to observe dense and policrome populations the roads margins usually with *Anacamptis papilionacea* subsp. *grandiflora* and *Anacamptis papilionacea* subsp. *papilionacea*.

2n=36

Synonyms: *Orchis longicornu* Poir.; *Anacamptis morio* (L.) R. M. Bateman, Pridgeon & M. W. Chase subsp. *longicornu* (Poir.) H. Kretzschmar, Eccarius & H. Dietr.

***Anacamptis palustris* (Jacq.) R.M.Bateman, Pridgeon & M.W.Chase, Lindleyana 12: 120 (1997).**

Distribution: Euri-Medit.

Phenology: V-VI

Habitat and ecology: mid-shade in a saline substrate at sea level.

Pollinators: It adopts a food deception strategy so a wide range of species are adopted as pollinators.

Discussion: The only population known for the island was discovered in 2011 and it rapidly decreases.

$2n=36,42$

Synonyms: *Orchis palustris* Jacquin; *Orchis laxiflora* subsp. *palustris* (Jacq.) Bonnier & Layens.

***Anacamptis papilionacea* (L.) R.M.Bateman, Pridgeon & M.W.Chase, Lindleyana 12: 120 (1997).**

Distribution: Medit.

Phenology: II-V

Habitat and ecology: Full sun, garrigues, meadows on a wide range of substrates to 1400m asl. Sometimes it prefers cool and shady locations.

Pollinators: *Anthophora crinipes*, *A. retusa*, *Bombus terrestris*, *Bombus humilis*, *Eucera hungarica*, *E.nigrescens*, *E. capsica*, *Eucera bidentata*, *Eucera tuberculata*, *Megachile parietina*, *Nomada imperialis*.

Discussion: Conspicuous populations are recorded throughout the island. Its pollination strategy is still uncertain.

$2n=32$

Synonyms: *Orchis papilionacea* L. subsp. *papilionacea*.

***Anacamptis papilionacea* subsp. *grandiflora* (Boiss.) Kreutz, Ber. Arbeitskreis. Heimische Orchid. 24(1): 148 (2007).**

Distribution: W-Medit.

Phenology: II-V

Habitat and ecology: Full sun, garrigues, meadows on a wide range of substrates to 1200m asl. Sometimes it prefers cool and shady locations.

Pollinators: *Anthophora crinipes*, *A. retusa*, *Bombus terrestris*, *Bombus humilis*, *Eucera hungarica*, *E.nigrescens*, *E. capsica*, *Eucera bidentata*, *Eucera tuberculata*, *Megachile parietina*, *Nomada imperialis*.

Discussion: Compared with *A. papilionacea* subsp. *papilionacea*, *Anacamptis papilionacea* subsp. *grandiflora* usually prefers sunnier and drier sites at lower altitudes.

2n=32

Synonyms: *Orchis expansa* Ten.; incl. *Orchis papilionacea* L. subsp. *grandiflora* (Boiss.) H. Baumann; *Orchis rubra* Jacq.; *Orchis papilionacea* L.

***Anacamptis pyramidalis* (L.) Rich., De Orchid. Eur.: 33 (1817).**

Distribution: Euri-Medit.

Phenology: IV-VI

Habitat and ecology: Full sun, garrigues, meadows on calcareous substrates to 1000m asl.

Pollinators: *Aporia crataegi*, *Zygaena minos*

Discussion: It is located especially in central-east and north-west part of the island. Not many populations are currently known but they are very conspicuous. A single white population is recorded on the hills of the municipality of Sanluri (Cagliari).

2n=36

Synonyms: *Orchis pyramidalis* L.

***Dactylorhiza* Neck. ex Nevski, Trudy Bot. Inst. Akad. Nauk S.S.S.R., Ser. 1, Fl. Sist. Vyssh. Rast. 4: 332 (1937), nom. cons.**

Etymology: the greek word *daktylos* means finger while *rhiza* means roots in referring to the fingered shape of tubers. Eurasian genus comprising c. 60 species, it usually prefers wetlands and don't show a clear soil preference. Flower are usually nectarless and food deception is adopted as pollination strategy.

***Dactylorhiza elata* subsp. *sesquipedalis* (Willd.) Soó, Nom. Nov. Gen. *Dactylorhiza*: 7 (1962).**

Distribution: W-Medit.

Phenology: VI-VII

Habitat and ecology: Mid-shade on alkaline wet substrate at 800m asl.

Pollinators: It adopts a food deception strategy so a wide range of species are adopted as pollinators.

Discussion: With less than 10 plants, it is currently it is recorded as the rarer orchid of Italy.

2n=80

Synonyms: *Orchis sesquipedalis* Willd.

***Dactylorhiza insularis* (Sommier) Ó.Sánchez & Herrero, Fl. Iber. 21: 98 (2005).**

Distribution: W-Medit.

Phenology: IV-VI

Habitat and ecology: full sun, mid-shade in meadows, grassland or guarigues from 500m to 1200m asl.

Pollinators:

Discussion: Compare to the Italian peninsula, in Sardinia this species is quite common especially in Monte Limbara.

$2n=60$

Synonyms: *Dactylorhiza romana* (Sebast.) Soó subsp. *bartonii* Huxley & P.F. Hunt; *Dactylorhiza sambucina* (L.) Soó subsp. *insularis* (Sommier) Soó; *Orchis insularis* Sommier.

***Himantoglossum* Spreng., Syst. Veg. 3: 675 (1826).**

Etymology: from Greek *imanto* strap and *glossa* in referring to the lip of *H. hircinum*.

Characteristics: Eurasian genus usually pollinated by bees via food deception. Since their distribution areas rarely overlap and the species have a different phenology, natural hybridization is quite rare. Plants are usually big and are the bigger species in European flora. Species don't show a preference in soil composition.

***Himantoglossum robertianum* (Loisel.) P. Delforge, Naturalistes Belges 80: 401 (1999).**

Distribution: Medit.

Phenology: I-V

Habitat and ecology: Meadows, garrigues, bush, up to 1000m asl.

Pollinators: *Apis mellifera*, *Bombus lucorum*, *B. terrestris*, *Xylocopa violacea*.

Discussion: It is extremely common and it is the first orchid to bloom.

$2n=36$

Synonyms: *Barlia longibracteata* (Biv.) Parl.; *Himantoglossum longibracteatum* (Biv.) Schltr.; *Himantoglossum robertianum* (Loisel.) P. Delforge; *Orchis longibracteata* Biv.; *Orchis robertiana* Loisel.

***Neotinea* Rchb.f., De Pollin. Orchid.: 29 (1852).**

Etymology: from greek *neos*, new and *-tineo* as tribute to the sicilian botanist Tineo (1791-1856).

For decades it was considered as a monospecific genus comprising exclusively *Neotinea maculata* (Desf.) Stearn, currently it includes species previously in *Orchis* Tourn. ex L. (Bateman *et al.*, 2003). Flowers are nectarless and the pollination strategy is food deception (Duffy *et al.*, 2009). Self-pollination was also recorded in *Neotinea maculata* (Desf.) Stearn (Duffy *et al.*, 2009).

***Neotinea lactea* (Poir.) R.M.Bateman, Pridgeon & M.W.Chase, Lindleyana 12: 122 (1997).**

Distribution: Medit.

Phenology: III-V

Habitat and ecology: Full sun and mid-shade on moist substrates up to 1000m asl.

Pollinators: It adopts a food deception strategy so a wide range of species are adopted as pollinators.

Discussion: It is the most widespread *Neotinea* in Sardinia, quite common throughout the island.

2n=42

Synonyms: *Orchis acuminata* Desf.; *Orchis corsica* Viv.; *Orchis parviflora* Ten.; *Orchis ricasoliana* Parl.; *Orchis tenoreana* Guss.; *Orchis lactea* Poir.

***Neotinea maculata* (Desf.) Stearn, Ann. Mus. Goulandris 2: 79 (1974 publ. 1975).**

Distribution: Medit.-Atl.

Phenology: III-V

Habitat and ecology: Garrigues, oakwood with deep soil up to 1300m asl.

Pollinators: It adopts a food deception strategy so a wide range of species are adopted as pollinators.

Discussion: *N. maculata* is common in the main woodland throughout the island.

2n=40

Synonyms: *Neotinea intacta* (Link) Rchb. f.; *Orchis intacta* Link; *Satyrium maculatum* Desf.; *Tinea cylindrica* Biv

***Neotinea tridentata* (Scop.) R.M.Bateman, Pridgeon & M.W.Chase, Lindleyana 12: 122 (1997).**

Distribution: Eurasiat.

Phenology: III-V

Habitat and ecology: Full sun on moist or dry substrate. Garrigues, meadows and oak wood up to 1300 m asl.

Pollinators: *Apis mellifera*, *Halictus patellatus*, *Osmia bicornis*, *O. niveata*.

Discussion: In Sardinia its distribution is limited to the Sarcidano and north-west. Populations are often rich.

2n=42

Synonyms: *Orchis commutata* Tod.; *Orchis conica* Willd.; *Orchis variegata* All.; *Orchis tridentata* Scop.

***Ophrys* L., Sp. Pl.: 948 (1753).**

Etymology: the paternity of its name is given to Plinius the old. It is generally derived from ophrus (=eyebrow) referring to petals or their use by ancient mediterranean culture as eyebrow dye. Reynaud (1986) reports that *Ophrys* is derived from *ophis* referring to similarity of the flower of *Neottia ovata* (L.) Mathias Joseph Bluff & Carl Anton Fingerhuth (at that time recorded as *Ophrys*) to a snake's head. A mainly mediterranean genus with controversial taxonomy (cit concetto di specie). This genus clearly prefers dry and calcareous soils in sunny positions. Flowers mimics

virgin female insect in shapes, colours and scent emitted, different species have a species-specific relation with pollinators and sex deception through pseudocopulation in the pollination strategy.

Ophrys annae J. Devillers-Terschuren & P. Devillers

Distribution: Endem. SA-CO

Phenology: III-VI

Habitat and ecology: Grassland, meadows, garrigues on calcareous and thin soils up to 900m asl.

Pollinators: *Osmia rufa* subsp. *rufa*

Discussion: discontinuously recorded on the island. Populations are often very rich.

2n=36,37,38

Synonyms: *Ophrys holoserica* subsp. *annae* (Devillers-Tersch. & Devillers) H.Baumann, Giotta, Künkele, R.Lorenz & Piccitto, J. Eur. Orch. 27: 217 (1995). *Ophrys fuciflora* subsp. *annae* (Devillers-Tersch. & Devillers) R.Engel & P.Quentin, Orchidophile (Asnières) 124: 205 (1996).

***Ophrys apifera* Huds., Fl. Angl.: 340 (1762).** *Ophrys apifera* var. *fulvofusca* M.P.Grasso & Scrugli, Orchidee (Hamburg) 38: 47 (1987).

Distribution: Euro-Medit.

Phenology: III-VI

Habitat and ecology: Garrigues, meadows roadside to 900 m asl.

Pollinators: self-pollination.

Discussion: Populations are quite conspicuous and dense. In Sardinia, this species is very variable and four variety are currently recorded: *O.apifera* Hudson var. *aurita* Moggridge as a bibliographic report, *O.apifera* Hudson var. *bicolor* (Naegeli) Nelson in the south-west area, *O.apifera* Hudson

var. *chlorantha* (Hegetschw) Richter in three populations in south west, center and north east and *O.apifera* Hudson var. *fulvofusca* Grasso et Scrugli in the central and north-east coast (south-west reports are bibliographic data not confirmed).

2n=36

Synonyms: *Ophrys arachnites* Mill.; *Ophrys rostrata* Ten.

***Ophrys bombyliflora* Link, J. Bot. (Schrader) 1799(2): 325 (1800).**

Distribution: Medit.

Phenology: III-V

Habitat and ecology: Meadows, garrigues and roadside up to 900 m asl.

Pollinators: *Eucera nigrescens*, *E. gracilipes*, *E. vulpa*.

Discussion: This species forms very rich populations.

2n=36

Synonyms: *Ophrys tabanifera* Will.

***Ophrys chestermanii* (J.J.Wood) Gölz & H.R.Reinhard, Mitt. Arbeitskreis Heimische Orchid.**

Baden-Württemberg 20: 115 (1988).

Distribution: Endem. SA

Phenology: III-V

Habitat and ecology: Deep, fresh soils in shady positions up to 600m asl.

Pollinators: *Bombus vestalis*

Discussion: *Ophrys annae* and *Ophrys chestermanii*: experiments run on these two endemic entities suggest that the gene flow is completely absent in nature and breeding crosses support the idea that these species are reproductively distinct (Lussu *et al.* 2018, Gogler *et al.* 2008).

$2n=36,37$

Synonyms: *Ophrys fuciflora* (F.W. Schmidt) Moench subsp. *chestermanii* (J.J. Wood) Blatt & M. Wirth.

***Ophrys eleonora* Devillers-Tersch. & Devillers, Naturalistes Belges 72: 100 (1991).**

Distribution: W-Medit.

Phenology: II-V

Habitat and ecology: Meadows, garrigues up to 1100m asl.

Pollinators: *Andrena morio* ♂.

Discussion: Common species especially in the south coast.

$2n=36$

Synonyms: incl. *Ophrys arnoldii* P. Delforge; incl. *Ophrys lojaconoi* P. Delforge; incl. *Ophrys sulcata* Devillers-Tersch. & Devillers, *Ophrys funerea* Viv., Fl. Cors. Prodr.: 15 (1824). *Ophrys fusca* subsp. *iricolor* (Desf.) K.Richt., Pl. Eur. 1: 261 (1890).

***Ophrys exaltata* subsp. *morisii* (Martelli) Del Prete, Webbia 37: 251 (1984).**

Distribution: Endem. SA-CO

Phenology: II-V

Habitat and ecology: Garrigues, meadows, woodland up to 1000m asl.

Pollinators: *Anthophora sicheli*.

Discussion: The hybrid formula suggested is *O. argolica* subsp. *crabronifera* × *O. sphegodes* (Govaerts 2003), we do not agree with the classification of this entities as hybrid because *O. argolica* subsp. *crabronifera* has never been recorded in Sardinia, in addition *O. exaltata* subsp. *morisii* is widespread and its distribution can not be considered as hybrid zone.

2n=36

Synonyms: *Ophrys aranifera* var. *morisii* Martelli, Monocot. Sardoae 1: 62 (1896), *Ophrys* × *morisii* (Martelli) G.Keller & Soó in G.Keller & al., Monogr. Iconogr. Orchid. Eur. 2: 42 (1931), *Ophrys argolica* subsp. *morisii* (Martelli) Kreutz, Kompend. Eur. Orchid.: 86 (2004), *Ophrys crabronifera* subsp. *morisii* (Martelli) H.Baumann & R.Lorenz, J. Eur. Orch. 37: 719 (2005).

***Ophrys funerea* Viv., Fl. Cors. Prodr.: 15 (1824).**

Distribution: Endem. SA-CO

Phenology: III-IV

Habitat and ecology: Garrigues and meadows up to 900 m asl.

Pollinators: *Andrena nigroaenea*♂.

Discussion: It is a very rare species located mainly on the central mountains of the island.

2n= Unknown.

Synonyms: *Ophrys funerea* Viv.; *Ophrys hespera* Devillers-Tersch. & Devillers; *Ophrys zonata* Devillers-Tersch. & Devillers.

***Ophrys fusca* Link, J. Bot. (Schrader) 1799(2): 324 (1800).**

Distribution: Medit.

Phenology: V

Habitat and ecology: Garrigues, roadside and meadows up to 900 m asl

Pollinators: *Colletes cunicularius*♂

Discussion: Very common species and populations are usually extremely rich.

2n=36

Synonyms: *Ophrys fuciflora* (F.W. Schmidt) Moench.

***Ophrys garganica* O.Danesch & E.Danesch, Pl. Syst. Evol. 124: 94 (1975).**

Distribution: Steno-Med.

Phenology: III-V

Habitat and ecology: Garrigues and roadside to 900 m asl.

Pollinators: *Andrena carbonaria*.

Discussion: Very variable species, one single population is currently known.

2n=36

Synonyms: Unknown.

***Ophrys incubacea* Bianca, Nov. Pl. Sp. Prope Hyblam: 8 (1842).**

Distribution: W-Medit.

Phenology: III-V

Habitat and ecology: Garrigues, meadows open wood and roadside to 960m asl.

Pollinators: *Andrena morio*.

Discussion: It is very common on the island, it is found in the most disparate environments forming numerous populations but not very dense

2n=36

Synonyms: *Ophrys atrata* Lindley; *Ophrys aranifera* Hudson subsp. *atrata* Arcangeli; *Ophrys sphegodes* Miller subsp. *atrata* (Lindley) E. Mayer

***Ophrys lutea* Cav., Icon. 2: 46 (1793).**

Distribution: Medit.-Atl.

Phenology: III-V

Habitat and ecology: Garrigues, meadows and roadside to 1000m asl.

Pollinators: *Andrena cinerea* ♂, *A. seneciocionis* ♂.

Discussion: *O. lutea* is not very common in Sardinia and it is often confused with *O. sicula*.

2n=36

Synonyms: *Ophrys phryganae* Devillers-Tersch. & Devillers; *Ophrys corsica* Soleirol ex G.Foelsche & W.Foelsche; *Ophrys lutea* Cav. subsp. *corsica* (Soleirol ex G.Foelsche & W.Foelsche) Kreutz; *Arachnites lutea* Tod.

***Ophrys normanii* J.J.Wood, Orchid Rev. 91: 385 (1983).**

Distribution: Endem. SA

Phenology: IV-V

Habitat and ecology: Garrigues, oak woods, roadside from 100m to 500m asl.

Pollinators: *Bombus vestalis* ♂.

Discussion: very rare. Due to its ambiguous morphology, for decades it has been considered a hybrid between the sympatric *O. chestermanii* and *O. tenthredinifera*. AFLP analysis, GC-EAD analyses and pollination experiments clarified that *O. normanii* is phylogenetically isolated by its putative parents and the sharing of *Bombus vestalis* as pollinator with *O. chestermanii* depends on convergent evolution (Gogler *et al.* 2008)

2n= Unknown.

Synonyms: *Ophrys x maremmae* O. et E. Danesch nsubsp. *woodii* (J.J. Wood) H. Baumann et Kunkele

Ophrys ortuabis M.P.Grasso & Manca, Orchidophile (Asnières) 151: 81 (2002).

Distribution: Endem. SA

Phenology: III-IV

Habitat and ecology: Dry garrigues dominated by *Rosmarinus officinalis* L.

Pollinators: *Andrena hypopolia*♂.

Discussion: Very rare species currently recorded in two populations, one on the east coast and one in the municipality of Laconi.

2n= Unknown.

Synonyms:

Ophrys × panattensis Scrugli, Cogoni & Pessei, Orchidee (Hamburg) 43: 225 (1992).

Distribution: Endem. SA

Phenology: IV-V

Habitat and ecology: Bush and garrigues up to 960 m asl.

Pollinators: *Osmia rufa* subsp. *rufa*.

Discussion: currently the hybrid formula suggested for *O. panattensis* is *O. morisii* × *O. scolopax*, however this hybridization should be investigated more because the *O. scolopax* group in Sardinia is represented by *O. scolopax* subsp. *conradiae* and *O. scolopax* subsp. *picta* which rarely co-occur with *O. morisii* and so *O. panattensis* distribution could not be considered as an hybrid zone between these putative species. Pollinator: *Osmia rufa* subsp. *rufa*. As suggested in Paulus and Gack 1995.

2n= Unknown.

Synonyms: *Ophrys* × *arachnitiformis* Gren. & Philippe, Mém. Soc. Émul. Doubs, sér. 3, 4: 391 (1860).

Ophrys scolopax* subsp. *conradiae (Melki & Deschâtres) H.Baumann, Giotta, Künkele, R.Lorenz & Piccitto, J. Eur. Orch. 27: 220 (1995).

Distribution: Endem. SA-CO

Phenology: IV-VI

Habitat and ecology: Meadows, garrigues, bush, open wood, woodland up to 960m asl.

Pollinators: Unknown.

Discussion: Very variable and rare species. Populations are often not very dense.

2n=36

Synonyms: *Ophrys conradiae* Melki & Deschâtres; *Ophrys scolopax* Cav. subsp. *sardoa* H. Baumann, Giotta, Künkele, R. Lorenz & Piccitto.

Ophrys scolopax* subsp. *picta (Link) Kreutz, *Kompend. Eur. Orchid.*: 114 (2004).

Distribution: Medit.

Phenology: IV-V

Habitat and ecology: Dry garrigues at 960m asl.

Pollinators: *Eucera barbiventris*.

Discussion: Extremely rare, found in a single population not recorded in the last years.

2n= Unknown.

Synonyms:

***Ophrys sicula* Tineo, Pl. Rar. Sicil., ed. 2: 13 (1846).**

Distribution: Med.

Phenology: III-V

Habitat and ecology: Dry garrigues, meadows, roadsides up to 900m asl.

Pollinators: *Andrena hesperia* ♂, *A. merula* ♂, *A. taraxaci* ♂.

Discussion: It is a very common orchid but it is usually confused with *O. lutea*.

2n= Unknow.

Synonyms: *Ophrys subfusca* subsp. *lepida* (S.Moingeon & J.-M.Moingeon) Kreutz, GIROS Orch.

Spont. Eur. 58: 7 (2015).

***Ophrys speculum* Link, J. Bot. (Schrader) 1799(2): 324 (1800), nom. cons.**

Distribution: Medit.

Phenology: IV-V

Habitat and ecology: Dry garrigues, meadows, roadsides up to 1000m asl.

Pollinators: *Dasyscolia ciliata* ♂.

Discussion: Species extremely common, populations usually counts hundreds of plants.

2n=36

Synonyms: *Ophrys ciliata* Biv.; *Ophrys vernixia* auct. non Brot.; *Ophrys vernixia* Brot. subsp. *ciliata* (Biv.) Del Prete.

Ophrys sphegodes* subsp. *praecox Corrias, Boll. Soc. Sarda Sci. Nat. 22: 325 (1983).

Distribution: Endem. SA-CO

Phenology: I-IV

Habitat and ecology: olive tree cultivations, roadside up to 730m asl.

Pollinators: *Andrena thoracica*.

Discussion: In Sardinia its distribution is limited to the north-west coast.

2n=36,37

Synonyms: *Ophrys praecox* (Corrias) Devillers-Tersch. & Devillers

Ophrys subfusca* subsp. *liveranii Orrù & M.P.Grasso, GIROS Notiz. 30: 9 (2005).

Distribution: Endem. SA

Phenology: III-V

Habitat and ecology: Pinewood up to 65m asl.

Pollinators: *Eucera nigrilabris* ♂

Discussion: It is recorded as single population in Gulf of Oristano.

2n= Unknow.

Synonyms:

***Ophrys tenthredinifera* Willd., Sp. Pl. 4: 67 (1805).**

Distribution: Med.

Phenology: III-V

Habitat and ecology: Dry garrigues, meadows, roadsides

Pollinators: *Eucera nigrilabris* ♂

Discussion: Following Devillers et al 2003 in Sardinia in the *O. tenthredinifera* group *O. aprilina* P. Devillers & J. Devillers-Terschuren and *O. neglecta* Parlato are also included. Following its taxonomy in Sardinia *O. neglecta* Parlato is the most common species of the group and the population found by Lai in 2004-2007 on the south-east coast should be considered ad *O. aprilina*.

2n=36

Synonyms: *Arachnites tenthredinifer* (Willd.) Tod., Orchid. Sicul.: 85 (1842).

***Orchis* Tourn. ex L., Sp. Pl.: 943 (1753).**

Etymology: Teofrasto (372-288 a.C.) used the term *orchis* in *Historia plantarum* to describe plants with roots similar to human male genitalia. A Euroasian genus, in which the monospecific genus *Aceras* L. has also been included (cit. Bateman). It occurs in a variety of habitats even if the the majority of species prefer grassland, macchia or forest. Except a few species, flowers are nectarless and pollination is through food-deception.

***Orchis anthropophora* (L.) All., Fl. Pedem. 2: 148 (1785).**

Distribution: Medit.-Atl.

Phenology: III-VI

Habitat and ecology: Full sun, mid-shade positions on dry or moist calcareous substrates. Garrigues, woodland edges up to 1100m asl.

Pollinators: It adopts a food deception strategy so a wide range of species are adopted as pollinators.

Discussion: Extremely common.

$2n=42$

Synonyms: *Aceras anthropophorum* (L.) R. Br.

***Orchis brancifortii* Biv., Stirp. Rar. Sicilia 1: 3 (1813).**

Distribution: Endem. SA-SI

Phenology: IV-VI

Habitat and ecology: Full sun on dry substrate. Garrigues from 200 to 1200m asl.

Pollinators: It adopts a food deception strategy so a wide range of species are adopted as pollinators.

Discussion: Currently the distribution of *O. brancifortii* is limited only to the central-east mountains.

$2n=42$

Synonyms: *Orchis quadripunctata* Cyrillo ex Tenore subsp. *brancifortii* (Bivona-Bernardi) E. G. Camus & A. Camus.

***Orchis italica* Poir. in J.B.A.M.de Lamarck, Encycl. 4: 600 (1798), nom. cons.**

Distribution: Medit.

Phenology: IV

Habitat and ecology: full sun on alkaline substrates.

Pollinators: It adopts a food deception strategy so a wide range of species are adopted as pollinators.

Discussion: In 2015 a single plant was found for the first time in the municipality of Ulassai (NU).

In the following years, other two populations were discovered: one in the municipality of Alghero (SS), 13 plants; and a single flowering plants in Castiadas (CA).

2n= Unknown.

Synonyms: *Orchis longicornis* Link, *Orchis longicuris subsp. longipenis* Font Quer & P.Palau, *Orchis undulatifolia* Biv., *Orchis tephrosanthos var. undulatifolia* (Biv.) Ker Gawl..

***Orchis mascula subsp. ichnusae* Corrias, Boll. Soc. Sarda Sci. Nat. 21: 403 (1982).**

Distribution: Endem. SA-CO

Phenology: III-VI

Habitat and ecology: Full sun or mis-shade position on dry or moist substrates. Grassland and garrigues from 200 m to 1400 m asl.

Pollinators: *Bombus hortorum*, *B. lapidarius*, *B. pratorum*, *B. ruderarius*, *B. rupestris*, *B. terrestris*.

Discussion: Species located in the central-east mountains (Sarcidano) and south-west mountains (Iglesiente). Local populations are very variable and conspicuous.

2n=42

Synonyms: *Orchis ichnusae* (Corrias) Devillers-Tersch. & Devillers; *Orchis olbiensis* Reut. ex Gren. subsp. *ichnusae* (Corrias) Buttler.

***Orchis provincialis* Balb. ex Lam. & DC., Syn. Pl. Fl. Gall.: 169 (1806).**

Distribution: Medit.

Phenology: III-VI

Habitat and ecology: Full sun or mid-shade cool positions. Woodland edges and grassland up to 1300 m asl.

Pollinators: *Bombus humilis*, *B. lapidarius*, *B. pratorum*, *B. ruderarius*, *B. rupestris*, *B. terrestris*.

Discussion: Species widespread but usually population are not very dense.

2n=42

Synonyms: *Orchis cyrilli* Ten.; *Orchis leucostachys* Griseb.

***Orchis purpurea* Huds., Fl. Angl.: 334 (1762).**

Distribution: Eurasiat.

Phenology: V-VI

Habitat and ecology: Full sun on dry substrate. Garrigues, meadows and bush up to 400 m asl.

Pollinators: It adopts a food deception strategy so a wide range of species are adopted as pollinators.

Discussion: Species extremely rare recorded only in the north-west meadows of the island.

2n=42

Synonyms: *Orchis fusca* Jacq.

***Platanthera* Rich., De Orchid. Eur.: 26 (1817), nom. cons.**

Etymology: from greek *platus* and *antheros* in referring to the shape of the anther. Holartic genus occurring in a very wide range of habitats. Plants are summergreen and leaves dry out in autumn.

Flowers are usually green-ish or white-ish in a dense to lax inflorescence. Noctuidae Latreille, 1809 are very often the usual pollinators.

***Platanthera algeriensis* Batt. & Trab., Bull. Soc. Bot. France 39: 75 (1892).**

Distribution: W-Medit.

Phenology: VI-VII

Habitat and ecology: Wet meadows, riverside from 600m to 1600m asl.

Pollinators: Unknown.

Discussion: Species recorded for the very first time in 1990 by Scrugli and Cogoni. It is located mainly on the Gennargentu mountains.

$2n=42$

Synonyms: *Platanthera chlorantha* (Custer) Reichenbach. subsp. *algeriensis* (Battandier et Trabut) Emberger.

***Platanthera kuenkelei* var. *sardoa* R.Lorenz, Akhalk., H.Baumann, Cortis, Cogoni & Scrugli, J. Eur. Orch. 44: 20 (2012).**

Distribution: Endem. SA

Phenology: V-VII

Habitat and ecology: Oak wood land with *Quercus ilex* and *Q. suber*.

Pollinators: Unknown.

Discussion: it was recorded for the very first time in 2011 in a forest dominated by *Quercus ilex* L. in San Leonardo De Sientes Fuentes (Borore, OR), molecular analyses showed a closer relationship with Algerian samples than with those from the Italian peninsula (cit. Lorenz).

2n= Unknown.

Synonyms: Unknown.

***Serapias* L., Sp. Pl.: 949 (1753).**

Etymology: the word *Serapias* derived from *Serapis*, egyptian god. Essentially mediterranean genus with few species in the Azores and Canary Islands. Sepals often connivent to form a hood from which the distal portion of the labellum departs. Labellum usually three lobed and divided in a proximal portion (hypochilum) and a distant portion (epichilum). Flower always nectarless and pollination through nest-deception.

***Serapias cordigera* L., Sp. Pl. ed. 2: 1345 (1763).**

Distribution: Medit.-Atl.

Phenology: III-V

Habitat and ecology: Meadows, garrigues, bush on dry substrates up to 600m asl.

Pollinators: *S. cordigera* is rather local but it forms big populations.

Discussion: it is a common species and usually populations are conspicuous.

2n=36

Synonyms:incl. *Serapias olbia* Verg.

***Serapias lingua* L., Sp. Pl.: 950 (1753).**

Distribution: Medit.-Atl.

Phenology: III-VI

Habitat and ecology: Roadside, meadows, garrigues up to 1200m asl. Very flexible. Despite the nest pollination syndrome typical of the genus *Serapias*, *S. lingua* is pollinated through sex deception.

Pollinators: *C. cucurbitina*.

Discussion: It is the most common *Serapias* in Sardinia and populations usually consist in more than one hundred plants.

$2n=72$

Synonyms: *Serapias oxyglottis* Willdenow.

***Serapias nurrica* Corrias, Boll. Soc. Sarda Sci. Nat. 21: 397 (1982).**

Distribution: W-Medit.

Phenology: IV-V

Habitat and ecology: Garrigues and pinewoods up to 200 m asl.

Pollinators: It adopts a nest deception strategy so solitary bees usually find its flowers as temporary refuge.

Discussion: Its distribution is limited in less than ten populations in the south-west coast (Iglesiente).

$2n=36$

Synonyms: Unknown.

***Serapias parviflora* Parl., Giorn. Sci. Sicilia 59: 66 (1837).**

Distribution: Medit.-Atl.

Phenology: III-VI

Habitat and ecology: Meadows, garrigues, bush on dry substrates up to 1000m asl.

Pollinators: It adopts a nest deception strategy so solitary bees usually find its flowers as temporary refuge.

Discussion: It is very common orchid on the island, it is usually found in the most disparate environments forming numerous but not very dense populations. *S. parviflora* often coexists with *S. lingua* with which it hybridizes (*Serapias* x *todaroi*).

2n=36

Synonyms: *Serapias occultata* J. Gay ex Cavalier.

ARTIFICIAL KEY TO THE ORCHIDS OF SARDINIA

1 Plants with leaves often reduced to brownish or green-purpleish bracts or plants with only two green opposite leaves and green flower with a bi-lobed lip 2

2 Floral bracts longer than the ovary. Lip entire, white with purple veins. Plant entirely brownish-violet, greenish-purple. 3

3 Well developed spur, arched and facing downwards, as long as the ovary.

Limodorum abortivum

3 Spur rudimentary (< 1mm) or completely absent.

Limodorum trabutianum

2. Floral bracts always shorter than the ovary. Lip deeply bi-lobed green or brownish, spur always absent. 4

4. Plants brownish and. Leaves extremely reduced and amplexicaul. Flowers yellowish-brown.

Neottia nidus-avis

4 Plants green and autotrophic. Two well developed green leaves. Flowers green or greenish.

Neottia ovata

1 Plants with several (sono sempre più di due?) well-developed green leaves. 5

5 Lip without spur. 6

6 Lip tri-lobed reddish. Lateral lobes long and narrow. Lobe median longer than the lateral ones, bifid. Hemispherical helmet, not acuminate, composed of yellow-green red veined sepals.

Orchis anthropophora

6 Lip different. 7

7 Lip resembling an insect: big, dark, velvety, hairy. 8

8 Lip apex simple, without any appendix. 9

9 Sepals and petals green, greenish or brownish. 10

10 Margins of the lip yellow 11

11 Wide yellow margin 12

12 Lip lateral lobes forming an angle of c. 65° with longitudinal axis. 13

13 Lip 14-18mm long.

Ophrys lutea

13 Lip 10 mm long.

Ophrys sicula

12 Lip lateral lobes forming with longitudinal axis more acute than 65°.

Ophrys subfusca subsp. liveranii

11 Yellow margins reduced to a narrow line 14

14 Lip \geq 13 mm, underside purple red.

Ophrys eleonora

14 Lip < 13 mm, underside brownish or yellowish. 15

15 Lip 12-13 mm long

Ophrys fusca

15 Lip < 10 mm. 16

16 Lip 7-10 mm x 5-8mm

Ophrys funerea

16 Lip 7-10 mm x 7- 8 mm.

Ophrys ortuabis

10 Lip margins not yellow, with dense brown hairs.

Ophrys speculum

8 Lip apex with an appendix (sometimes folded backwards or reduced) 17

17 Lip apex evident.

18 Gynostemium simple. 19

19 Lip appendix folded backwards .

Ophrys bombyliflora

19 Lip appendix folded upwards 20

20 Lip 9-15 mm long

21 Lip margins folded upwards

Ophrys tenthredinifera

21 Lip margins folded backwards

Ophrys annae

20 Lip 16-26 mm long

22 Sepals broadly rounded

Ophrys normanii

22 Sepals elongated

Ophrys chestermanii

18 Gynostemium apex beak-like 23

23 Gynostemium apex sharp and elongated

Ophrys apifera

23 Gynostemium apex short 24

24 Lip entire 25

25 Lip margins pointing downwards

Ophrys panattensis

25 Lip margins folded backwards

Ophrys exaltata* subsp. *morisii

24 Lip deeply three-lobed 26

26 Lip 9-13 x 9.5mm.

Ophrys scolopax* subsp. *conradiae

26 Lip 7-12 x 7-11 mm.

Ophrys scolopax* subsp. *picta

17 Lip apex extremely reduced 27

27 Lip margins folded upwards

Ophrys garganica

27 Lip margins folded downwards 28

28 Lip with two big swellings (>4mm) at its base

Ophrys incubacea

28 Lip swellings small (< 4mm) or absent at its base

Ophrys sphegodes* subsp. *praecox

7 Lip not resembling an insect 29

29 Lip divided into two parts by a constriction: a basal part (hypochile) and a distal part (epichile) resembling a tongue 30

30 Petals and sepals fused together forming an helmet. 31

31 Hypochile (7-14mm x 14-17mm) with a single callosity, whole, shiny and purple almost black, with a coffee bean shape. Epichile (7-17mm x 4-11mm) pendant, pinkish

Serapias lingua

31 Hypochile with two callosities 32

32 Epichile lanceolate (5-10 mm x 3-5 mm), much narrower than the hypochile (6-10mm x 9-11mm), typically facing backwards. Hood greenish-grey or pale lilac

Serapias parviflora

32 Epichile bigger than 5-10 mm x 3-5 mm, hypochile bigger than 6-10mm x 9-11mm 33

33 Linear and divergent callosities resembling a letter V. Epichile widened at the base, heart-shaped and similar in sizes to hypochile (15- 35mm x 8-25mm)

Serapias cordigera

33 Lamellar callosities slightly arcuate (to resemble two round brackets). Epichile 10-14x 6-10 always much narrower than hypochile ovate-lanceolate, with marginal line typically lighter, gray-whitish. Hypochile 10-17mm x 6-10mm.

Serapias nurrica

30 Petals and sepals not fused together 34

34 Flowers erect, white or pink 35

35 Flowers purplish pink

Cephalanthera rubra

35 Flowers white 36

36 Leaves elliptic. Inflorescence composed of 3-8 flowers

Cephalanthera damasonium

36 Leaves lanceolate. Inflorescence composed of 8-20 flowers

Cephalanthera longifolia

34 Flowers pendant, or parallel to the ground. 37

37 Inflorescence dense. 38

38 Inflorescence that stretches up to $\frac{3}{4}$ of the stem. Plant from 40 to 60 cm.

Epipactis tremolsii

38 Inflorescence generally less than half the stem. Plant tall 20-80 cm.

Epipactis helleborine

37 Inflorescence lax. 39

39 Hypochile triangular when spread.

Epipactis palustris

39 Hypochile cup-shaped. 39

40 Hairy stem.

Epipactis microphylla

40 Hairless stem. 41

41 Epichile with two small and pinkish bosses at its base.

Epipactis muelleri

41 Epichile with two big white and pinkish bosses at its base.

Epipactis exilis

29 Lip entire. Flowers arranged in a spiral, white and extremely small (< 3mm) 40

40 Base of the lip greenish. Stem entirely tomentose. Dense and narrow inflorescence along the stem.

Spiranthes spiralis

40 Lip completely white. Stem tomentose only in the upper part.

Spiranthes aestivalis

5 Lip with spur 41

41 Two distinct pollinic lodges (thecae)

44 Flowers greenish or cream-ish.

Platanthera algeriensis

44 White flowers. Lateral sepals lanceolate and deeply falciform

Platanthera kuenkelei subsp. *kuenkelei* var. *sardoa*

43 Single pollinic lodge (theca). 45

45 Three-lobed lip with the median shorter and divided in two segments. Spur short, rounded at the apex and directed downwards.

Himantoglossum robertianum

45 Lip otherwise. 46

46 Lateral tepals folded on the column forming an helmet 47

47 Lip larger than longer 48

48 Lip entire 49

49 Lip concave longer than wide, pinkish-white to purple, lighter along the central axis. Usually unveined.

Anacamptis papilionacea subsp. *papilionacea*

49 Lip wider than long, from pinkish-white to pink. Darker veins well marked, diverging in a radial pattern from the longitudinal axis.

Anacamptis papilionacea subsp. *grandiflora*

48 Lip trilobed 50

50 Flowers green, very small

Gennaria diphylla

50 Flowers from white striped to deep purple

Anacamptis longicornu

48 Lip longer than larger, three lobed 51

51 Median lobe entire

Anacamptis fragrans

51 Median lobe bilobe or bifid 52

52 Floral bracts longer than the half of the ovary 53

53 Leaves spotted

Neotinea maculata

53 Leaves unspotted 54

54 Inflorescence cylindrical.

Neotinea lactea

54 Inflorescence first conical then globose.

Neotinea tridentata

52 Floral bracts shorter than the half of the ovary

55 Tepals blackish fused together

Orchis purpurea

55 Tepals whitish pink veined

Orchis italica

46 Lateral tepals folded backwards 56

56 Leaves distributed on all the stem 57

57 Floral bracts longer than the ovary 58

58 Straight spur, generally horizontal and sometimes ascending. Flowers yellow or pale yellow.

Dactylorhiza insularis

58 Spur slightly arched and facing downwards. Flowers usually violet.

Dactylorhiza elata subsp. *sesquipedalis*

57 Floral bracts shorter than the ovary 59

59 Lip entire, spur filiform

Anacamptis pyramidalis

59 Lip three-lobed 60

60 Lip larger than longer, median lobe shorter than lateral

Anacamptis laxiflora

60 Lip longer than larger, median lobe longer than lateral

Anacamptis palustris

57 Leaves forming a basal rosette 61

61 Spur filiform.

Orchis brancifortii

61 Spur cylindrical or sack-like 62

62 Spur cylindrical 63

63 Spur ascendant, 13-19 mm long. Flowers from white to pale yellow with purpleish spots in the center in the center of the lip. Leaves spotted.

Orchis provincialis

63 Spur horizontal, 9-14 mm long. Flowers from pink-ish to purple with darker spots in the center of the lip. Leaves unspotted.

Orchis mascula subsp. *ichnusae*

61 Spur sack-like

Anacamptis collina

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Table 1. Species list and databases review. In TRY Plant Trait Database columns refers to ObsNum: Number of Observations, ObsGRNum: number of geo-referenced observations, ObsPubNum: number of public observations, MeasNum: number of measurements, MeasGRNum: number of geo-referenced measurements, TraitNum: number of traits.

| Species | IUCN red list | | GenBank items | TRY Plant Trait Database | | | | |
|---|--------------------------------|-------------------|---------------|--------------------------|----------|---------|-----------|----------|
| | Status | Pop. trend | | ObsNum | ObsGRNum | MeasNum | MeasGRNum | TraitNum |
| <i>Anacamptis</i> | | | | | | | | |
| <i>A. collina</i> | | | 6 | | | | | |
| <i>A. fragrans</i> | | | 2 | | | | | |
| <i>A. laxiflora</i> | | | 70 | | | | | |
| <i>A. longicornu</i> | | | 38 | | | | | |
| <i>A. papilionacea</i> var. <i>papilionacea</i> | | | 4 | | | | | |
| <i>A. papilionacea</i> var. <i>grandiflora</i> | | | 4 | | | | | |
| <i>A. palustris</i> | <i>Least Concern</i> ver 3.1 | <i>decreasing</i> | 24 | | | | | |
| <i>A. pyramidalis</i> | | | 25 | 244 | 31 | 447 | 87 | 120 |
| <i>Cephalanthera</i> | | | | | | | | |
| <i>C. damasonium</i> | | | 86 | | | | | |
| <i>C. longifolia</i> | | | 106 | 329 | 57 | 827 | 334 | 111 |
| <i>C. rubra</i> | | | 13 | 1 | 469 | 4 | 113 | |
| <i>Dactylorhiza</i> | | | | | | | | |
| <i>D. elata</i> subsp. <i>sesquipetalis</i> | <i>Near Threatened</i> ver 3.1 | <i>decreasing</i> | | | | | | |
| <i>D. insularis</i> | | | 2 | | | | | |
| <i>Epipactis</i> | | | | | | | | |

| | | | | | | | | |
|-----------------------|----------------------|-------------------|------|------------|-----------|------------|-----------|------------|
| <i>E. gracilis</i> | | | | | | | | |
| <i>E. helleborine</i> | | | 1734 | 509 | 117 | 1458 | 738 | 145 |
| <i>E. microphylla</i> | | | 41 | 100 | 12 | 236 | 8 | 81 |
| <i>E. muelleri</i> | <i>Least Concern</i> | <i>decreasing</i> | 13 | 74 | | 164 | | 74 |
| <i>E. palustris</i> | <i>Least Concern</i> | <i>decreasing</i> | 162 | 271 | 25 | 427 | 25 | 120 |
| <i>E. tremolsii</i> | | | 1 | | | | | |
| Gennaria | | | | | | | | |
| <i>G. diphylla</i> | | | 11 | | | | | |
| Himantoglossum | | | | | | | | |
| <i>H. robertianum</i> | | | 41 | | | | | |
| Limodorum | | | | | | | | |
| <i>L. abortivum</i> | | | 97 | 93 | | 234 | | |
| <i>L. trabutianum</i> | | | 1 | | | | | |
| Neotinea | | | | | | | | |
| <i>N. lactea</i> | | | 7 | | | | | |
| <i>N. maculata</i> | | | 16 | | | | | |
| <i>N. tridentata</i> | | | 9 | | | | | |
| Neottia | | | | | | | | |
| <i>N. nidus-avis</i> | <i>Least Concern</i> | <i>decreasing</i> | 84 | 434 | 21 | 701 | 23 | 127 |
| <i>N. ovata</i> | | | 34 | | | | | |
| Ophrys | | | 84 | | | | | |
| <i>O. annae</i> | | | 0 | | | | | |

| | | | | | | | | |
|--|--|--|-----|---|--|--|--|--|
| <i>O. apifera</i> | | | 102 | | | | | |
| <i>O. bombyliflora</i> | | | 16 | 1 | | | | |
| <i>O. chestermanii</i> | | | 0 | | | | | |
| <i>O. eleonorae</i> | | | | | | | | |
| <i>O. exaltata</i> subsp. <i>morisii</i> | | | 9 | | | | | |
| <i>O. funerea</i> | | | 5 | | | | | |
| <i>O. fusca</i> | | | 79 | | | | | |
| <i>O. garganica</i> | | | 64 | | | | | |
| <i>O. incubacea</i> | | | 17 | | | | | |
| <i>O. lutea</i> | | | 26 | | | | | |
| <i>O. normanii</i> | | | 0 | | | | | |
| <i>O. ortuabis</i> | | | 0 | | | | | |
| <i>O. panattensis</i> | | | 0 | | | | | |
| <i>O. scolopax</i> subsp. <i>conradiae</i> | | | | | | | | |
| <i>O. scolopax</i> subsp. <i>picta</i> | | | 1 | | | | | |
| <i>O. sicula</i> | | | 11 | | | | | |
| <i>O. speculum</i> | | | 18 | | | | | |
| <i>O. sphegodes</i> subsp. <i>praecox</i> | | | 0 | | | | | |
| <i>O. subfusca</i> subsp. <i>liveranii</i> | | | 0 | | | | | |
| <i>O. tenthredinifera</i> | | | | | | | | |
| Orchis | | | | | | | | |
| <i>O. anthropophora</i> | | | 61 | | | | | |

| | | | | | | | |
|---|------------------------------|----------------|----|---|----|--|----|
| <i>O. brancifortii</i> | <i>Least Concern ver 3.1</i> | <i>stable</i> | 2 | | | | |
| <i>Orchis mascula</i> subsp. <i>ichnusae</i> | | | 0 | | | | |
| <i>O. provincialis</i> | | | 26 | 1 | | | |
| <i>O. purpurea</i> | | | 29 | | | | |
| <i>Platanthera</i> | | | | | | | |
| <i>P. algeriensis</i> | | | 4 | | | | |
| <i>P.kuenkelei</i> subsp. <i>kuenkelei</i> var. <i>sardoa</i> | | | 8 | | | | |
| <i>Serapias</i> | | | | | | | |
| <i>S. cordigera</i> | | | 12 | 2 | 3 | | 2 |
| <i>S. lingua</i> | | | 19 | 6 | 17 | | 9 |
| <i>S. nurrica</i> | <i>Near Threatened</i> | <i>unknown</i> | 5 | | | | |
| <i>S. parviflora</i> | | | 11 | 3 | 19 | | 12 |
| <i>Spiranthes</i> | | | | | | | |
| <i>S. aestivalis</i> | | | 12 | | | | |
| <i>S. spiralis</i> | | | 47 | | | | |

Table 2. Hybrids recorded in Sardinian orchids.

| Hybrid | Parental species | Distribution |
|--|--|--------------|
| <i>Anacamptis</i> × <i>bornemannii</i> Asch. | <i>Anacamptis papilionacea</i> × <i>Anacamptis longicornu</i> | W-Medit. |
| <i>Anacamptis</i> × <i>caccabaria</i> Verguin | <i>Anacamptis laxiflora</i> × <i>Anacamptis papilionacea</i> | Medit.-Atl. |
| <i>Anacamptis</i> × <i>sarcidani</i> Scrugli et Grasso | <i>Anacamptis laxiflora</i> × <i>Anacamptis longicornu</i> | Endem. SA |
| <i>Ophrys</i> × <i>barbaricina</i> M. Allard et M.P.Grasso | <i>Ophrys speculum</i> × <i>Ophrys morisii</i> | Endem. SA |
| <i>Ophrys</i> × <i>cosana</i> H. Baumann et Kunkele | <i>Ophrys bombyliflora</i> × <i>Ophrys incubacea</i> | W-Medit. |
| <i>Ophrys</i> × <i>daissiorum</i> (H. Baumann, Giotta, Künkele, Lorenz & Piccitto) P. Delforge | <i>Ophrys chestermanii</i> × <i>Ophrys morisii</i> | Endem. SA |
| <i>Ophrys</i> × <i>domus-maria</i> M.P. Grasso | <i>Ophrys apifera</i> × <i>Ophrys morisii</i> | Endem. SA |
| <i>Ophrys</i> × <i>fernandii</i> Rolfe | <i>Ophrys bombyliflora</i> × <i>Ophrys speculum</i> | W-Medit. |
| <i>Ophrys</i> × <i>heraultii</i> G. Keller ex Schrenk | <i>Ophrys tenthredinifera</i> × <i>Ophrys speculum</i> | Medit. |
| <i>Ophrys</i> × <i>laconensis</i> Scrugli et Grasso | <i>Ophrys exaltata</i> subsp. <i>morisii</i> × <i>Ophrys tenthredinifera</i> | Endem. SA |
| <i>Ophrys</i> × <i>maladroxiensis</i> Scrugli, Todde e Cogoni | <i>Ophrys exaltata</i> subsp. <i>morisii</i> × <i>Ophrys annae</i> | Endem. SA |
| <i>Ophrys</i> × <i>manfredoniae</i> O. & E. Danesch | <i>Ophrys incubacea</i> × <i>Ophrys tenthredinifera</i> | W-Medit. |
| <i>Ophrys</i> × <i>sommieri</i> E.G. Camus ex Cortesi | <i>Ophrys bombyliflora</i> × <i>Ophrys tenthredinifera</i> | Medit. |
| <i>Ophrys</i> × <i>spanui</i> P. Delforge | <i>Ophrys annae</i> × <i>Ophrys tenthredinifera</i> | Endem. SA-CO |
| <i>Ophrys</i> × <i>sulcitana</i> Scrugli, Todde e Cogoni | <i>Ophrys annae</i> × <i>Ophrys bombyliflora</i> | Endem. SA |
| <i>Ophrys</i> × <i>tavignanensis</i> H. & J.M. Mathé & M. Pena | <i>Ophrys eleonora</i> × <i>Ophrys incubacea</i> | W-Medit. |
| <i>Orchis</i> × <i>penzigiana</i> A. Camus nsubsp. <i>sardoa</i> Scrugli et Grasso | <i>Orchis provincialis</i> × <i>Orchis mascula</i> subsp. <i>ichnusae</i> | Endem. SA |
| <i>Serapias</i> × <i>ambigua</i> Rouy | <i>Serapias cordigera</i> × <i>Serapias lingua</i> | Medit.-Atl. |
| <i>Serapias</i> × <i>cortoghiana</i> , Grasso M.P | <i>Serapias nurrica</i> × <i>Serapias cordigera</i> | W-Medit. |
| <i>Serapias</i> × <i>semilingua</i> E.G. Camus et al. | <i>Serapias lingua</i> × <i>Serapias parviflora</i> | Medit.-Atl. |

Table 3. List of endemisms and their distribution.

| Chorological rank | Taxa |
|--------------------------|---|
| Endem. SA | <i>Ophrys chestermanii</i> |
| Endem. SA | <i>Ophrys normanii</i> |
| Endem. SA | <i>Ophrys ortuabis</i> |
| Endem. SA | <i>Ophrys panattensis</i> |
| Endem. SA | <i>Ophrys subfusca</i> subsp. <i>liveranii</i> |
| Endem. SA-CO | <i>Ophrys annae</i> |
| Endem. SA-CO | <i>Ophrys funerea</i> |
| Endem. SA-CO | <i>Ophrys morisii</i> |
| Endem. SA-CO | <i>Ophrys scolopax</i> subsp. <i>conradiae</i> |
| Endem. SA-CO | <i>Ophrys sphegodes</i> subsp. <i>praecox</i> |
| Endem. SA-CO | <i>Orchis mascula</i> subsp. <i>ichnusae</i> |
| Endem. SA-SI | <i>Orchis brancifortii</i> |
| Endem. SA-TU | <i>Platanthera kuenkelei</i> var. <i>sardoa</i> |

Table 4. Phenology of Sardinian orchids during the year.

| | I | II | III | IV | V | VI | VII | VIII | IX | X | XI | XII |
|---|---|----|-----|----|---|----|-----|------|----|---|----|-----|
| <i>Anacamptis</i> | | | | | | | | | | | | |
| <i>A. collina</i> | | | ■ | ■ | ■ | ■ | | | | | | |
| <i>A. fragrans</i> | | | | | ■ | ■ | ■ | ■ | | | | |
| <i>A. laxiflora</i> | | | | ■ | ■ | ■ | ■ | | | | | |
| <i>A. longicornu</i> | | | ■ | ■ | ■ | ■ | ■ | | | | | |
| <i>A. palustris</i> | | | | | ■ | ■ | ■ | | | | | |
| <i>A. papilionacea</i> subsp. <i>grandiflora</i> | | | ■ | ■ | ■ | ■ | ■ | | | | | |
| <i>A. papilionacea</i> subsp. <i>papilionacea</i> | | | ■ | ■ | ■ | ■ | ■ | | | | | |
| <i>A. pyramidalis</i> | | | | ■ | ■ | ■ | ■ | ■ | | | | |
| <i>Cephalanthera</i> | | | | | | | | | | | | |
| <i>C. damasonium</i> | | | | | | ■ | ■ | ■ | | | | |
| <i>C. longifolia</i> | | | | | ■ | ■ | ■ | ■ | | | | |
| <i>C. rubra</i> | | | | | | ■ | ■ | ■ | ■ | | | |
| <i>Dactylorhiza</i> | | | | | | | | | | | | |
| <i>D. elata</i> subsp. <i>sesquipedalis</i> | | | | | | ■ | ■ | ■ | ■ | | | |
| <i>D. insularis</i> | | | | | ■ | ■ | ■ | ■ | | | | |
| <i>Epipactis</i> | | | | | | | | | | | | |
| <i>E. exilis</i> | | | | | | | ■ | ■ | ■ | | | |
| <i>E. helleborine</i> | | | | | ■ | ■ | ■ | ■ | ■ | ■ | | |
| <i>E. microphylla</i> | | | | | ■ | ■ | ■ | ■ | ■ | | | |
| <i>E. muelleri</i> | | | | | | | ■ | ■ | ■ | | | |
| <i>E. palustris</i> | | | | | | | ■ | ■ | ■ | ■ | | |
| <i>E. tremolsii</i> | | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | | | |
| <i>Gennaria</i> | | | | | | | | | | | | |
| <i>G. diphylla</i> | | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | | | |
| <i>Himantoglossum</i> | | | | | | | | | | | | |

Figure 1. Orchid genera of the flora of Sardinia.

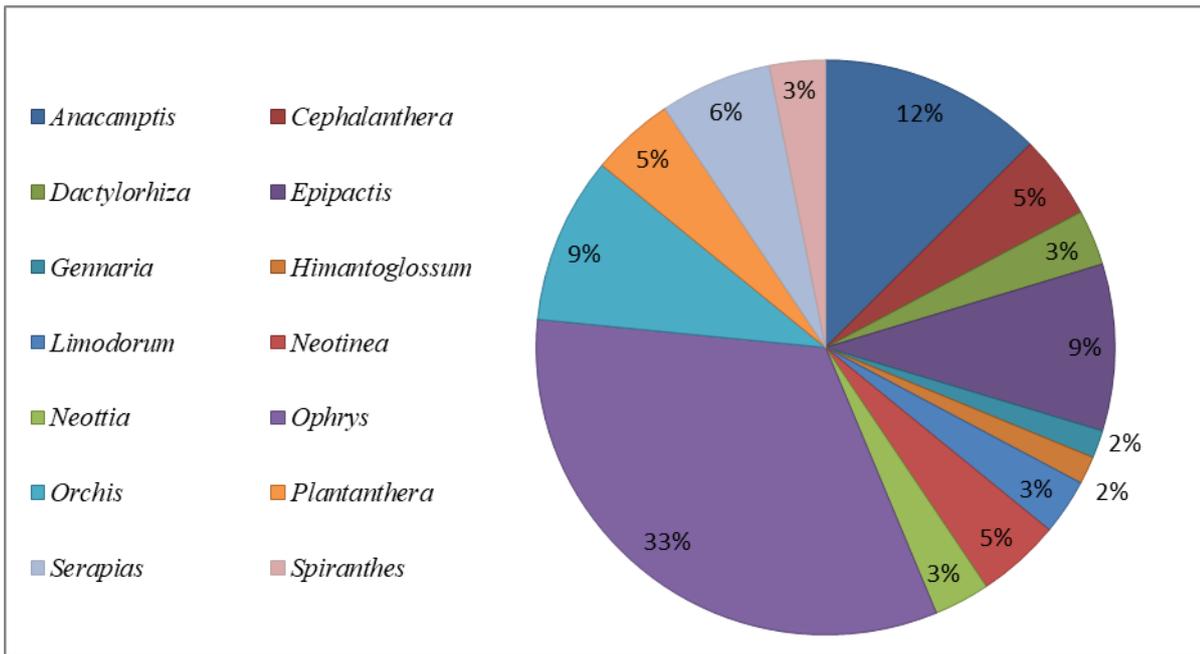
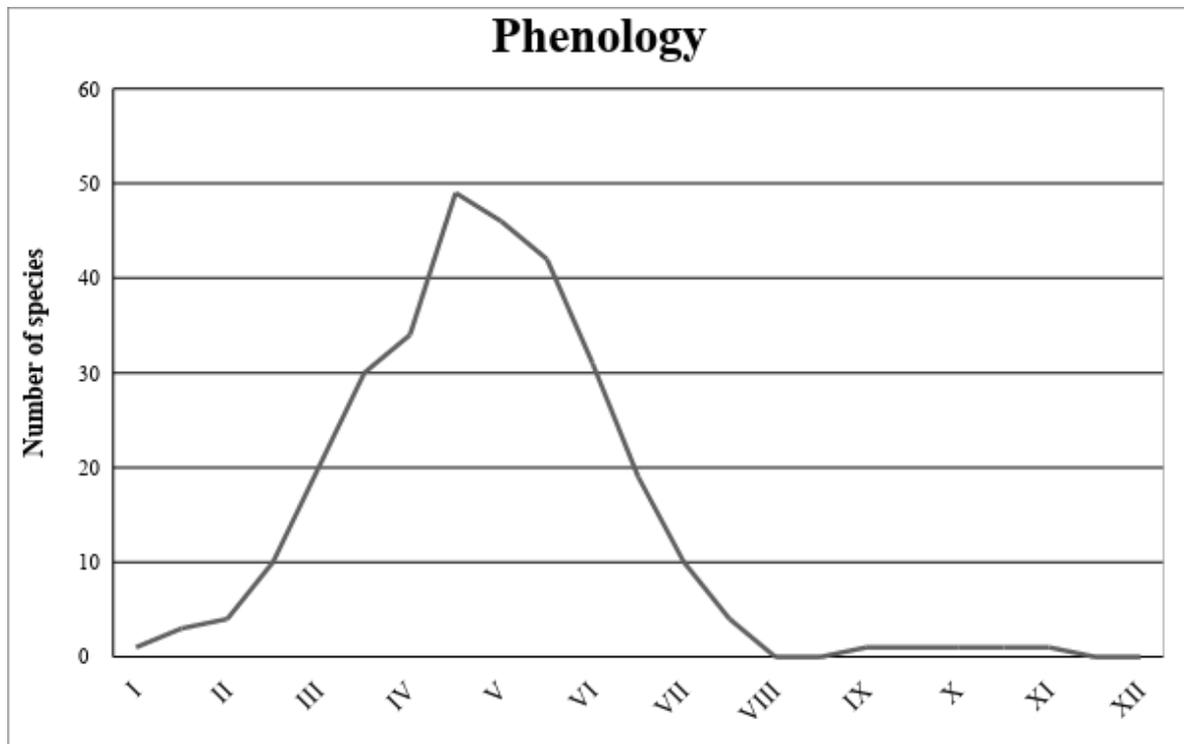


Figure 2. Number of blooming species during the year.



APPENDIX I

Table.1. Chronological review of the scientific literature concerned Sardinian orchids.

| Literature | Summary | Methods | Results | Area covered | Citations |
|---------------------------------|--|--|--|---------------------------|-----------|
| Bartolucci <i>et al.</i> , 2018 | An updated check-list of the vascular flora native to Italy | Literature check-list | National Flora check-list | Floristics | 0 |
| Bateman, Sramkó, & Paun, 2018 | Phylogeny of the genus <i>Ophrys</i> | Integration next-generation sequencing and morphological cladistic techniques | Monophyly of the genus is supported, but microspecies are barely supported | Phylogeny | |
| Lussu <i>et al.</i> , 2018 | Species boundaries between <i>Ophrys annae</i> and <i>O. chestermanii</i> | Analyses of reproductive isolation mechanisms in close related endemisms | The gene flow between the two species is absent. | Systematics | 0 |
| Ongaro <i>et al.</i> , 2018 | Patterns of distribution of Sardinian orchids in a changing world | Bioinformatics | A widening of distribution of the species analysed | Climate changes | 0 |
| Breitkopf <i>et al.</i> , 2015 | Investigation on the radiation in <i>Ophrys</i> | Comparative Phylogenetic techniques | Pollinators shifts promoted radiation in bee orchids | Phylogeography | |
| Gögler <i>et al.</i> , 2015 | Species boundaries between <i>Ophrys chestermanii</i> and <i>O. normanii</i> | Phenology, biometry, video monitoring, cross pollination experiments, germination tests, phylogeny | Column structure as boundary to the gene flow between the species studied | Systematics and phylogeny | 3 |
| Turco <i>et al.</i> , 2015 | Cytology of six <i>Ophrys</i> species | Feulgen staining | Definition of the chromosomal number of the species studied | Cytology | 1 |
| Cela <i>et al.</i> , 2014 | Seed | SEM and light | Support to the monophyly of the | Taxonomy | 4 |

| | | | | | |
|-------------------------------|--|--|--|---------------------|----|
| | micromorphology of 19 taxa of the genus <i>Ophrys</i> | microscope | genus, but not section within the genus | | |
| Zitari <i>et al.</i> , 2012 | Distribution of <i>Anacamptis longicornu</i> | Phylogeny, population genetics | Distribution of <i>A. longicornu</i> is caused by allopatry and limited gene flow | Phylogeography | 3 |
| Gögler <i>et al.</i> , 2011 | Species boundaries between <i>Ophrys chestermanii</i> and <i>O. normanii</i> | Behavioural tests, GC-EAD, GC-MS analysis | <i>Ophrys</i> scent is evolved to mimic pheromones emitted by virgin female of pollinators to induce male pseudocopulation | Pollination biology | 5 |
| Pavarese <i>et al.</i> , 2011 | Genetic structure and phylogeographic relationship of <i>Platanthera bifolia</i> var. <i>kuenkelei</i> | Population genetics | Updated distribution of <i>P. bifolia</i> var. <i>kuenkelei</i> which is more closely related to the Tunisian <i>P. bifolia</i> var. <i>kuenkelei</i> than to the geographically closer <i>P. bifolia</i> populations in Italy | Phylogeography | 9 |
| Fenu <i>et al.</i> , 2010 | <i>Dactylorhiza elata</i> subsp. <i>sesquipedalis</i> | On the biology of <i>Dactylorhiza elata</i> (Poir.) Soó subsp. <i>sesquipedalis</i> (Willd.) Soó | Update of rarer italian orchid | Floristics | 0 |
| Cortis <i>et al.</i> , 2009 | Species boundaries between <i>Ophrys iricolor</i> and <i>O. incubacea</i> | AFLP of the species and hybrids, pollination fitness | Parental species and hybrids produced a similar odour bouquet hybrids produce lower fruit and seed set than parental species, many were mostly first-generation hybrids | Pollination biology | 46 |
| Duffy <i>et al.</i> , 2009 | Comparison of Molecular and ecologic factors in <i>Neotinea maculata</i> | Vegetation communities, breeding system and genetic | The species is autogamous, similar genetic distance was found | Phylogeography | 36 |
| Devey <i>et al.</i> , 2009 | Phylogeography within the <i>Ophrys fuciflora</i> group | AFLP genetic fingerprinting technique to evaluate levels of genetic variation | Genetic diversity is higher in Kent than in other regions. Gene flow occur between populations in closer geographical proximity than those that are morphologically | Phylogeography | |

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|---------------------------------------|---|--|--|---------------------|----|
| | | | more similar. | | |
| Gögler <i>et al.</i> , 2009 | Species boundaries between <i>Ophrys chestermanii</i> and <i>O. normanii</i> | Behavioural tests, GC-MS analysis, genetic analyses using AFLP and plastid markers | Sympatric <i>Ophrys</i> not-so-close-related converged to the same pollinators. Unexpected relevance of postmating barriers in <i>Ophrys</i> | Pollination biology | 29 |
| Devey <i>et al.</i> , 2008 | Phylogeny of the genus <i>Ophrys</i> | AFLP of ITS marker | Ten genetically distinct groups were identified within the genus | Phylogeny | |
| Bellusci <i>et al.</i> , 2008 | Phylogeny of 14 species of the genus <i>Serapias</i> | Maximum parsimony analyses using the markers trnL-trnF, trnS-trnG, rps16, and atpF | Molecular relationships does not reflect morphological assessments. | Phylogeography | 20 |
| Bocchieri, Iiriti, & Pontecorvo, 2008 | Distribution of vascular plants in a specific area of Sardinia | Distribution and environments identification | Updated Flora of a microhabitats, capes and island | Floristics | 0 |
| Stökl <i>et al.</i> , 2008 | Species boundaries between <i>Ophrys iricolor</i> and <i>O. lupercalis</i> | Behavioral, GC-EAD, chemical, morphological, and genetic methods (AFLPs) | The scent variation in <i>Ophrys</i> as first step of speciation and extinction of a species | Pollination biology | 46 |
| Bacchetta, Pontecorvo, & Vacca, 2007 | Flora of Monte Arcuentu | Retrieval, identification and census the flora of a defined area. | Updated Flora of a specific area | Floristics | 0 |
| Gamarra <i>et al.</i> , 2007 | Seed morphology of the genus <i>Neotinea</i> | Electron microscopy | Support the monophyly | Cytogenetics | 16 |
| Stökl <i>et al.</i> , 2007 | Comparison of the scent produced by <i>Ophrys iricolor</i> and pheromones produced by female of <i>Andrena morio</i> , its pollinator | GC-EAD, GC/MS | Compounds produced by orchids and its pollinators overlap.. | Pollination biology | 30 |
| D'Emerico <i>et al.</i> , | Karyotype of 11 | Chromomycin A3 | First karyotype description of | Cytogenetics | 27 |

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|---|---|--|---|---------------------------|----|
| 2005 | species of the genus <i>Ophrys</i> | (CMA) staining | the species | | |
| Pellegrino <i>et al.</i> , 2005 | Species boundaries between <i>Orchis mascula</i> and <i>O. provincialis</i> | Artificial crosses, morphological observations, karyological and molecular analyses with nuclear and plastidial markers. | Hybridization between the species leads to a discussion on the role of postzygotic boundaries in food deceptive species . | Systematics and Phylogeny | 18 |
| D'Emérico <i>et al.</i> 2002 | Cytogenetic of six <i>Orchis</i> species | Chromomycin A3 (CMA) staining | Support the monophyly | Cytogenetics | 11 |
| Bullini <i>et al.</i> , 2001 | Genetic population of <i>Dactylorhiza insularis</i> | Genetic population | The hybrid origin of the western <i>D. insularis</i> (<i>D. mmana</i> and <i>D. sambucina</i>) | Phylogeography | 25 |
| Cafasso, Pellegrino, Caputo, Scrugli, Cozzolino, 2001 | Genetic structure of insular endemic species of Sardinia and Sicily | Molecular analyses using ITS | Different level of genetic structure are recorded in insular species. | Systematics | 4 |
| D'Emérico <i>et al.</i> 2001 | Evolution of ribosomal genes in Sardinian orchid species and hybrids | Ribosomal genes analysis (18S-25S rDNA and one pair of 5S rDNA) | Implication for understanding evolutionary trends. | Cytogenetics | 4 |
| D'Emérico, Pignone, & Scrugli, 2000 | Karyology of the genera <i>Cephalanthera</i> and <i>Listera</i> | Giemsa C-banding | Role of Robertsonian rearrangements and quantitative heterochromatin variation in karyotype reorganization | Cytology | 6 |
| D'Emérico, Pignone, & Scrugli, 2000a | Karyology of the genus <i>Serapias</i> | Giemsa C-banding | A recent origin for the genus <i>Serapias</i> | Cytology | 18 |
| D'Emérico, Grünanger, Scrugli, Pignone, 1999 | Karyology of the genera <i>Cephalanthera</i> and <i>Epipactis</i> | Giemsa C-banding | Supporting a possible palaeo-polyloid origin of the genera analyzed | Cytology | 13 |
| Arduino <i>et al.</i> , 1995 | Intraspecific variation in <i>Orchis papilionacea</i> | Multilocus electrophoresis | Intraspecific differences are more related with geographic distribution than | Taxonomy | 0 |

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|--------------------------------|--|---|---|--------------|----|
| | | | geneticepipactis exilis | | |
| Baumann H. <i>et al.</i> 1995 | Description of <i>Ophrys chestermanii</i> | | Description of a new species | Taxonomy | 0 |
| Cogoni <i>et al.</i> 1992 | Analyses of 38 endophytes of Sardinian orchids | | Identification of orchid hyphae | Cytogenetics | 0 |
| Cogoni, Riess & Scrugli, 1992 | Mychorrhiza in <i>Neottia nidus-avis</i> | | Identification of orchid mychorrhiza | Cytology | 0 |
| Corrias <i>et al.</i> 1991 | <i>Anacamptis longicornu</i> in Sardinia | Genetic, morphological and chorology | Description of the species | Taxonomy | 43 |
| Scrugli 1990 | Description of <i>Platanthera algeriensis</i> | | Record of a new species for the italian flora | Floristics | 1 |
| Scrugli, Cogoni, & Riess, 1990 | Mychorrhiza in the genus <i>Limodorum</i> | | Identification of orchid mychorrhiza | Cytology | 0 |
| Scrugli <i>et al.</i> 1988 | Orchids flora of Sarcidano | Retrieval, identification and census of orchids of Sarcidano, a historical region of central Sardinia | check-list of the orchid of Sarcidano | Floristics | 4 |
| Scrugli & Grasso 1986 | Description of <i>Orchis</i> × <i>sarcidani</i> | Morphometric analysis | First record of the hybrid <i>O. laxiflora</i> x <i>O. longicornu</i> | Taxonomy | |
| Scrugli & Grasso 1984 | Description of <i>Ophrys</i> × <i>laconensis</i> | Morphometric analysis | First record of the hybrid <i>O. morisii</i> x <i>O.tenthredinifera</i> | Taxonomy | |
| Scrugli & Grasso 1979 | Orchids of Sardinia | Retrieval, identification and census of orchids of Sardinia | First check-list of the orchid of Sarcidano | Floristics | |

Table 2. Summary of papers.

| Topic | Tot. papers | % | Tot. citations |
|---------------------------|--------------------|----------|-----------------------|
| Cytology | 6 | 14% | 38 |
| Taxonomy | 6 | 14% | 47 |
| Pollination biology | 5 | 12% | 156 |
| Cytogenetics | 5 | 12% | 48 |
| Floristics | 7 | 16% | 5 |
| Phylogeography | 7 | 16% | 58 |
| Systematics and Phylogeny | 4 | 9% | 21 |
| Systematics | 2 | 5% | 4 |
| Climate changes | 1 | 2% | 0 |

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APPENDIX II

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Chapter 4

***Ophrys annae* and *Ophrys chestermanii*: an impossible love between two orchid sister species.**

Lussu M., De Agostini A., Marignani M, Cogoni A., Cortis P. (2018) *Ophrys annae* and *Ophrys chestermanii*: an impossible love between two orchid sister species.

Nordic Journal of Botany DOI: 10.1111/njb.01798

Abstract

The biological concept of species is founded on the presence of barriers that limit the gene flow and species delimitation represents a vivid topic in orchids. Studies on reproductive isolation in Mediterranean orchids often examine gene flow between co-occurring species providing a relevant understanding of how species boundaries are maintained. In the sexually deceptive *Ophrys* genus, the role of postmating barriers has been traditionally thought to be weak and, despite the relevant morphological and ethological differences, low phylogenetic diversity has been recorded. In contrast, not many studies are focused on the relevance of each barriers in closely related species in island condition. Using a morphological approach and experimental crosses to evaluate postmating barriers, here, we clarify the taxonomic position of two sardinian endemic orchid sister-species, *Ophrys annae* and *O. chestermanii*, currently ascribed in the *bornmuelleri* group. In *Ophrys*, due their hyper specialized pollination syndrome, premating barriers are often considered more relevant than postmating barriers. We found that the two endemisms differ substantially in 16 functional traits adopted. We further detected asymmetric results in manual crosses suggesting that postmating barriers may act as reinforcement of the premating barriers. Our results suggest that in closely related orchid species of the *Ophrys* genus, gene flow is limited also by postmating barriers which have a relevant act in keeping the two reproductively isolated.

Keywords: *Ophrys*, reproductive barriers, gene flow, speciation

Introduction

The debate around the concept of species has always been particularly vibrant. In 1859 Charles Darwin wrote: ‘No one definition has satisfied all naturalists; yet every naturalist knows vaguely what he means when he speaks of a species’ (Darwin 1859). In biology, this concept is the evolutionary starting unit (Mayr 1957) and the discussion on its definition is still open. Traditionally, a species was defined by the morphological analysis from which phylogenetic distance was derived. Grouping together living forms on the basis of morphological similarity was the revolutionary method used by Linneus to organize them in an innovative pattern. This approach can clearly muddle up the relation between organisms and often leads to different and competitive results as consequence of biological events such as convergent evolution (Sanderson et al. 1996, West-Eberhard 2003, Chirat et al. 2013).

Darwin proposed the importance of biogeography and ecological factors as mechanisms to explain not only biodiversity but also actual patterns of species distribution. In chapter twelve of the *Origin of Species* entitled ‘Geographic Distribution’, he reported that all oceanic islands visited during his massive travel on the *Beagle* lack of terrestrial mammals species, except for those introduced by humans, he also noticed that an endemic species on an island would often have strong similarities with a species on the mainland closest to the island in question. Hence, he suggested the explanation that these similarities were based on a common ancestor. Ecological speciation is a crucial mechanism of speciation (Schluter 2009) and it is defined as the evolution of reproductive isolation between populations, or subsets of a single population, as a result of ecologically based divergent natural selection (Schluter 2000, 2001, Rundle and Nosil 2005, Funk 2009). In the last century Dobzhansky (1937) and Mayr (1942) defined species and speciation by the criterion of reproductive isolation instead; Mayr postulated: ‘A *species is a group of interbreeding (or potentially interbreeding) individuals, that is reproductively isolated from other groups of interbreeding individuals*’ hence this concept is based on the reduction of the gene flow between populations taking each population to an independent evolutionary process. Zygotic barriers are

identified by Mayr on the basis of their action in relation of the fertilisation. More recently, the discovery of the DNA and its implication in systematics have clarified many evolutionary processes as the reconstruction of the phylogeny of Angiosperms (Judd et al. 2008) and from its discovery we are able to answer to the question when biodiversity has evolved (Mergeay and Santamaria 2012).

In the plant kingdom the innovation of the structure of the flower to attract pollination is a key element of diversification (Stebbins 1970, Ollerton et al. 2011). In detail, in orchids, deceptive pollination is actually considered the most relevant mechanism of diversification and speciation (Schiestl 2005). Within Orchidaceae, the sexually deceptive genus *Ophrys* is globally recognized as a model system to investigate plant-pollinator relationship (Devey et al. 2008) and its plasticity and rapidity of adaptation to pollinators is considered fundamental in the process of diversification through ecological adaptation (Xu et al. 2011).

Due to its particularly intricate biogeographic history, especially during Tertiary and Quaternary, the Mediterranean basin is globally recognized as a hotspot of biodiversity with more than 25000 vascular plant species (Thompson 2005). Here, the number of species included within the genus *Ophrys* ranges from ten macrospecies described on the base of internal transcribed spacer (ITS) DNA sequencing (Devey et al. 2008) to 19 subspecies with 75 subspecies based on a morphological approach (Pedersen and Faurholdt 2007), up to more than 250 species when a more geographical approach is adopted (Delforge 2016) showing that the ‘species problem’ (Mayr, 1957) is absolutely still vivid (Bateman 1997, Hey 2001, Bateman et al. 2003, Bateman et al. 2011, Vereecken 2009, Vereecken and Schiestl 2009, Vereecken and Mc Naeil 2010, Vereecken et al. 2011, Cortis et al. 2009). Reproductive barriers and their relevance in diversification of Mediterranean deceptive orchids have been recently investigated in detail: the hypothesis that floral scent plays a key role in attracting pollinators, especially between closely related species, is corroborated so that, if floral isolation is sufficiently strong, ecological speciation based on pollinator shift may happen rapidly (Cozzolino et al. 2005, Scopece et al. 2007, Xu et al. 2011, Schiestl et al. 1999).

Sardinia is the second largest island in the Mediterranean basin and with approximately 19 species of *Ophrys*, represents the ideal situation to bring a solid contribution to better understand orchids' evolution in island conditions. In this study, we focus on endemic putative species of sexually deceptive orchids *Ophrys annae* and *O. chestermanii*. As sexually deceptive orchids (Scopece et al. 2007), we expect that the studied species produce a low fruit set. The aim of this research is to clarify morphological and ecological characters of *O. annae* and *O. chestermanii*, two sardinian endemisms that were used here to observe the evolution mechanisms in island condition. In particular, we paid attention to what can be considered a barrier to gene flow in closely related taxa. We updated the distribution using herbarium samples and original field data; we also investigated the prezygotic barriers analyzing floral morphological traits and the natural fitness and seed viability of manually cross-pollinated flowers. We posed two main questions: despite the observed low genetic diversity i) are the taxa morphologically different? ii) are these two closely related species sufficiently reproductively isolated to keep their identity on secondary contact? To answer this latter question, inter- and intra-specific crosses were conducted to quantify postmating barriers. This information could elucidate the knowledge on the gene flow in a group of plants where species boundaries are kept mainly by prezygotic barriers.

Materials and Methods

Species studied and data sampling

Within the genus *Ophrys*, the *bornmuelleri* group encompasses 24 species that in Sardinia are represented by the endemics *O. annae* and *O. chestermanii*. The two species have a clear monophyletic origin as showed by genetic analyses (Gogler et al. 2009) but differences on scent compounds to attract pollinators are documented (Gogler et al. 2009, Devey et al. 2008).

O. annae J. Devillers – Terschuren & P. Devillers is 10-40 cm tall, with a compact inflorescence consisting of 4-8 showy flowers. It is recorded as endemic of Sardinia and Corsica (Delforge 2006). This orchid grows on calcareous substrates in sunny and rocky fields, open scrubs or garigue and in

open fields in forests dominated by *Quercus ilex* (Lai, 2008). Populations of *O. annae* are locally not very sizeable and isolated plants are not rare (Lai 2008). The main pollinator of *O. annae* is a bee, *Osmia rufa* (Megachilidae). Two hybrids are recorded for this species: *Ophrys* × *maladroxiensis* Scrugli, Todde & Cogoni, 1992 with *O. exaltata morisii* (Martelli) Del Prete, 1984 and *Ophrys* × *spanui* Delforge, 1997 with *O. tenthredinifera* Willd., 1805.

Ophrys chestermanii (J. J. Wood) Gözl & H. R. Reinhard is a Sardinian endemism orchid 10-30 cm tall, lax inflorescence with 2-6 big and showy flowers. This species prefers calcareous or schistous substrates in humid and shady environments such as the high scrubs of *Quercus ilex* (Delforge, 2006). The solitary bee *Bombus vestalis* (Apidae) is recorded as the only pollinator of *O. chestermanii*. For long time *O. chestermanii* and *O. tenthredinifera* Willd., 1805 were considered putative parents of *O. normanii* J.J. Wood but recent genetic analysis, behavioural studies on pollination and chemotaxonomic results do not support this hypothesis (Gogler et al. 2009). In contrast to *O. annae*, its distribution is very restricted but it locally forms fairly large populations (Lai 2008).

To update the distribution (figure 1) of the two species all regional, national and foreign monographs were taken into account (Terraciano 1914, Wood 1982, Wood 1983, Devillers – Terschuren & P. Devillers 1992, Delforge 2006, Delforge 2016). In detail, herbarium research was carried out at the herbarium of the University of Cagliari (CAG), the University of Sassari (SASSA). During the fieldwork (2016 and 2017 blooming seasons) we confirmed and integrated the bibliographic data to update the actual distribution of the two entities. All new sites were recorded using a GPS.

To collect Plant Functional Traits (PFTs, Garnier et al 2016) of the two species, a specific sampling was performed during flowering periods from March to June in 2016 and 2017. Because of the rarity of those species, in each population examined, only 20 plants for each species were chosen for this research.

O. annae was collected in Laconi (OR), Loc. Dominariu, at 929 m s.l.m. (509971E, 4415910N; WGS 84/pseudo Mercator). This population is located on a carbonatic plateau (limestones and dolomites) where a very thin and dry soil is located. The dominant vascular plants are grasses, *Pyrus pyraster* L. Burgsd. and *Quercus ilex* L. (Bacchetta et al. 2009). *O. chestermanii* was studied in Domusnovas (CA), Loc. Sa Duchessa, at 418 m s.l.m. (466880E, 4356898N; WGS 84/pseudo Mercator). This area is characterized by sedimentary metamorphites (metasandstones, metashales, metalimestones, metadolomites etc.) with a mature soil formed by a rich organic component, with a tall mediterranean vegetation, dominated by *Quercus ilex* L. and *Arbutus unedo* L. (Bacchetta et al. 2009).

For each taxon we randomly chose 20 individuals and tagged them. We collected a total of 40 flowers (1 flower collected in each of the 40 individuals) in a solution 70% ethanol for morphometric analyses. For each plant we determined plant and inflorescence height, total number of flowers, number of fruits. Statistical differences between functional traits were tested using Mann Whitney U-test. Fruit production were used to calculate fitness as the ratio between the number of fruits and the number of sexually mature flowers (Scopece et al. 2007).

To determine differences and similarities among the species, we performed statistical analyses with a total of 16 flower functional traits of 25 plants of *O. chestermanii*, 25 flowers of *O. annae* and 24 flowers of *O. tenthredinifera* actually ascribed to the *O. tenthredinifera* group (Breitkopf et al. 2014) as outgroup. The same traits were used to perform principal component analyses (PCA; varimax rotation). Principal Component Analysis (PCA) (Fig 2), was applied to discriminate samples from the species investigated. PCA is an unsupervised multivariate data analysis method with which the multivariate space in which objects are distributed can be reduced, so as to visualize similarities and/or differences within multivariate data of morphological measurements. The dataset of the 74 samples investigated was thus submitted to PCA, in a targeted approach based on the 16 morphological functional traits previously characterized listed in Table 1 and 2. The plots and loading plots were built with the aim of discriminating the three species. We used the software R.

To evaluate the presence of postzygotic barriers, 22 plants of each species were preserved in a greenhouse at the Hortus Botanicus Kalaritanus (Cagliari) and used for tests on manual cross-pollination. We choose not to use more than two flowers for each plant to avoid excessive stress. At the time of fruits maturity (late May to early June), we collected all capsules and let them dehydrate for at least a month. For each capsule, we calculated the mean of embryo seeds based on 500 randomly chosen seeds for each capsule.

Within the interspecific crosses, we quantified the fruit production (FP) in the two directions of interspecific crosses as the ratio between fruits produced and the number of pollinated flowers. We defined Seeds Vitality (SV) as the ratio between the sum of embryo seeds counted in both crosses and the total of seeds counted.

To calculate the reproductive isolation we used the formula $RI = 1 - 2 * (H / (C + H))$ as in Sobel and Chen (2014). In detail, postmating prezygotic (FP) was calculated as $RI_{\text{postm. prezy.}} = 1 - 2 * (H / (C + H))$ and embryo mortality calculated as $RI_{\text{emb}} = 1 - 2 * (H / (C + H))$

According to Moyle et al. 2004, all results vary between 0 (no reproductive isolation) and 1 (complete reproductive isolation). Mann Whitney U-test were performed to test a quantitative difference in seeds production in both intraspecific and interspecific crosses.

Results

We collected three sites for *O. annae* and two for *O. chestermanii*; a total of 11 herbarium samples were analysed and 50 from the fieldwork (25 for each species). We confirmed the endemic distribution of the entities with a wider distribution of *O. annae*, distributed across the island and two populations of *O. chestermanii*. No new sites were discovered for both species.

The observational data demonstrates that in the 100% of the sites studied the two entities never occur together: *O. chestermanii* sensitively prefers more shady positions with deep soil (we have never found plants in sun-taking positions except for a few hours a day), while *O. annae* always grows in sunny position with very dry and thin soil.

A total of 40 wild plants were analyzed in this study. Morphometric investigations show a global trend of *Ophrys chestermanii* to be bigger than *Ophrys annae* (Table 1). *Ophrys chestermanii* is sensibly taller, but *Ophrys annae* produces a greater number of flowers per plant (see Table 1). The same trend results from floral traits analyses (Table 2). In particular, the differences between the dimensions of the labellum and the stigmatic cavity, which are directly linked with pollination, are relevant. Labellum area is smaller in *Ophrys annae* than in *Ophrys chestermanii* (mean=179.812). Analyses on stigmatic cavity show that this flower's structure is less variable than other floral part (sd=0.303 and 0.594) (Table 2). The Mann Whitney U-test result significant at $p < 0.05$. for the following flower functional traits: vertical sepal length (p value= 0.03572), labellum length (p value= 0.00054), stigmatic cavity width (p value= 0.01684) and plants functional traits as plant height, flowers per plant and first flower height (Table 3). Principal Component Analysis (PCA) (Fig 2), was applied to discriminate samples from the species investigated. The Principal Component Analysis (PCA) produced two PCs with an eigenvalue above one explaining 78.33% of the total variance. As shown in Fig.1, the first component (PC1) that explains 50.26% of the variation, individually separates *Ophrys chestermanii* and *Ophrys annae* from *Ophrys tenthredinifera*. Conversely, PC2 (28.07% of the variation) discriminates between *Ophrys chestermanii* and *Ophrys annae* from *Ophrys tenthredinifera*.

Fruits production and seeds viability tests were conducted on 44 manual pollinations. Total fertility is demonstrated by intraspecific crosses. In contrast, interspecific crosses in both directions, *Ophrys annae* X *Ophrys chestermanii* and *Ophrys chestermanii* X *Ophrys annae*, reveal that the first cross produced 16 capsules on 16 crosses and a mean of embryo seeds of 66.2075, the latter lead to 4 fruits on 16 crosses.

Although *Ophrys annae* produced more flowers per population (106 flowers vs 68 in *Ophrys chestermanii*), fruits production recorded was 4 in *Ophrys annae* and 10 in *Ophrys chestermanii* and the fitness was 0.037 and 0.147 respectively.

The percentage of fruits production is 100% in intraspecific crosses and in 100% in the cross *Ophrys annae* X *Ophrys chestermanii* and 25% in *Ophrys chestermanii* X *Ophrys annae* (Tab.4). Percentage of embryo seeds vary from 78.46 in *Ophrys chestermanii* X *Ophrys chestermanii* and 57.39 in *Ophrys annae* X *Ophrys annae* (Table1). All measures of isolation varied between 0 (no isolation) and 1 (complete isolation). The embryo mortality has the value of 0.01. Reproductive isolation ($RI_{\text{postm. prezy.}}$) value is 0.23 and RI_{emb} is -0.0061. No significant differences were recorded in both crosses (intraspecific cross Mann–Whitney U test=121, P=0.41294, intraspecific Mann–Whitney U test= 62.5, P= 0.30153).

Conclusions

Our study showed that *Ophrys chestermanii* and *Ophrys annae* are clearly morphologically different and suggests that the two phylogenetic closely related endemic entities are two well defined species. In fact, to support this hypothesis these entities differ significantly for all the morphological characteristics studied, especially for flower traits. Particularly relevant the differences of the lengths of labella considered by different authors as an evidence of a pollinator-mediated speciation (Benitez-Vieyra et al. 2009, Xu et al. 2011). In Angiosperms, pollinators-driven diversification is an important way of speciation (Xu et al. 2011) and these entities might represent a valid example of how the ability to adapt to new pollinators is a prerequisite in biodiversity in the *Ophrys* genus, reflecting what was already investigated in the orchid genus *Chiloglottis* (Mant et al. 2002). The record of two different species of pollinators ascribed to different families, i.e., Apidae and Megachilidae, is another major barrier to the gene flow between these plants. The shift to so different pollinators could be a mechanism for enhancing reproductive isolation as well as hyper-specialization to a particular pollinator, and it can be interpreted as the need to occupy an ecological niche after there has been a colonization of the island and it might be taken into account in *Ophrys* species definition because it leads a strong limit to the gene flow (Peakall et al. 2013).

The continental species of reference, *Ophrys bornmuelleri*, has a distribution that mainly includes the Anatolian peninsula. Of the 24 species currently ascribed in the *bornmuelleri* group (Delforge 2016), nine (as *Ophrys bornmuelleri* and *Ophrys mesopotamica*) are distributed in Anatolia to the north of the Tigris and Euphrates rivers, ten in Greece and the islands of the Aegean Sea, one in Cyrenaica (Libya, Africa), one is endemic to Sicily, one in Italian peninsula, and finally *Ophrys annae* and *Ophrys chestermanii* (which have a more western distribution), are endemic of Sardinia and Corse and Sardinia respectively (Breitkopf et al. 2015).

The lack of a substantial genetic difference between these two entities (Gogler et al. 2009) might be explained by a rapid and recent speciation of the genus *Ophrys* occurred in the late Pliocene or early to mid-Pleistocene (Bateman et al. 1997, Bateman et al. 2003, Chase et al. 2003, Inda et al. 2012). During this geological period, the Mediterranean sea level was lower and Sardinia and Corsica were a single island closely linked to the Italian peninsula by the Tuscan archipelago. This geographical situation might suggest the current distribution of the entities considered in this study: coming from the southeast of the Mediterranean, a progenitor might have climbed the Italian peninsula and arrived in Corsica and Sardinia through the bridge represented by the Tuscan archipelago as explained for other species (Thompson 2005). Finally in Sardinia *Ophrys chestermanii* might have been adapted and differentiated to colonise a more sciaphilous and wooded ecological context, in a process of secondary adaptation. In fact, compared to the others species of the *bornmuelleri* group that generally prefer sunny position with very dry and thin soil as *Ophrys annae* does, it is permissible to think that the adaptation to more sciaphilous positions might be a secondary adaptation, also observing the limited distribution of *Ophrys chestermanii* and its peculiar distinct ecological characteristics.

Numerous studies emphasize the importance of premating barriers as major limitations in gene flow in *Ophrys* especially between closely related taxa (Scopece et al. 2007), and also our study well supports this hypothesis: we have found that these entities never occur in sympatry. In fact, their distribution within the island is very differentiated, the species occupy two completely

different ecological niches. However, the barriers that contribute to maintain separated two species are multiple (Dobzhansky 1937; Mayr 1942, 1947, 1963; Coyne 1992; Schluter 2001; Ramsey et al. 2003; Husband and Sabara 2003; Kay 2006), but sometime it is not easy to identify the role covered by each one during speciation (Scopece 2007). Due to their early presence during the life cycle, prezygotic barriers have a reflection on postzygotic and are often considered the most relevant; however there are several studies that demonstrate an equal role played by the postzygotic (Schemske 2000; Ramsey et al. 2003). Within deceptive orchids, comparative studies underlining postzygotic barriers are more dominant in the food-deceptive than in sexually deceptive, where the gene flow is limited by the extremely specialized pollination systems, and so the pollinator could be considered a barrier strong enough to keep species boundaries. As we expected from literature (Scopece et al. 2007), our analyses on postmating barriers show that intraspecific breeding always produces capsules, but hybridization tests surprisingly do not always lead to fruit formation, however, we found that if the fruit is formed, the percentage of embryo seeds is comparable to that of the intraspecific ones. We recorded that the *Ophrys annae* X *Ophrys chestermanii* cross produces a sensibly lower number of fruits by suspecting that there may be mechanisms of recognition of pollen of *Ophrys annae* by the stigmatic cavity of *Ophrys chestermanii* that may act before fruit production. This barrier might allow the reinforcement of the reproductive isolation mainly based on prezygotic isolation between the entities involved. To be considered such, reinforcement firstly acts in the parental generation avoiding heterospecific matings and secondly avoids fruit formation to not dissipate energy in a hybrid prole (Levin 1970, Grant 1981). This might explain the fruit production in our crosses contributing to the idea that gene flow between the two entities is restricted and that populations of both taxa are effectively reproductively isolated from each other. The absence of fruit production is caused when the ovary is not penetrated by the pollen tubes, but which factor is responsible of the asymmetric fruits production in these two sardinian endemisms has not been tested. Postzygotic isolations as embryo mortality, hybrid inviability and hybrid sterility are not always considered as relevant as the prezygotic (Schemske 2000) mainly as a

consequence that they appear later in time. In contrast with the fact that in *Ophrys* chimera individuals are very often found in nature (Pedersen and Faurholdt, 2007), during our review and our fieldwork, we did not find any plants that could be morphologically classified as hybrid, so we were not able to test hybrid viability and their sterility in natural environment as postmating barriers (Harrison 1993) that, alone or in combination, are crucial to maintain reproductive isolation in deceptive orchids (Cozzolino and Scopece 2008, Gogler et al. 2015). This lack could be interpreted as the result of strong isolation between the two species.

In conclusion, the evidences presented here suggest that, despite a low genetic diversity, taxa analyzed are morphologically and ecologically different. The results of the current study focused on the evaluation of reproductive isolation support the recognition of two taxa as species. Our analyses show that in a pollination syndrome where prezygotic barriers play a relevant role, two closely related species with an extremely low genetic diversity (Gogler et al. 2009) maintain a significant reproductive separation thank to the occurrence of postzygotic barriers. Our results also corroborate the idea that an exclusively genetic approach could not be fully exhaustive in explaining biodiversity (Bateman et al. 2011, Vereecken et al. 2011). Hence, morphological, geographical and ethological traits should be taken into account to substantially clarifying the mechanisms of species evolution in the sexually deceptive syndrome. In the light of this innovative result, it will be interesting to investigate more postzygotic barriers between closely related taxa within the genus *Ophrys*. This might be helpful to elucidate the fascinating mechanisms of their evolution.

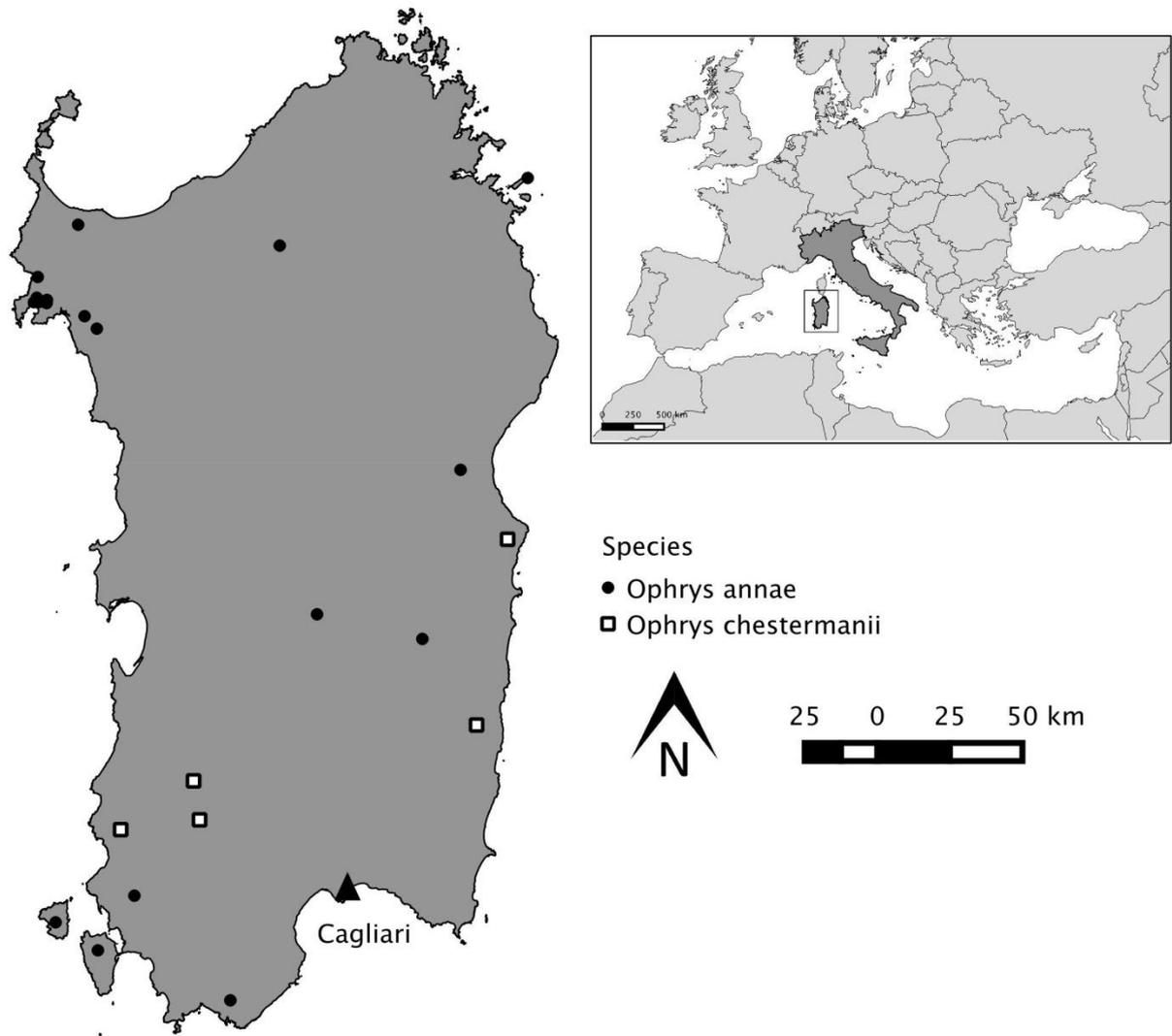


Figure 1 The distribution of the two species *O. annae* and *O. chestermanii*

| | <i>Ophrys annae</i> | <i>Ophrys chestermanii</i> |
|--------------------------------|---------------------|----------------------------|
| Plant Functional Traits | mean ± S.D. | mean ± S.D. |
| Plant height | 13.8 ± 4.183 | 21.1 ± 6.52 |
| First flower height | 8.6 ± 2.56 | 15 ± 4.855 |
| Inflorescence height | 5.2 ± 2.166 | 6.1 ± 3.462 |
| Flowers per plant | 5.3 ± 2.028 | 3.2 ± 0.994 |
| Fruits per plant | 0.2 ± 0.41 | 0.5 ± 0.76 |
| Flowers per population | 5.3 ± 2.028 | 3.4 ± 0.994 |
| Fruits per population | 0.2 ± 0.41 | 0.5 ± 0.76 |
| | | |
| Fitness | 0.04% | 0.15% |

Table 1. Plant Functional Traits analyses: fitness per population was calculated as the percentage of ratio between number of fruits and number of flowers sexually mature.

| | <i>Ophrys annae</i> | | <i>Ophrys chestermanii</i> | |
|--|---------------------|------------------|----------------------------|------------------|
| | length | width | length | width |
| Floral traits (mm) | mean \pm S. D. | mean \pm S. D. | mean \pm S. D. | mean \pm S. D. |
| Right sepal | 9.5 \pm 1.18 | 4.8 \pm 0.65 | 12.2 \pm 2.12 | 5.7 \pm 1.11 |
| Left sepal | 9.1 \pm 1.3 | 5 \pm 0.68 | 12.5 \pm 2.23 | 5.9 \pm 0.92 |
| Vertical sepal | 8.7 \pm 1.27 | 4.5 \pm 0.72 | 11.8 \pm 1.75 | 4.9 \pm 0.88 |
| Right petal | 3.6 \pm 0.68 | 2.1 \pm 0.79 | 4.8 \pm 0.97 | 2.1 \pm 0.74 |
| Left petal | 3.7 \pm 0.79 | 2 \pm 0.56 | 4.9 \pm 0.93 | 1.9 \pm 0.56 |
| Labellum | 7.4 \pm 1.00 | 10.7 \pm 1.07 | 11.9 \pm 1.31 | 17.2 \pm 1.6 |
| Stigmatic cavity | 2.2 \pm 0.23 | 1.3 \pm 0.18 | 3.1 \pm 0.34 | 1.7 \pm 0.28 |
| Labellum area (mm ²) | 39.9 \pm 8.49 | | 103.1 \pm 18.03 | |
| Stigmatic cavity area (mm ²) | 1.5 \pm 0.3 | | 2.55 \pm 0.59 | |

Table 2. Floral morphometric analysis.

| | <i>U value</i> | <i>Z-Score</i> | <i>p-value</i> |
|-------------------------|----------------|----------------|----------------|
| Right sepal length | 858.5 | 1.85309 | 0.06432 |
| Right sepal width | 994 | 1.1541 | 0.25014 |
| Left sepal length | 886.5 | -1.79392 | 0.07346 |
| Left sepal width | 941.5 | 1.5388 | 0.12356 |
| Vertical sepal length | 826.5 | 2.09843 | 0.03572 |
| Vertical sepal width | 1109.5 | 0.30776 | 0.75656 |
| Right petal length | 1030 | 0.89031 | 0.37346 |
| Right petal width | 1095 | 0.24192 | 0.81034 |
| Left petal length | 886.5 | 1.94182 | 0.05238 |
| Left petal width | 1105 | -0.34073 | 0.72786 |
| Labellum area | 913 | 1.74764 | 0.08012 |
| Labellum length | 663 | 3.45757 | 0.00054 |
| Labellum width | 1115 | -0.26746 | 0.78716 |
| Stigmatic cavity area | 816.5 | 2.45476 | 0.01428 |
| Stigmatic cavity heigth | 933.5 | 1.59742 | 0.1096 |
| Stigmatic cavity width | 825.5 | 2.38881 | 0.01684 |
| Plant height | 69.5 | 3.51651 | 0.00044 |
| First flower height | 45.5 | 4.16571 | < 0.00001 |
| Inflorescence hiegth | 179 | 0.55453 | 0.58232 |
| Flowers per plant | 122.5 | -2.77263 | 0.0056 |
| Fruits per plant | 164 | 0.96028 | 0.33706 |

Table 3. Mann Whitney U Test results.

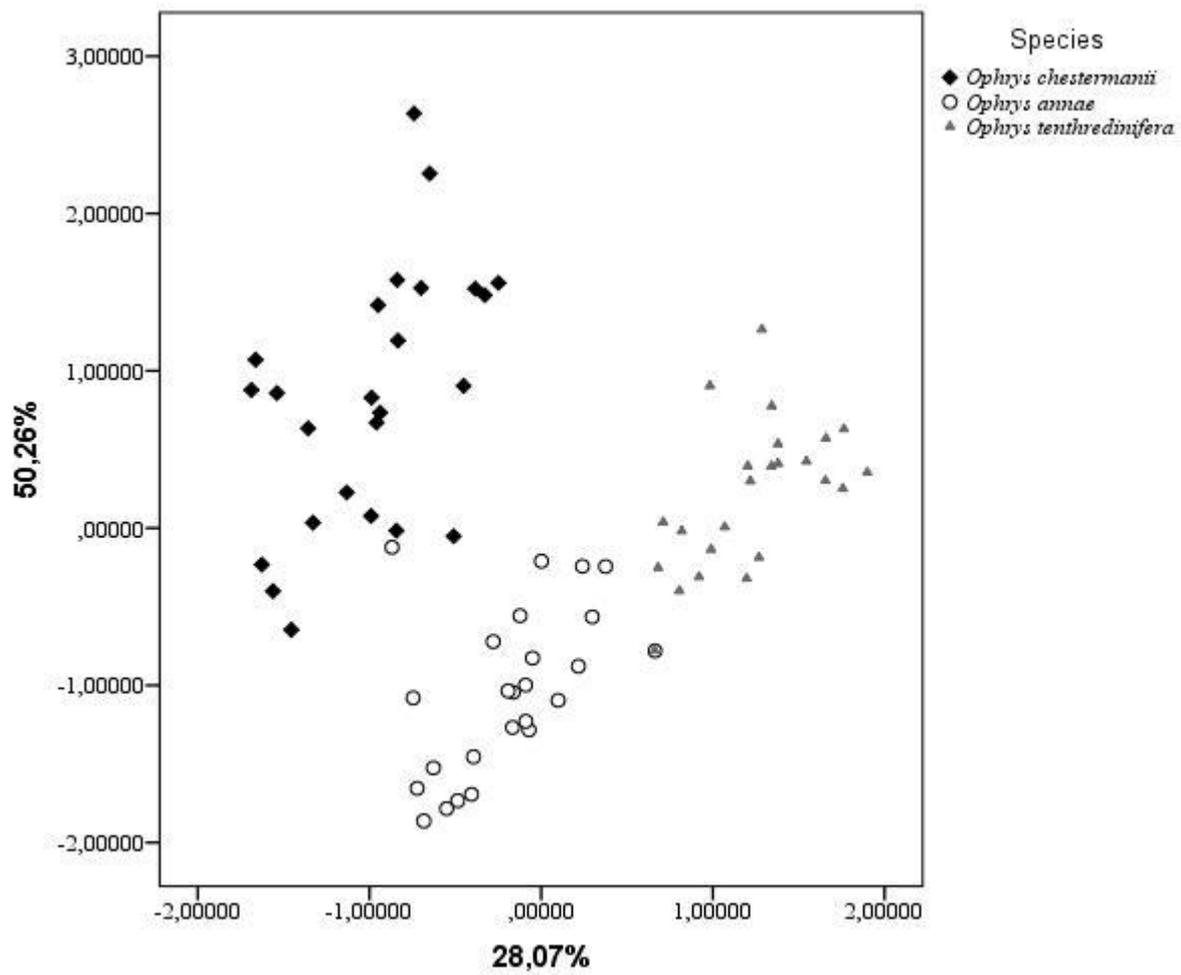


Fig 2. Principal Component Analysis (PCA) of the plant functional traits. Each point represents an examined individual.

| | | Fruits | Embryo Seeds mean \pm s.d. |
|----------------------------------|---|---------------|--|
| Interspecific crosses | <i>Ophrys annae</i> X <i>Ophrys chestermanii</i> | 100% | 66.62 \pm 17.479 |
| | <i>Ophrys chestermanii</i> X <i>Ophrys annae</i> | 25% | 70.89 \pm 8.998 |
| Intraspecific crosses | <i>Ophrys chestermanii</i> X <i>Ophrys chestermanii</i> | 100% | 78.46 \pm 11.246 |
| | <i>Ophrys annae</i> X <i>Ophrys annae</i> | 100% | 57.39 \pm 24.492 |

Table 4. Percentage of fruits production, mean and standard deviation in interspecific and intraspecific crosses.

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Chapter 5

Does size really matter? A comparative study on floral traits in two different orchid's pollination strategies

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Abstract

“Lock and key hypothesis” assumes that male and female genitalia match in a unique system to prevent interspecific crosses. This hypothesis is largely investigated in animals, while there is a distinct lack of studies on plants. Nevertheless few plants such as orchids, display an extremely complex floral morphology.

Here we apply a comparative approach to examine the variation of floral functional traits in food and sex deceptive orchids. To understand if a specific deceptive strategy is related with a specific variance of floral traits. We have evaluated the variation of sterile and fertile traits among species and subsequently examined the correlations between male and female reproductive organs of the same species with the aim to investigate the role of "lock and key" hypothesis in deceptive orchids. Accordingly our results show that the functional morphology of fertile traits plays a pivotal role in limiting the gene flow in species that grow in sympatry. In particular it was observed that the Reproductive Standardization Index (RSI) is significantly different in the two pollination strategies and that the correlation between pollinarium length and stigmatic cavity length is stronger in food deceptive species when compared to the sex deceptive ones.

These results reveal that the “lock and key hypothesis” contributes to keep boundaries in plants with very complex floral morphology.

Keywords: Reproductive Standardization Index, lock and key hypothesis, deceptive pollination, floral traits, pollinators.

Introduction

The lock and key hypothesis assumes that species-specific morphological matching between male and female genitalia promotes prezygotic reproductive isolation between closely related species. The biological concept of species is based on one or more barriers that at different levels affect the gene flow, leading to the rise of reproductive isolation mechanisms (RIMs) between populations promoting speciation (Dobzhansky 1937, Mayr 1943, Lussu et al 2018). In animals, the lock and key hypothesis compares the female genitalia to a lock, and male genitalia to a key: the combination between the key with the right lock leads to successful coupling (Shapiro and Porter 1989, Coyne and Orr 2004). What emerges is that barriers are essential in successful coupling and in order to define a given barrier it is necessary to fulfil two conditions: first the male and female reproductive organs must match, second the correlation between the morphology of the two sexes should be expressed (Masly 2012).

This hypothesis has been investigated actively in sympatric and parapatric animal species (Schick 1965, Tanabe and Sota 2008) and several recent investigations have shown that this mechanism contributes to keep reproductive isolation as, for instance, in the millipede genus *Parafontaria* (Xystodesmidae) (Tanabe and Sota 2008).

In Angiosperms, a great specialisation of floral traits is often associated with a dominance of prezygotic barriers compared to postzygotic barriers, although their co-occurrence leads to the maintenance of species borders (Darwin 1862, Philips et al. 2017). Although a highly specialised pollination strategy allows benefits and a reduction of pollen loss, it also brings different negative side effects such as, for instance, an increasing risk of extinction (Waser 1996).

In flowering plants studies on the role of mechanical isolation as prezygotic barrier are often associated with ethological investigations on pollinators (Grant 1994, Jersàková et al. 2009). Grant (Grant 1994) identified two different conditions of mechanical isolation: in the first, plant species isolation is maintained by adopting different pollinators; in the second, plant species share the same pollinators but they allocate pollen in different parts of pollinator's body. The astonishing diversity

of Orchidaceae is accompanied by a precise morphology of floral traits (Dressler 1993, Rudall and Bateman 2002) that make these plants unique and immediately recognizable. Orchid flowers are often described as adaptive attractions to different pollinators (Cozzolino and Widmer 2005, Vandewoestijne et al. 2008). Many authors investigated the role of perianth in pollinator attraction, reproductive isolation or speciation and concluded that morphological and chemical traits often contribute to limit gene flows between orchid species (Dafni 1983, Dressler 1993, Cozzolino and Widmer 2005). Little is known however, about the occurrence and the relevance of the lock and key hypothesis in orchids. The majority of orchids offers some kind of rewards to their pollinators but nearly one third relies on deceptive strategies (Cozzolino and Widmer 2005, Jersáková et al. 2009). In her review on food deception Jersáková et al. (Jersáková et al. 2009) described mechanical barrier as “the “lock and key” system whereby pollinarium adheres to orchid stigmata means that orchids can easily share pollinators with other plants without losing pollen to their stigmata”.

Food deceptive orchids are, in this context, particularly interesting because they mimic traits such as colors, scents or spur typical of species that offer a reward (Peter and Johnson 2006, Scopece et al. 2009) or mimic a well defined rewarding species (Johnson 2000, Anderson and Johnson 2005) devising interesting mechanisms to prevent the pollinator from discovering their deception. Usually, food deceptive orchids are generalist mimics and they are often pollinated by various pollinators (Robertson 1928) then prezygotic barriers are very labile when they occur in sympatry. Pollinators are mainly naïve insects or recently emerged ones or those who explore different nectar resources (Dafni 1983, Cozzolino and Widmer 2005).

In sex deception, plant species attract insects (especially males) imitating shapes, colors, and molecules secreted by sexually mature females (Paulus 2006). This relationship is very often species-specific, thus many orchids species are pollinated by one or few closely related insect species (Gögler et al 2009; Dormont et al. 2014; Gögler et al. 2015) hence usually displaying a batesian mimics. Several studies confirmed that sex deception strongly limits the gene flow also

between closely related species representing a strong and precise prezygotic barrier (Cortis et al. 2009; Vereecken et al. 2011; Xu et al. 2012, Whitehead and Peakall 2014, Gögler et al. 2015; Lussu et al. 2018). The European genus “*Ophrys*” was used by Grant (Grant 1994) to name a very precise condition of ethological mechanism.

In this study, we investigate the role of the *lock and key hypothesis* as RIM in orchids. In particular we hypothesise the sizes of the stigmatic cavity and the size of the pollinarium could covary because of their ecological role in reproduction. According to this, their correlation is expected to be stronger in sex deceptive orchids than in those species that adopt a more generalist food deception. Using a morphological and functional approach on eight sympatric orchid species as sample of the two deception strategies, we addressed the following questions:

1. Do fertile and sterile traits vary within each deception strategy? Since deceptive plants prevent pollinator from associating the floral traits with the deception, we hypothesised that in food deceptive species sterile traits vary more than the fertile ones because visual traits are primarily involved in this deception strategy.

2. Are pollinarium and the stigmatic cavity morphologies correlated? Does the sexually deception show a stronger correlation?

3. Is there a morphological correspondence between stigmatic cavity and pollinarium promoted by natural selection?

- 4.

Material and methods

For this study we selected eight of the most representative Mediterranean widespread and unrewarding orchid species with an overlapping phenology and distribution. We have excluded from this study endemic taxa or poorly ethologically studied species ascribed to phylogenetically unresolved clades. We compiled a list of eight species, four representative of the food deception strategy and four representative of the sex-deception strategy (for a complete list of species, see S1

Table). In order to avoid individuals affected by hybridization or introgression phenomena we have selected only samples from isolated populations. The data gathering was conducted during the blooming season of 2017 in Sardinia (Italy): food deceptive orchids are the first to bloom reaching a peak between March and April, instead sex deceptive orchids have the tendency to bloom later, getting a peak between April and May. For each species twenty plants were haphazardly chosen and one fresh, healthy, mature and undamaged flower for each plant was collected. Length of stigmatic cavity and pollinarium were measured in field after removing the flower from the plant. In absence of information about caudicle movement after the removal of the pollinarium, its length was measured after 2,5 minutes, the necessary time for the caudicle to bend in the ideal position (Darwin 1862, Peter and Johnson 2006). Pollinaria were extracted with an inoculating loop and their lengths was measured from the base of the viscidium to the top of pollinium. After removing the pollinarium, each flower was preserved in 70% ethanol solution. In laboratory, dissections were conducted under stereoscopic microscope then petals, sepals and labellum were digitalized on graph paper. For each trait length, width and area were measured using ImageJ (<https://imagej.nih.gov/ij/>). Fitness was calculated following the formula $W = n^{\circ} \text{fruits} / n^{\circ} \text{flowers}$ (Tremblay 2011).

In order to summarize the relationship between the stigmatic cavity length and the pollinarium one, we calculated the ratio between stigmatic cavity length and pollinarium length (Reproductive Standardization Index, RSI). To assess the difference within and between food and sex deceptive groups we calculated means, standard deviations and coefficients of variation. Analysis of variance (ANOVA) was performed to assess differences of RSI between and within pollination strategy. To test the hypothesis that plant functional traits differed between the two deception strategies we performed a Permutational multivariate analysis of variance (PERMANOVA (Anderson 2001)) by testing the simultaneous response of all the measured traits, only the fertile and only the sterile traits comparing the food vs sex deceptive groups. We used Euclidean distance on standardized data, 999 unrestricted permutations of raw data using

correct permutable units; the pairwise tests were corrected for multiple comparison. Pearson correlation coefficient were calculated to estimate morphological relation between male and female traits within each strategy and the relationship between RSI with fitness. Significance was assessed with generalized linear model and binomial distribution and logit link, fitness (W) was used as response variable and fertile traits and RI as independent variables. All the statistical analyses were performed using R Studio (R Core Team, 2017).

Results

We examined 160 flowers over the duration of this study. Within sterile traits intraspecific variation of the labellum is generally more variable than the other floral traits (Table 1). Coefficients of variation of sterile traits are 0,136 in food deceptive strategy and 0,26 in sexually deceptive strategy. On the other hand, coefficient of variations of fertile traits are 0,24 and 0,119 in food deceptive strategy and sexually deceptive strategy respectively (Fig 2). Details on each species are shown in S1 Table. PERMANOVA analysis showed that the deception strategy is significant in generating differences between plant functional traits among individuals (Total traits $F_{(1,158)} = 28,4$, $p < 0,001$; Sterile traits; $F_{(1,158)} = 35,95$; $p < 0,001$ and Fertile traits $F_{(1,158)} = 5,54$; $p < 0,001$). Considering RSI, statistically significant differences between the two deception strategies were detected $F_{(1,158)} = 24,8$, $p < 0,001$. Generally, results showed significant correlation between stigmatic cavity length and pollinarium length in the same species ($r = 0,546$, $p < 0,001$), a tendency that was confirmed at the pollination strategy level, (Fig 1), but no correlation was detected between RSI and fitness ($p > 0,05$).

At family level, multivariate analysis shows that there is evidence that RI, stigmatic cavity length and pollinarium length are significant predictors of fitness (Table 2). Moreover, when decoupled, in sex deceptive strategy no statistical support was detected, while in food deceptive strategy stigmatic cavity length and pollinarium length were both found to affect fruit set.

Discussion

Reproductive Isolation Mechanisms (RIMs) are the fundamentals of the biological concept of species because they limit gene flow and maintain species boundaries (Dobzhansky 1937). To date, several RIMs have been identified in plants, with Orchidaceae being one of the most emblematic families in pollination biology.. In this study we have analysed the features of the intra and inter specific variation of floral functional traits in food and sexually deceptive orchids, evaluating whether these matched the predictions of the lock and key hypothesis and if it is more expressed in one of the two considered strategies.

The lock and key hypothesis has been largely supported in animals, especially insects, but poorly studied in plants. In the present work several lines of evidence support the validity of this hypothesis also for orchids.

Based on the idea that flowers of deceptive mediterranean orchids vary in different traits such as flowers colors to prevent the association of floral traits and frauds (Anderson 2001, Ackerman et al. 2011; Stejskal et al. 2015), we have hypothesized that traits variance might be different in the two strategies. Generally, variation in sterile traits was found to be larger than the variation of the fertile portions. We have found that the trait with the highest variance in both strategies is the area of labellum. In orchids, labellum plays a key role in pollinator attraction (Bell et al. 2010), because in both strategies it is directly involved in plant-pollinator communication in order to avoid geitonogamy. In sexually deceptive species labellum is involved in scent production and its morphology is a trigger for pseudocopulation, thus even significant changes in its morphology might be not so relevant as it concerns the chemical communication with the pollinators, in fact even a small labellum is able to produce the scent necessary to attract pollinators and induce pseudocopulation. A great intraindividual variance in the labellum size might play a complementary role to fragrances and color variability (Dormont et al. 2014) in avoiding the recognition of deception and so promoting outcrossing. Indeed, in food deceptive strategy, adopting a high variation of colors and floral scents (Dormont et al. 2014), to avoid pollinators to learn easily to recognize deception. Although several pollinators are generally

recorded for these species such as for instance *Anacamptis longicornu* (Poir.) R.M.Bateman, Pridgeon & M.W.Chase, stabilizing selection on floral traits might be caused by most frequent and efficient species (Stebbins 1970). As previously demonstrated for other floral traits such as colors (Zitari et al. 2012) here we propose that negative frequency-dependent selection might be involved in keeping the high variability of labellum in both strategies (Gigord et al. 2001).

The core of the lock and key hypothesis is the size correspondence between male and female genitalia. Our results show a positive correlation between stigmatic cavity and pollinarium length in both strategies, yet this correlation was stronger in food deceptive species. .

We hypothesized a size correspondence between pollinarium and stigmatic cavity in orchids due to their peculiar plant-insect relation and their unique flower morphology. There is no study, to our knowledge, that analyzes purely the “lock and key” combination in these plants. Although a general low variability in floral traits our results showed correlation between pollinarium and stigmatic cavity sizes and differences between food and sexually deception strategy. In plants with complex flower structures such as orchids, mechanical isolation represent a barrier and our findings are congruent with the fact that an interaction between other mechanism of reproductive isolation prevent hybridisation. In the most specialized species, as the sexually deceptive *Ophrys*, a powerful tool to keep boundaries are floral volatile compounds that attract one or few pollinators (Ayasse et al. 2000, Cozzolino and Widmer 2005, Gögler et al. 2015). By contrast, in food deceptive species in which prezygotic barriers are weaker, boundaries are kept by postzygotic barriers such as different chromosomal number or as reduced hybrid fitness. We did not find any significant selection to promote the lock and key correlation. Given the potential importance of this relation, not significant selection on these traits is surprising. Nevertheless, the fact that we did not find any statistically significant association between RSI and fruit set does not mean that this trait is not under selection . It has been reported that speciation is fast when selection is significant, in orchids this has been identified in the diversification of the genus *Ophrys* in which pollinator mediated selection can drive diversification in a short time (Breitkopf et al. 2015, Lussu et al. 2018), we might conclude that

perhaps stigmatic cavity and pollinarium are not under a strong selection if compared to those traits involved in the deception strategies. It could be interesting in the future to study this correlation of time, observing the fitness variation in different years, also considering the effect of annual fluctuation of environmental variables.

We have demonstrated the role of morphological differences of pollinarium and stigmatic cavity in reproductive isolation in species with complex floral morphologies and elaborated pollination strategy. Our results help to elucidate the importance of the lock and key hypothesis for reproductive isolation among orchids. In general, more examples of natural selection on species with complex floral traits such as in *Orchidaceae* or *Asclepiadoideae* (Muola et al. 2011) are necessary to evaluate the role of floral morphology in keeping species boundaries. Possible future investigations should focus on comparative studies focused on hybrid fecundity, their floral compounds and seed vitality in sympatric species.

Table 1. Means, standard deviations and coefficients of variation for plants functional traits in food and sex deceptive strategies.

Fig 1. Correlation among the length of the stigmatic cavity vs pollinarium length in the two deceptive strategies: in food deceptive strategy (black dots) the correlation is higher than in sexual deceptive strategy (white squares).

Fig 2. Coefficient of variations of fertile traits, fitness and RI. W, fitness; CsL Stigmatic cavity Length, ScW Stigmatic cavity Width, PL Pollinarium Length.

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| | <i>Food deceptive strategy</i> | | | <i>Sexually deceptive strategy</i> | | |
|------------------------------|--------------------------------|-----------|-----------|------------------------------------|-----------|-----------|
| | Mean | SD | CV | Mean | SD | CV |
| <i>Fertile traits</i> | | | | | | |
| Stigmatic cavity Length | 2,930 | 0,410 | 0,148 | 2,449 | 0,463 | 0,189 |
| Stigmatic cavity Width | 2,538 | 0,255 | 0,103 | 2,706 | 0,810 | 0,299 |
| Stigmatic cavity Area | 7,6775 | 1,42 | 0,2 | 6,721 | 2,626 | 0,391 |
| Pollinarium Length | 3,260 | 0,310 | 0,095 | 3,298 | 0,536 | 0,162 |
| Mean | | | 0,136 | | | 0,26 |
| RSI | 0,901 | 0,141 | 0,15 | 0,752 | 0,132 | 0,172 |
| Total of fertile traits | 2,405 | 1,068 | 0,444 | 2,313 | 1,084 | 0,468 |
| <i>Sterile traits</i> | | | | | | |
| Labellum Height | 11,393 | 1,128 | 0,108 | 10,183 | 1,506 | 0,148 |
| Labellum Width | 12,605 | 1,360 | 0,108 | 11,047 | 1,850 | 0,167 |
| Labellum Area | 114,438 | 21,03 | 0,190 | 114,253 | 32,408 | 0,284 |
| Left sepal Length | 10,710 | 0,985 | 0,093 | 9,213 | 1,996 | 0,217 |
| Left sepal Width | 4,948 | 0,698 | 0,145 | 4,882 | 1,041 | 0,213 |
| Dorsal sepal Length | 9,653 | 0,950 | 0,105 | 8,425 | 1,400 | 0,166 |
| Dorsal sepal Width | 4,195 | 0,535 | 0,135 | 4,824 | 1,135 | 0,235 |
| Left sepal Length | 10,493 | 0,990 | 0,098 | 9,126 | 2,091 | 0,229 |
| Left sepal Width | 4,955 | 0,523 | 0,110 | 4,914 | 1,151 | 0,234 |
| Right petal Length | 8,640 | 0,705 | 0,080 | 5,175 | 1,625 | 0,314 |
| Right petal Width | 3,053 | 0,390 | 0,130 | 2,034 | 0,619 | 0,304 |
| Left petal Length | 8,420 | 0,923 | 0,113 | 5,220 | 1,612 | 0,309 |
| Left petal Width | 3,015 | 0,420 | 0,140 | 2,031 | 0,605 | 0,298 |
| Mean | | | 0,119 | | | 0,24 |
| Total of sterile traits | 6,851 | 3,738 | 0,545 | 5,584 | 2,842 | 0,508 |

Table 1. Means, standard deviations and coefficients of variation for plants functional traits in food and sex deceptive strategies.

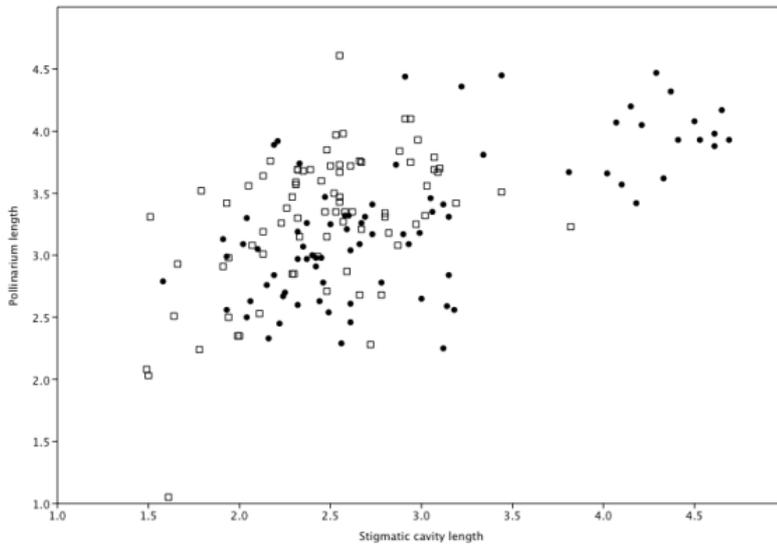


Fig 1. Correlation among the length of the stigmatic cavity vs pollinarium length in the two deceptive strategies: in food deceptive strategy (black dots) the correlation is higher than in sexual deceptive strategy (white squares).

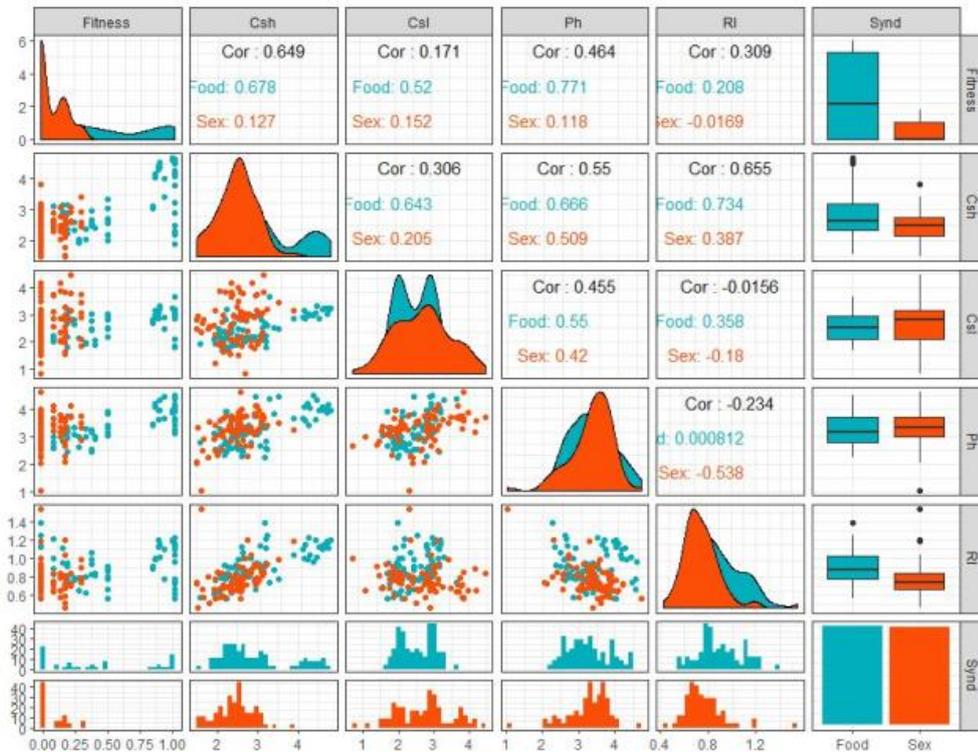


Fig 2. Coefficient of variations of fertile traits, fitness and RI. W, fitness; CsL Stigmatic cavity Length, ScW Stigmatic cavity Width, PL Pollinarium Length.

Supporting information

S1 Table. Biological characteristics of species and locality of populations studied.

S2 Table. Plant functional traits measures of food deceptive species investigated.

S3 Table. Plant functional traits measures of sexually deceptive species investigated.

| Species | Pollination deception | Pollinators recorded | References | Location | W |
|--|-----------------------|---|---|--------------------------|-------|
| <i>Anacamptis collina</i> | Food | <i>Apis mellifera</i> (Linnaeus 1758) ♀ <i>Andrena</i> sp. <i>Eucera</i> sp. | Dafny and Ivri 1979 | 39°14'33.3"N 9°06'46.9"E | 0,393 |
| <i>Anacamptis longicornu</i> | Food | <i>Andrena albopunctata</i> (Rossi 1792) ♀♂ <i>A. ovatula</i> (Kirby, 1802) ♀♂ <i>Apis mellifera</i> (Linnaeus 1758) ♀ <i>B. rupestris</i> (Fabricius 1793) ♀♂ <i>B. sylvarum</i> (Linnaeus 1761) ♀♂ <i>B. sylvestris</i> (Lepeletier 1832) ♀♂ <i>B. terrestris</i> (Linnaeus 1758) ♀♂ <i>B.vestalis</i> (Geoffroy 1785) ♀♂ <i>Bombus lapidarius</i> (Linnaeus 1758) ♀♂ <i>Eucera hungarica</i> (Friese 1895) ♀♂ <i>Halictuspatellatus</i> (Morawitz 1873) ♀♂ <i>Lasioglossum xanthopus</i> (Kirby 1802) ♀♂ <i>Osmia bicornis</i> (Linnaeus 1758)♀♂ | Cozzolino and Widmer 2005 Zitari et al. 2012 | 39°51'11.3"N 9°06'40.8"E | 0,072 |
| <i>Anacamptis papilionacea</i> subsp. <i>grandiflora</i> | Food | <i>Anthophora crinipes</i> (Smith, 1854) ♀♂ <i>A. retusa</i> (Linnaeus 1758) ♀♂ <i>Bombus terrestris</i> (Linnaeus 1758) ♀♂ <i>Bombus humilis</i> (Illiger 1806) ♀♂ <i>Eucera hungarica</i> (Friese 1895) ♀♂ <i>E.nigrescens</i> (Pe ´rez 1879) ♀♂ | Delforge 2006, Scopece et al. 2009 | 39°14'33.3"N 9°06'46.9"E | 0,314 |

| | | | | | |
|-----------------------------------|------|---|--|--------------------------|-------|
| | | <i>E. capsica</i> (Morawitz 1873) ♀♂ <i>Eucera bidentata</i> (Pe 'rez 1879) ♀♂ <i>Eucera tuberculata</i> (Fabricius 1793) ♀♂ <i>Megachile parietina</i> (Geoffroy 1785) ♀♂ <i>Nomada imperialis</i> (Schmiedeknecht 1882) ♀♂ | | | |
| <i>Himantoglossum robertianum</i> | Food | <i>Apis mellifera</i> (Linnaeus 1758) ♀ <i>Bombus lucorum</i> (Linnaeus 1758) ♀♂ <i>B. terrestris</i> (Linnaeus 1758) ♀♂ <i>Xylocopa violacea</i> (Linnaeus 1758) ♀♂ | Bateman et al. 2016, Claessens et Kleynen 2011 | 39°14'33.3"N 9°06'46.9"E | 0,952 |
| <i>Ophrys eleonora</i> | Sex | <i>Andrena morio</i> ♂ | Delforge 2006 | 39°14'33.3"N 9°06'46.9"E | 0,282 |
| <i>Ophrys sicula</i> | Sex | <i>Andrena hesperia</i> ♂ <i>A. merula</i> ♂, <i>A. taraxaci</i> ♂, <i>A. torda</i> ♂ | Delforge 2006 | 39°14'33.3"N 9°06'46.9"E | 0,200 |
| <i>Ophrys speculum</i> | Sex | <i>Dasyscolia ciliata</i> (Fabricius, 1787) ♂ | Delforge 2006 | 39°14'33.3"N 9°06'46.9"E | 0,240 |
| <i>Ophrys tenthredinifera</i> | Sex | <i>Eucera nigrilabris</i> ♂ | Delforge 2006 | 39°14'33.3"N 9°06'46.9"E | 0,257 |

S1 Table. Biological characteristics of species and locality of populations studied.

| Food Deception | | | | | | | | |
|-------------------------|---------------------------|-------|------------------------------|------|--------------------------------|-------|-----------------------------------|-------|
| Variable\Species | <i>Anacamptis collina</i> | | <i>Anacamptis longicornu</i> | | <i>Anacamptis papilionacea</i> | | <i>Himantoglossum robertianum</i> | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Stigmatic cavity Length | 2,43 | 0,41 | 2,58 | 0,43 | 2,55 | 0,32 | 4,16 | 0,48 |
| Stigmatic cavity Width | 2,11 | 0,31 | 2,18 | 0,18 | 2,79 | 0,29 | 3,07 | 0,24 |
| Stigmatic cavity Area | 5,16 | 1,36 | 5,63 | 1,1 | 7,16 | 1,34 | 12,76 | 1,88 |
| Labellum Height | 9,57 | 0,84 | 5,68 | 0,84 | 15,57 | 1,28 | 14,75 | 1,55 |
| Labellum Width | 10,39 | 1,08 | 8,94 | 1,05 | 15,92 | 1,74 | 15,17 | 1,57 |
| Labellum Area | 82,68 | 14,36 | 40,04 | 8,87 | 170,87 | 27,15 | 164,16 | 33,75 |
| Pollinode Length | 3,28 | 0,39 | 2,68 | 0,22 | 3,07 | 0,32 | 4,01 | 0,31 |
| Spur Length | 6,18 | 1,2 | 10,42 | 0,86 | 10,34 | 1,33 | 6,49 | 0,85 |
| Left sepal Length | 10,62 | 1,22 | 5,92 | 0,57 | 14,66 | 0,95 | 11,64 | 1,2 |
| Left sepal Width | 3,87 | 0,37 | 2,64 | 0,5 | 6,55 | 0,89 | 6,73 | 1,03 |
| Vertical sepal Length | 9,66 | 0,74 | 5,31 | 0,79 | 12,58 | 1,15 | 11,06 | 1,12 |
| Vertical sepal Width | 4,05 | 0,41 | 2,25 | 0,37 | 4,15 | 0,8 | 6,33 | 0,56 |
| Left sepal Length | 10,38 | 1,03 | 5,62 | 0,65 | 14,37 | 1,33 | 11,6 | 0,95 |
| Left sepal Width | 3,95 | 0,45 | 2,41 | 0,34 | 6,46 | 0,92 | 7 | 0,38 |
| Right petal Length | 8,59 | 0,73 | 4,7 | 0,44 | 11,29 | 1,01 | 9,98 | 0,64 |
| Right petal Width | 3,14 | 0,33 | 1,53 | 0,23 | 3,94 | 0,59 | 3,6 | 0,41 |
| Left petal Length | 8,67 | 0,86 | 4,69 | 0,62 | 10,87 | 1,21 | 9,45 | 1 |
| Left petal Width | 3,14 | 0,36 | 1,62 | 0,26 | 3,53 | 0,67 | 3,77 | 0,39 |
| Numbers of individuals | 20 | | 20 | | 20 | | 20 | |

S2 Table. Plant functional traits measures of food deceptive species investigated.

| Sexually Deception | | | | | | | | |
|-------------------------|-------------------------|-------|----------------------|------|------------------------|------|-------------------------------|-------|
| Variable\Species | <i>Ophrys eleonorae</i> | | <i>Ophrys sicula</i> | | <i>Ophrys speculum</i> | | <i>Ophrys tenthredinifera</i> | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Stigmatic cavity Length | 2,7 | 0,46 | 1,95 | 0,39 | 2,51 | 0,32 | 2,64 | 0,24 |
| Stigmatic cavity Width | 3,72 | 0,37 | 2,38 | 0,7 | 1,87 | 0,26 | 2,86 | 0,27 |
| Stigmatic cavity Area | 9,99 | 1,69 | 4,62 | 1,62 | 4,72 | 1,05 | 7,55 | 1,11 |
| Labellum Height | 11,5 | 1,18 | 8,19 | 0,68 | 10,54 | 0,84 | 10,51 | 0,78 |
| Labellum Width | 12,41 | 1,23 | 9,33 | 0,82 | 9,64 | 0,92 | 12,81 | 0,88 |
| Labellum Area | 125,17 | 22,54 | 61,93 | 9,43 | 97,44 | 8,45 | 111,37 | 15,14 |
| Pollinode Length | 2,61 | 0,59 | 2,61 | 0,59 | 3,25 | 0,29 | 3,56 | 0,32 |
| Left sepal Length | 11,81 | 1,14 | 7,28 | 0,58 | 8,08 | 1,18 | 9,69 | 0,96 |
| Left sepal Width | 6,08 | 0,46 | 4,2 | 0,41 | 3,8 | 0,27 | 5,45 | 0,7 |
| Vertical sepal Length | 9,94 | 0,88 | 7,03 | 0,67 | 7,69 | 0,99 | 9,04 | 0,71 |
| Vertical sepal Width | 6,23 | 0,6 | 3,94 | 0,43 | 3,82 | 0,44 | 5,31 | 0,62 |
| Left sepal Length | 11,82 | 1,19 | 7,04 | 0,71 | 7,84 | 0,85 | 9,8 | 1,06 |
| Left sepal Width | 6,31 | 0,57 | 4,13 | 0,47 | 3,82 | 0,49 | 5,4 | 0,72 |
| Right petal Length | 7,66 | 0,88 | 4,84 | 0,57 | 4,4 | 0,75 | 3,98 | 0,62 |
| Right petal Width | 2,14 | 0,27 | 1,76 | 0,26 | 1,4 | 0,32 | 2,82 | 0,35 |
| Left petal Length | 7,68 | 0,76 | 4,72 | 0,41 | 4,42 | 0,92 | 3,88 | 0,43 |
| Left petal Width | 2,1 | 0,24 | 1,7 | 0,26 | 1,45 | 0,26 | 2,89 | 0,37 |
| Numbers of individuals | 20 | | 20 | | 20 | | 20 | |

S3 Table. Plant functional traits measures of sexually deceptive species investigated.

Chapter 6

General conclusions

It is very often said that science rises more questions than it answers. This contribution demonstrates the potential of evolutionary study to rise vibrant philosophical questions that should be the core of any scientific work. If the initial question was how many *species* are currently in Sardinia, the reader did not find a certain answer. The cause of this uncertainty lies in the complexity of life on Earth, of which orchids are an iconic model. My final list included 62 species and 14 genera. Results from literature investigations clearly showed the relevance of sardinian studies to implement knowledge on patterns of evolution in orchids, but also that this knowledge is often fragmented in different biological topics. The main result is the potential of island orchids in investigations of biological processes and ecological dynamics.

In the other two papers, first I investigated this complexity and how it links two close related endemisms that share a common past but evolved RIMs to limit the gene flow. The two sister species investigated are *Ophrys annae* and *Ophrys chestermanii*, endemic of Sardinia. I found that these species differ in 16 morphometric characters. Intra- and interspecific crosses demonstrate the role of postzygotic barriers to reinforce prezygotic reproductive isolation mechanisms in limiting gene flow in a specialized pollination syndrome. In addition, ecological analyses suggested that the two endemisms have different habitat preferences underlying its role as a strong limit to gene flow.

I reported the *lock and key theory* as a potential RIM in plant kingdom trying to link an established zoological hypothesis into botany. Morphometric comparison showed that RSI was significantly different in the two pollination strategies; the correlation between pollinium length and stigmatic cavity length was stronger in food deceptive species when compared to the sex deceptive ones. A possible explanation of this difference might be the weakness of prezygotic barriers in food deceptive strategy. In contrast, in sexually deceptive species this correlation was weaker perhaps

because in these species prezygotic barriers that occur earlier such as pollinator's specificity are stronger.

In summary, these three works are a modest sample to elucidate that mechanisms of speciation are extremely variable in space and time and they should not be considered fixed because life on earth is continuously under evolution. In plants with peculiar flower morphology and complex phylogenetic history such as orchids, RIMs are crucial to maintain borders between close related taxa. Indeed, evolution follows intricate and fascinating patterns leading to astonishing and often fragile organisms; hence it is necessary invoke the ethic role of our species, *Homo sapiens*, to discover and understand biodiversity in its entirety.



Orchis mascula subsp. *ichnusae* Corrias

