UNIVERSITÀ DELLA CALABRIA

FACOLTÀ DI SCIENZE MATEMATICHE, FISICHE E NATURALI

DOTTORATO DI RICERCA IN BIOLOGIA VEGETALE

SCUOLA DI DOTTORATO "LIFE SCIENCES"

XXV CICLO

SETTORE DISCIPLINARE: 05/A1

"EVOLUTIVE SIGNIFICANCE OF HYBRIDIZATION IN MEDITERRANEAN DECEPTIVE ORCHIDS"

Coordinatore:

Prof. Aldo Musacchio

Supervisore:

Prof. Giuseppe Pellegrino

Dottoranda:

Dott. Alessia Luca

Anno Accademico 2011-2012

TABLE OF CONTENTS

Aí míei adorați nípoți

INTRODUCTION

The Orchidaceae is one of the largest plant families on Earth, including almost 10% (approximately 20,000 species) of all flowering plant species (Dressler 1993; Dixon et al., 2003); It is rivalled only by the Asteraceae, which contains approximately 23 000 species (Bremer, 1994).

Orchids show a wide diversity of epiphytic and terrestrial growth forms and have successfully colonized almost every habitat on earth. Indeed, they occur throughout the world from the cold subartic regions to elevations above 4000 m asl and even within highly developed urban regions (Brown, 2002). Areas of particularly high orchid abundance closely follow areas of high plant diversity, or biodiversity 'hotspot' (Parsons and Hopper, 2003). Despite the fact that orchids are so widespread and adaptable, many species are rare or under threat of extinction (Koopowitz et al., 2003; Swarts and Dixon, 2009). The life cycle of terrestrial species is closely linked to seasonal changes in temperature and soil moisture conditions (Dixon, 1991).

It is their staggering variation in floral form that has long attracted the interest of evolutionary biologists. Indeed, orchid flower shows different colours, shapes, scents and energy-rich rewards and is related to orchid pollination systems. Notably, the highest percentage of species with features related to deceptive pollination occurs in Orchidaceae, a family renowned for elaborate floral structures and specialized interactions with pollinators (Ackerman, 1986; Schiestl, 2005; Jersáková, Johnson and Kindlmann, 2006).

Pollination systems in orchids are often mistakenly assumed to be the outcome of co-evolutionary processes (van der Pijl and Dodson, 1966; Dressler, 1968; Dodson, 1975). Co-evolution between orchids and their pollinators is probably uncommon (Szentesi, 2002) and most of the evolution is unilateral on the orchid side without any evolutionary changes in the pollinator (Williams, 1982).

Deceptive pollination systems are prevalent among Euro-Mediterranean terrestrial orchids (Cozzolino and Widmer, 2005), most of which (e.g. *Orchis* L., *Anacamptis* Rich., *Dactylorhyza* Neck.) have developed a food-deceptive and generalist strategy. Members of the genus *Ophrys* L. have evolved a sexually deceptive and specialized pollination strategy, their flowers mimic either the

shape and/or pheromones of the female of the pollinator species and thereby attract the male (Schiestl, 2005).

The pollination is related to reproductive isolation. In general, reproductive isolation among orchid species is based on several mechanisms that may act during the pre and/or post-mating stages (Templeton, 1989; Marques et al., 2007; Rieseberg and Willis, 2007; Widmer et al., 2009). The pre-mating stage may be distinct in a pre-pollination and a post-pollination pre-zygotic step (Ramsey et al., 2003). Differences in blooming times, floral topology and pollinator behaviour are examples of pre-pollination mechanisms (Grant 1994). The post-pollination pre-zygotic barriers occur at level of pollen–stigma and pollen–style (Franklin-Tong, 1999), whereas post-zygotic barriers are due to karyological divergence and hybrid sterility (Cozzolino et al., 2004).

But hybrid could not represent a dead end population, but may have a role as potential reserve of adaptive variability and is an unusual stage along the speciation process.

Members of this family grow in a wide range of habitats and have a substantial variety of life history strategies ranging from epiphytic to terrestrial, and from evergreen to nongreen species. All orchids are initially myco-heterotrophic (Leake, 1994) but most eventually produce leaves and become photosynthetic. But also photosynthetic orchids in adult stage show a interaction with mycorrhizal fungi.

ORCHID SYSTEMATIC

Orchids are a part of the Spermatophyte (or Phanerogames) branch, in which they belong to the Angiosperm branch, plants with a closed ovary containing the ovules, in the division Magnoliophyta (i.e. *Magnolia* and tulip trees, *Liriodendron tulipifera*), class Liliopsida (i.e. Lilies), order Asperagales. There are some 170,000 species of Angiosperms placed in different families and orders grouped into two classes. Orchids are members of the class Monocotyledonae, plants with a single embryonic seed leaf (cotyledon) at germination, and normally with simple, entire leaves, with parallel veins. They are distinct from class Dicotyledonae, wich have two cotyledons, with branching or fanned veins and 5- or 4-part flowers (e.g. magnolias, aristolochias, buttercups, roses).

Orchidaceae is one of the two largest families of flowering plants; the other is Asteraceae (Compositae). The higher-level classification of Orchidaceae has traditionally been based on the construction of the fused gynoecium and androecium (gymnostemium or column), which is unique to the family. The number of anthers has been the primary trait emphasised, which has resulted in the family being split into three groups, often recognised as subfamilies. Those with two anthers or one anther were placed in Diandrae and Monandrae, respectively. The taxonomy of this family is in constant flux. The assumption of all classifications prior to the advent of the use of DNA data in classification, including the most recent one based on morphology (Dressler, 1993), is that Monandrae were monophyletic, and of course because they include the type genus, some part of them at least must be called Orchidoideae. Dressler (1993) recognised five subfamilies: Apostasioideae, Cypripedioideae, Orchidoideae, Spiranthoideae and Epidendroideae (the last by far the largest). Dressler (1993) accepted a broad concept of Orchidaceae, which included both Apostasiaceae and Cypripediaceae. There was no category that included all of the monandrous orchids, but instead they were split into three subfamilies. As far as relationships among these five subfamilies, it was assumed that the lack of complete androecial/gynoecial fusion was a good indication that the apostasioids were sister of the rest, followed by the cypripedioids. Dressler (1993) believed that Orchidoideae and Spiranthoideae were sister taxa and most likely this pair were sister to Epidendroideae.

The first DNA studies, those of Chase et al. (1994) and Cameron et al. (1999), put a somewhat different slant onto the patterns. First of all, these studies confirmed that the apostasioids shared a unique genetic relationship to the rest of the orchids, thus making their treatment as a separate family unnecessary. Second, it was not clear that monandrous orchids were monophyletic. Third, Spiranthoideae were embedded in Orchidoideae, thus making the later unnatural. And fourth, the vanilloid orchids were an unexpected major clade, thus justifying their treatment as a distinct subfamily.

Subsequent DNA studies (Cameron, 2004; Freudenstein et al., 2004) have confirmed these patterns, and a new formal classification was proposed by Chase et al. (2003). A cladogram version of this classification is given in Figure. 1,

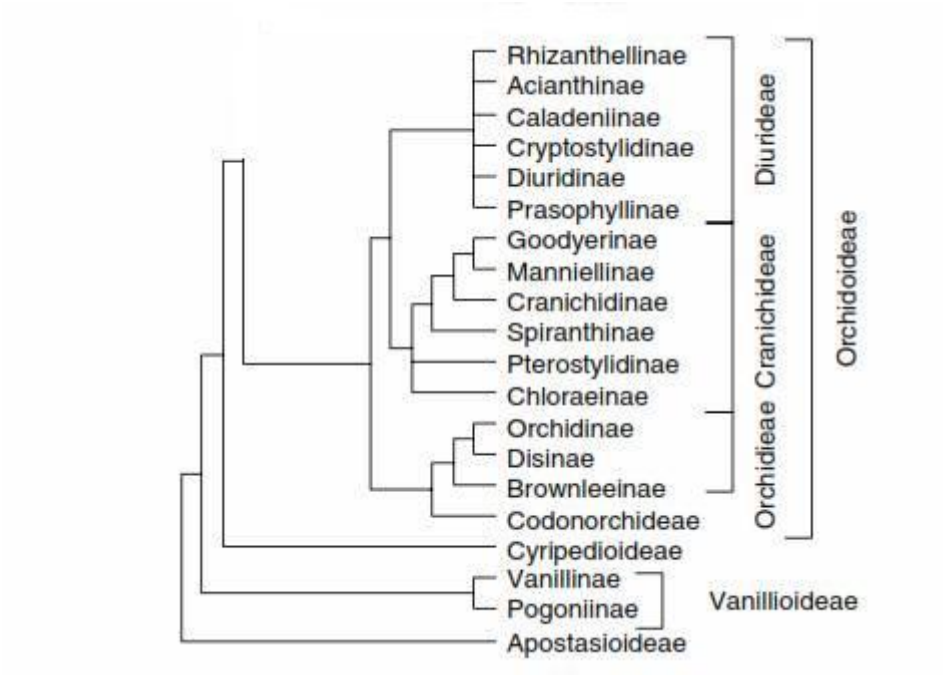

Fig. 1 Systematic cladogram

its main features are:

1. Vanilloideae are sister to all other subfamilies except Apostasioideae, which means that the reduction to a single anther occurred at least twice.
2. Most of the spiranthoid orchids, labelled here as Cranichidae, are embedded in the orchidoids and hence are treated as a tribe in Orchidoideae.
3. Other previous members of Spiranthoideae, tribe Tropidieae are members of Epidendroideae, so the terminal anther character upon which Spiranthoideae were based is not reliable.
4. There is a lack of resolution among the tribes and subtribes of Epidendroideae except for Epidendreae, Vandeae and Cymbidieae.

A question is whether there are morphological characters (synapomorphies) to support the clades observed in the DNA-based trees, the answer is positive. A cladistic analysis of orchid morphological data produced similar results (Freudenstein and Rasmussen, 1999), and that analysis provides characters for a large number of the groups identified by the DNA studies. For example, Orchidoideae as here defined have no fibres in their leaves, and Vanilloideae have a peculiar form of column.

The taxonomy of this family is complex, as new studies continue to identify more classificatory elements. The Orchidaceae is currently placed in the order Asparagales by the APG III system (APG III, 2009). Five subfamilies are recognised. The cladogram has been made according to the APG system: (Table. 1).

Tab. 1 APG III- system classification

SOTTOFAMIGLIA	TRIBÙ	SOTTOTRIBÙ
Apostasioideae	--	--
Cypripedioideae	--	--
Vanilloideae	Pogonieae	---
	Vanilleae	---
Epidendroideae	Arethuseae	Arethusinae
		Coelogyninae
	Calypsoeae	---
	Collabieae	Collabiinae
	Cymbidieae	Catasetinae
		Coeliopsidinae
		Cymbidiinae
		Cyrtopodiinae
		Eriopsidinae
		Eulophiinae
		Maxillariinae
		Oncidiinae
		Stanhopeinae
		Vargasiellinae
		Zygopetalinae
	Dendrobieae	Dendrobiinae
	Epidendreae	Bletiinae

		Chysinae
		Coeliinae
		Laeliinae
		Pleurothallidinae
		Ponerinae
	Gastrodieae	---
	Malaxideae	---
	Neottieae	---
	Nervilieae	Nerviliinae
		Epipogiinae
	Podochileae	Eriinae
		Thelasinae
	Sobralieae	---
	Triphoreae	Diceratostelinae
		Triphorinae
	Tropidieae	---
	Vandeae	Aerangidinae
		Aeridinae
		Angraecinae
		Polystachyinae
	Xerorchideae	---
	incertae sedis	Agrostophyllinae
Orchidoideae	Chloraeeae	---
	Codonorchideae	---
	Cranichideae	Achlydosinae

		Cranichidinae
		Galeottiellinae
		Goodyerinae
		Manniellinae
		Pterostylidinae
		Spiranthinae
	Diseae	Brownleeinae
		Coryciinae
		Disinae
		Huttonaeinae
		Satyriinae
	Diurideae	Acianthinae
		Caladeniinae
		Cryptostylidinae
		Diuridinae
		Drakaeinae
		Megastylidinae
		Prasophyllinae
		Rhizanthellinae
		Thelymitrinae
	Orchideae	Orchidinae

ORCHID FLORAL MORPHOLOGY

The primary characteristics that distinguish the orchids as a group are found in the flower (Figure. 2). Few orchids show a single flower, many orchids have a inflorescence with a large number of flowers. Orchid flower has a bilaterally symmetry (zygomorphic symmetry), and two whorls of sterile elements, forming perianth. Above pedicel the outer whorl has three sepals, while above and inside the sepals there is the inner whorl showing three petals. Sometimes the sepals and petals are very similar and thus called tepals. The perianth protects the flower and attracts pollinators.

The upper medial petal, called the labellum or lip, is always modified and enlarged. The inferior ovary or the pedicel usually rotates 180 degrees (resupination), so that the labellum, goes on the lower part of the flower, thus becoming suitable to form a platform for pollinators.

Inside are the sexual portions of the flower. The sexual portions of the orchid flower are quite different from other generalized flowers, and they tend to characterize the family.

Orchid flowers primitively have three (genus *Neuwiedia* and *Apostasia*) or two (Cipripedioideae) stamens, while all of the other orchids retain only the central stamen. Male reproductive organs vary widely in angiosperms, due to the number of pollen grains in pollen dispersal units (PDUs), a term used to indicate the different ways in which ripe pollen is presented for dispersal (Pacini, 1997). On the basis of the number of aggregated pollen grains and sticking modalities, Pacini and Franchi (1998) recognized 13 PDU types in Angiosperms: ten in monocots (Pacini and Franchi, 2000) and eight in orchids (Pacini and Hesse, 2002), four of which are typical of this group. Orchidaceae is the Angiosperm family with the greatest number of PDU types. Orchids possess: monad pollen with pollenkitt, monad pollen grouped by elastoviscin; isolated tetrads, aggregated pollen tetrads, tetrads grouped by elastoviscin (soft pollinium), tetrads grouped in a compact pollinium.

In the centre of the flower is the pistil, which consists of an enlarged three-carpelate inferior ovary. The majority of the orchids retain only a single anther at the apex of the column. The filaments, anthers, style, and stigma are reduced in

number and are usually fused into a single structure called the gynostemium or column.

The stigma, usually a shallow depression on the inner sides of the column, is composed of three stigmatic lobes (as in the typical monocot flower); however, the three lobes are fused together in the orchids. In the majority of the orchids, a portion of one of the three stigma lobes forms the rostellum, a flap of tissue that projects down in front of the anther separating the stigma and the anther. As the visiting insect backs out of the flower, it brushes the rostellum, which is covered with sticky stigmatic liquid. The pollinia are then picked up from the anther and adhere to the body of the insect. Some primitive species have no rostellum, and the pollinia simply stick to stigmatic liquid that is first smeared on the back of the insect. A further specialization occurs in more advanced orchids in which the caudicles of the pollinia are already attached to the rostellum and a portion of it comes off as a sticky pad called a viscidium. In the most advanced genera a strap of nonsticky tissue from the column connects the pollinia to the viscidium. This band of tissue is called the stipe and should not be confused with the caudicles, which are derived from the anther. Orchids that have a stipe also have caudicles that connect the pollinia to the apex of the stipe. The pollinia, stipe, and viscidium are called the pollinarium.

The ovary typically develops into a dehiscent capsule by three or six longitudinal slits, remaining closed at both ends. The ovules are arranged along the ridges inside the ovary and do not develop until some time after the flower has been pollinated, thereby contributing to the long delay between pollination and the opening of a ripened pod. There are several types of nectaries in the orchids, including extrafloral types that secrete nectar on the outside of the buds or inflorescence (flower cluster) while the flower is developing. Shallow cuplike nectaries at the base of the lip are common. Some nectaries are in long spurs that develop either from the base of the lip.

Members of the *Epidendrum* complex have long tubular nectaries embedded in the base of the flower alongside the ovary. Nectaries on the side lobes of the lips are known, and general nectar secretion along the central groove of the lip is common. The infiorescence is often a spike (flowers stalkless) or a raceme (flower stalked); rarely it comprises a solitary flower (*Cypripedium*, *Calypso*). The inflorescence can be dense, near lax or lax, often becoming laxer as

flowering proceeds. The shape for the infiorescence is variable: cylindrical, conical (*Anacamptis*), ovoid, spiral (*Spiranthes*), unilateral (some *Epipactis*). Flower always have bracts, small leaves inserted into the axis of the inflorescence, at the base of the pedicel or ovary; these may be green or colorated as a sepal (*Serapias*), large and leaf-like (*Dactylorhiza*, *Epipactis*), or reduced to membranous scales (most *Orchis*).

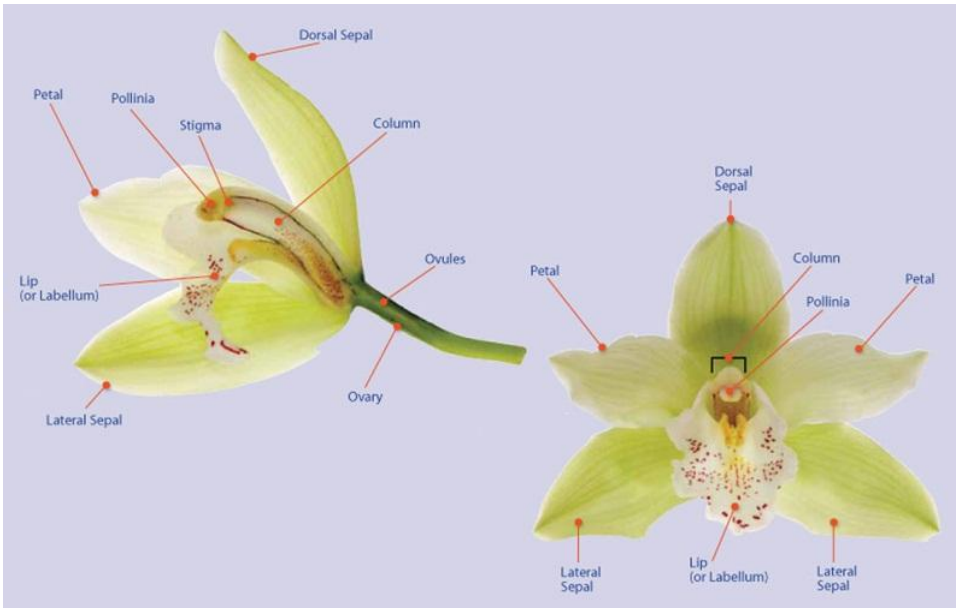

Fig. 2 Orchid floral anatomy

ORCHID AERIAL PARTS

As in most monocotyledons, the stems of European orchids are never branched; they are nerly always erect, more or less circular in section, rerely angular, solid or hollow, hair-less or hairy. The leaves are like those of other monocotyledons, complete, never composite or divided, with parallel longitudinal veins forming a visible network (*Goodyera*). In most of the saprophytic orchids the leaves, performing no function, have been reduced to scales or a sheath. When the leaves are developed, they may be clustered at the base of the stem in a basal rosette (as in most *Ophrys*) or spaced out along the stem, in which case they can arranged in a spiral , in two opposite ranks inserted at the same level (opposite), or alternately at different levels (alternate); the upper cauline leaves can be very small, resembling bracts. In some genera there are only a few leaves, sometimes only two (*Plantathera*, *Gennaria*) or evenjust one (*Malaxis*, *Calypso*).

ORCHID UNDERGROUND PARTS

All the orchids in Europe are geophytes, except of 2 genera rapresenting the sub-tribe Liparinae (*Liparis* and *Malaxis*), which can be considered to be epiphytes. Their underground parts comprise various forms of roots; roots proper, normally in the form of slender, cylindrical, umbranched filaments, whitish or brownish, an underground stoloniferous stem or a rhizome producing aerial stems, or root-tuber (tuberous roots), organs for storing food that allow the growth of a new plant, but which are not true tuber and certainly not bulbs, albeit that these terms are in common use (Figure. 3).

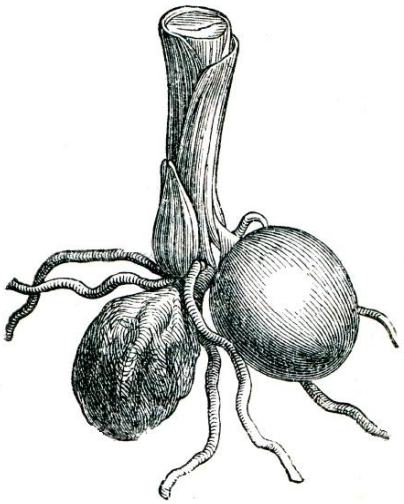

Fig. 3 Tuber and roots

In species with root-tubers, the roots are always placed at its tip. Root- tubers are varied: complete and ovoid (*Orchis*, *Ophrys*), palmate, flattened and long-digitate or spindle-shaped. There are generally 2 tubers on each plant at flowering time; however, certain species have 3 or more, often attached to the stem by a long underground stalk (*Serapias lingua*). Between the roots and aerial parts, there is normally a long underground stem, the neck, covered in whitish, brownish, or sometimes purplish scale-leaves.

ORCHID REPRODUCTION AND LIFECYCLE

The life cycle of terrestrial species is closely linked to seasonal changes in temperature and soil moisture conditions (Dixon, 1991). Most terrestrial species commence growth with early winter rains of April to early May (Dixon, 1991). It is at this time that there is a hight amount of organic matter to provide a substrate for the saprophytic fungi that form symbiotic relationship with orchids (Rasmussen, 1995). Nutrients stored in the parent tuber of the orchid are utilized to begin the production of roots and first leaves. Reinfection of the adult plant by a mycorrhiza provides supplementary nutrients for continued leaf growth and production of a replacement tuber (Ramsay et al., 1986; Dixon, 1991; Rasmussen, 1995). The maturation of the mycorrhizome is a slow process and varies greatly between species. In most species it takes around four years before the first leaf is produced, but in others, such as the Burnt Orchid (*Orchis ustulata*) it may take as long as fifteen years (Lang, 1980). The mycorrhizal infection of the developing orchid is at first parasitic, but as the plant matures its dependence on the fungus is reduced. The degree to which the mycorrhizal infection continues once the plant has reached maturity also varies greatly depending on the species in question. Some species, such as the Bee orchid (*Ophrys apifera*), eventually expel the fungus, while others retain the infection. In the most extreme case, the saprophytic species such as the Birds nest orchid (*Neottia nidus-avis*), are entirely dependant on the nutrients they derive from the fungus throughout their lives.

Orchid floral longevity is generally considered to be long in comparison to other plants (Primack, 1985; Clayton and Aizen, 1996). Specific flower opening times are dependent on the species of concern and are likely to be linked to availability of pollinators and pollinations mechanisms. Durations of flower opening varies and flowers may stay open for up to three weeks without a pollination event occurring. Following the successful deposition of pollinaria on the stigma, the pollen tubes grow down the style reaching the ovules a few days later (Rasmussen, 1995). Fertilisation takes places shortly afterwards and the development of the seed proceeds (Rasmussen, 1995). Orchid fruits produce many thousands of seeds (Rasmussen, 1995; Arditti and Ghani, 2000). The production of orchid seed is an immense drain on the plants resources and it is

the low frequency of successful pollination and germination that drives this over-compensatory reproductive strategy (Rasmussen, 1995) (Figure. 4).

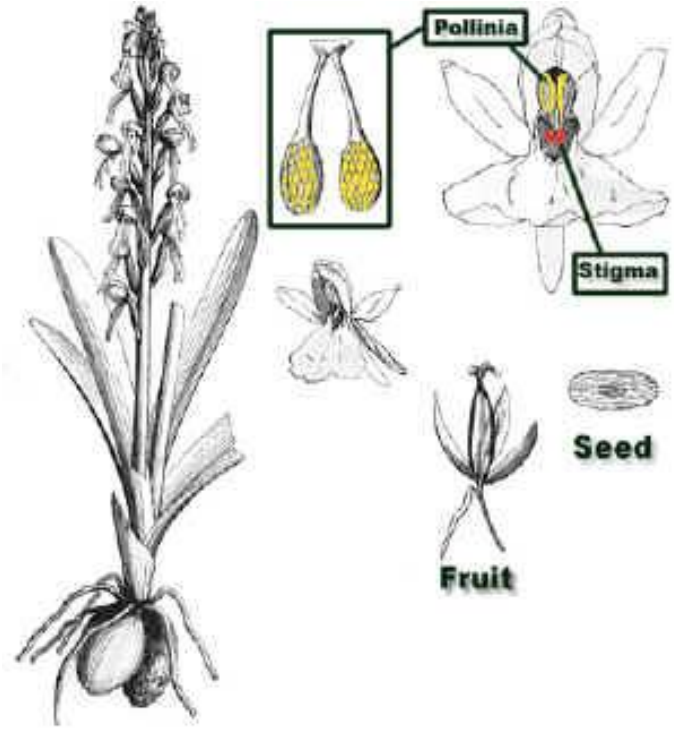

Fig. 4 Seed and fruit

Orchid seeds are among the smallest known seeds in the plant kingdom (Rasmussen, 1995). Terrestrial orchid species typically have very simple seeds consisting of a long, tapering air filled testa characteristic of anemochorous seed (Arditti and Ghani, 2000). The small shape, size and considerable air space within the seed mean that orchid seed can remain in the air for long periods of time , thus aiding long distance dispersal (Arditti and Ghani, 2000). Orchid seeds possess a very small embryo with the majority being devoid of a cotyledon or an endosperm (Arditti and Ghani, 2000). The occurrence of mycotrophy in orchid seed germination is required due to the lack of sustenance contained within the seed (Rasmussen, 1995; Arditti and Ghani, 2000).

POLLINATION

The complex mechanisms which orchids have evolved to achieve cross-pollination were investigated for the first time by Charles Darwin in his book "*Fertilisation of* Orchids" (1862).

Pollinators are often visually attracted by the shape and colours of flowers. In addition, the flowers may produce attractive odours (Dobson 1994). Although absent in most species, nectar may be produced in a spur of the labellum, on the point of the sepals or in the septa of the ovary, the most typical position amongst the Asparagales. In orchids that produce pollinia, pollination happens as some variant of the following. When the pollinator enters into the flower, it touches a viscidium, which promptly sticks to its body, generally on the head, proboscis or abdomen (Figure.5). While leaving the flower, it pulls the pollinium out of the anther, as it is connected to the viscidium by the caudicle or stipe. The caudicle then bends and the pollinium is moved forwards and downwards. When the pollinator enters another flower of the same species, the pollinium has taken such position that it will stick to the stigma of the second flower, just below the rostellum, pollinating it.

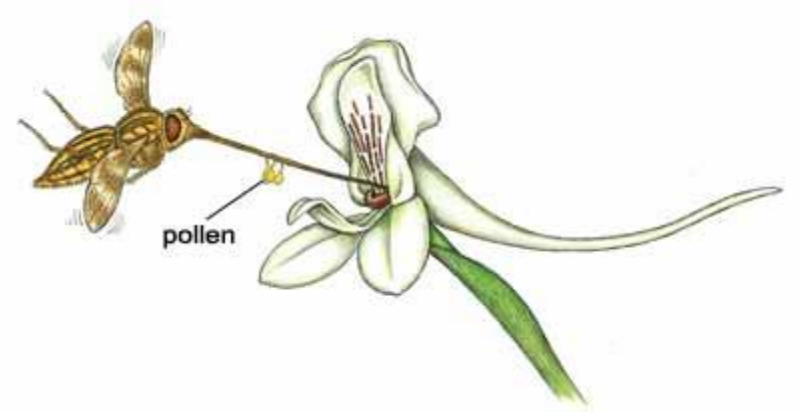

Fig. 5 Pollination

Some orchids mainly or totally rely on self-pollination, especially in colder regions where pollinators are particularly rare. The caudicles may dry up if the flower has not been visited by any pollinator, and the pollinia then fall directly on the stigma (Ren, 2011).

The orchid rewardless implement a deceptive pollination. The mechanisms of deception include generalized food deception, food-deceptive floral mimicry, brood-site imitation, shelter imitation, pseudoantagonism, rendezvous attraction and sexual deception (Jersakova, 2006). In this last case (sexual-deception), the flowers mimic female insect mating signals, especially their pheromones, and are pollinated by the lured male insects, which often try to copulate with the flower. The sexual response ranges from a less advanced stage, in which the orchids deceive pollinators mainly by olfactory cues (Bino, Dafni and Meeuse, 1982; Stoutamire, 1983), towards highly adapted flowers which elicit ' pseudocopulation ' by male insects (Correvon and Pouyanne, 1916; Pouyanne, 1917; Coleman, 1927; Ames, 1937; Kullenberg, 1961; Priesner, 1973; Kullenberg and Bergström, 1973, 1976b; Vogel, 1976; Kullenberg, Borg-Karlson and Kullenberg, 1984; Vöth, 1984; Paulus and Gack, 1990; Peakall and Beattie, 1996; Ayasse et al., 2000, 2003; Schiestl and Ayasse, 2002; Schiestl et al., 1999, 2000, 2003). Roy and Widmer (1999) and Schiestl (2005) extend the concept of Batesian mimicry in plants to cover not only food-deceptive floral mimicry (see above), but also floral mimicry of insects (sexual deception), on the basis that deceptive mimics in both systems should experience negative frequency-dependent pollination success. Dressler (1981) suggested that rendezvous attraction might have been the first step in evolution towards pseudocopulation. This would be followed by a stage in which flowers emit signals releasing at least certain phases of the male sexual behaviour (Bergström, 1978). This step is represented in the East Mediterranean species *Orchis galilaea*, which is pollinated exclusively by males of *Lasiglossum marginatum* (syn. Halictus marginatus), while females visit the flowers of other plant families (Bino et al., 1982). The behavior of the males landing on dark spots on the labellum suggests that the strong, musk-like scent of the flowers is similar to that of the pheromone of the females. This intermediate state also appears in the South Australian species Caladenia patersonii pollinated by tiphiid males (Stoutamire, 1983). However, sexual deceit in this species appears to be mixed with generalized food deception, as the flowers are pollinated also by other insects of both sexes, including bees and syrphid flies searching for food (Stoutamire, 1983). Orchid flowers that elicit ' pseudocopulation ' by male insects possess not only sex-pheromone-like

odours, but also visual and tactile cues (Bergström, 1978). The odour plays a key role in the long-range attraction of males to the flower (Kullenberg, 1961; Peakall, 1990; Schiestl et al., 1999). During pseudocopulation the pollinia become attached to the male's head or abdomen and are transferred to a flower of another plant during the next copulation attempt (Borg-Karlson, 1990). The pheromone-like odour of orchids is often even more attractive for male insects than that of their own females, but males can learn to avoid areas containing orchids or females can increase their attractiveness by walking away from the orchid colony (Wong and Schiestl, 2002; Wong, Salzmann and Schiestl, 2004). Sexual deception imposes strong specialisation in orchids as insect pheromones are generally highly species specific (Paulus and Gack, 1990). The specialisation ranges from species that lure few pollinator taxa (Paulus and Gack, 1990; Schiestl et al., 1999, 2000) to species pollinated exclusively by one pollinator (Schiestl et al., 2003; Schiestl, Peakall and Mant, 2004). True sexual deception is found only in the orchid family, although exploitation of mate-seeking behaviour through petal ornamentation that resembles insects has been reported in plants belonging to other families (Johnson and Midgley, 1997; Johnson and Dafni, 1998). Unrelated orchid genera that exploit mating behaviour of pollinators by mimicking attraction cues of female insects evolved independently in Europe, Australia, Africa and South America. Pseudocopulation is found in Europe only in the genus Ophrys (Kullenberg, 1961; Paulus and Gack, 1990; Schiestl et al., 1999), while in southern Australia at least ten orchid genera (Coleman, 1928; Stoutamire, 1975, 1983; Peakall, Beattie and James, 1987; Peakall, 1990; Dafni and Bernhardt, 1990; Bower, 1996; Schiestl et al., 2004), in South America five genera (van der Pijl and Dodson, 1966; Dod, 1976; Singer, 2002; Singer et al., 2004), and the Central American genus *Lepanthes* (Blanco and Barboza, 2001, 2005) are involved in sexual deception. Sexual deception has also been reported in two African Disa species (Steiner, Whitehead and Johnson, 1994). *Ophrys* L. is a genus of sexually deceptive orchids, which mainly occurs in the Mediterranean area. In *Ophrys*, the labellum is adapted to have a colour, shape and odour which attracts male insects via mimicry of a receptive female. In this pollination system, floral odor is the key factor for specific pollinator attraction (Schiestl et al. 1999, 2003; Mant et al. 2005a,b; Peakall et al. 2010). One of the major

characteristics of sexual deception is its high specificity, with each species of *Ophrys* only attracting one or very few species of male insects as pollinator(s) (Paulus and Gack 1990b). Therefore, different *Ophrys* species, which are mostly genetically compatible and crossable, are potentially isolated from each other due to ethological floral isolation, that is, the nonsharing of pollinator species (Ehrendorfer 1980; Paulus and Gack 1990b; Schiestl and Ayasse 2002; Scopece et al. 2007; Schiestl and Schlüter 2009).

Pollination happens as the insect attempts to mate with flowers. Many neotropical orchids are pollinated by male orchid bees, which visit the flowers to gather volatile chemicals they require to synthesize pheromonal attractants. Each type of orchid places the pollinia on a different body part of a different species of bee, so as to enforce proper cross-pollination. An underground orchid in Australia, *Rhizanthella slateri*, is never exposed to light, and depends on ants and other terrestrial insects to pollinate it. *Catasetum*, a genus discussed briefly by Darwin, actually launches its viscid pollinia with explosive force when an insect touches a seta, knocking the pollinator off the flower. After pollination, the sepals and petals fade and wilt, but they usually remain attached to the ovary.

ORCHID HYBRIDIZATION AND REPRODUCTIVE BARRIERS

In Mediterranean food deceptive orchids, hybridization is a common phenomenon, as a natural consequence of their unspecific pollination system (Cozzolino et al., 2006). In contrast to tropical orchids, surprisingly high levels of natural hybridization have been documented among orchid species and genera of the Mediterranean region (over 200 records, see Willing and Willing, 1977 and Willing and Willing, 1985). This exceptional number of reported natural hybrids (almost all species appear to be intercrossable with each other) contrasts sharply with the widespread perception of a highly specialized pollination biology in orchids. This loss of specificity seems to be the consequence of the evolution of deceptive pollination mechanism in many Mediterranean orchids (Dafni, 1984). Whenever parental species and hybrid co-occur and bloom during overlapping periods, they may share common pollinators and similar soil preferences, i.e., biotic and abiotic factors (Arnold, 1997; Waser, 2001; Mallet, 2005; Cozzolino et al., 2006). Flowering plants possess various reproductive isolation mechanisms, acting before or after pollination or even in combination (Cozzolino et al., 2004; Moccia et al., 2007; Raguso, 2008; Stökl et al., 2008), which limit hybridization. For example, divergence in floral traits (different pollination syndromes) leads to attraction of different pollinators and hence to reproductive isolation between species such as *Iris* spp. (Hodges et al., 1996), *Penstemon* spp. (Castellanos et al., 2004), *Mimulus* spp. (Ramsey et al., 2003), and numerous orchid species (van der Cingel, 1995; Cozzolino et al., 2004; Moccia et al., 2007; Stökl et al., 2008). Most orchids emit characteristic bouquets of volatile compounds, widely varying among species in their composition. Each orchid species has a restricted range of pollinators as result of floral morphology and scent (van der Cingel, 1995; Stökl et al., 2008), a specificity that contributes to premating isolating mechanisms between co-occurring orchid species (van der Cingel, 1995; Waser, 2001; Cozzolino et al., 2004; Scopece et al., 2007). In the case of most European orchids, extensive observations over several decades have identified confirmed pollinators, i.e., insects acting efficiently as pollen vectors (van der Cingel, 1995; Schatz, 2006). Although orchids often exhibit strong ecological isolation for pollination (van der Cingel, 1995; Cozzolino et al., 2004), hybrids

are frequent (Cozzolino and Widmer, 2005). Their frequent occurrence in sympatry with parental species suggests that the latter can share pollen vectors (Schatz, 2006). More generally, barriers preventing cross-pollination in orchids are not completely effective (Dafni, 1987; van der Cingel, 1995; Schatz, 2006), so that prezygotic isolation is not absolute, e.g., in the Mediterranean species from the genus *Orchis* (van der Cingel, 1995; Aceto et al., 1999; Cozzolino and Widmer, 2005; Schatz, 2006). It has been suggested that these frequent hybridization events may also play a relevant role in Mediterranean orchid speciation and may provide clues to evolution within this orchid group (Ehrendorfer, 1980; Van der Pijl and Dodson, 1966). If this is true, it would imply that the significance of hybridization for conservation issues may be very different in orchids compared to many other taxa, and that hybridization may represent an advantage rather than a threat to biodiversity in this particular case. Consequently, specific conservation strategies should be designed to protect hybrid populations and individuals in order to maintain them as invaluable sources of heritable variation for future evolution. On the contrary, if hybrid plants do not represent a first step in orchid speciation but simply a natural outcome of their peculiar pollination biology, then conservation priorities should focus mostly on the parental species instead of hybrids. Of course, assessing the conservation status of hybrid specimens should also take into account whether hybridization is a rare phenomenon, or whether it represents a renewable (or even repeatable) event that results naturally from sympatric co-occurrence. Despite the large number of hybrid records reported for Mediterranean orchids, only a relatively small number of studies have analyzed orchidhybridzones in detail. In particular, only few studies have investigated hybrid populations rather than single hybrid specimens to provide information on the genetic architecture of orchidhybridzones, on the fate of hybrid generations and on levels of genetic introgression between parental taxa (Arduino et al., 1996, Pellegrino et al., 2000 and Pellegrino et al., 2005). These molecular studies have revealed that the majority of hybrid individuals were F1's but did not address the question of the ecological impact of hybridization on parental taxa and its consequences for their conservation management. In fact, hybridization may represent a threat for

parental taxa not only by promoting genetic mixing but also by reducing their fitness through costly reproductive efforts (Arnold, 1997).

ORCHID MYCORRHIZAE

Orchidaceae, more than any other plant family, have a high proportion of threatened genera. The persistence of these plants is linked to abiotic and biotic factors that act in a linear sequence of interactions dependent on their level of criticality for growth, development and reproductive success. For example, for most ground orchids, the presence and vitality of mycorrhiza in soil around plants have a more immediate impact on plant persistence than other factors. The great taxonomic diversity of Orchidaceae is often attributed to their specialization to particular habitats, pollinators and mycorrhizal associations (Swarts and Dixon, 2009). The evolution of orchid mycorrhizae is linked to extreme specialization, since orchids plants produce an abundant number of microscopic seeds, with limited storage materials, for dispersal into specialized habitats in different environments. These associations have hyphal coils in host cells with very few morphological signs, which renders hard to assess whether the fungi are specialized root inhabitants or plain invaders, in contrast with AM (arbuscular mycorrhizae) and ECM (Ectomycorrhizae) which display the host-fungus interface with highly specialized hyphae (Brundrett, 2002) (Figure. 6).

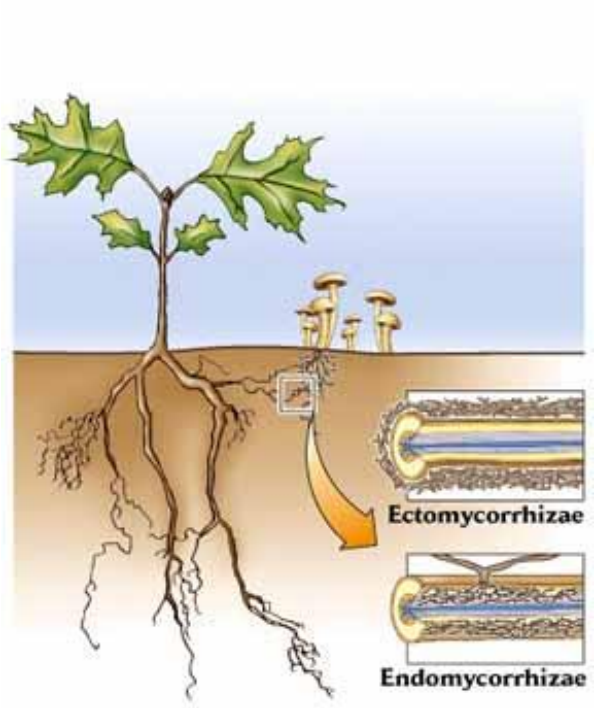

Fig. 6 Rappresentation of ecto- and endomycorrhizae

Orchid seeds are very small with minimal nutrient reserve, therefore upon germination, fungal hyphae promptly penetrate the cell walls of the orchid and form characteristic coils, called pelotons, within the cells. Growth of the fungus is restricted to cortical cells, probably by the deposition of phenolic compounds and the production of anti-fungal substances (Shimura et al., 2007). Differently from other mycorrhizas pelotons are subsequently “digested", and through this process the orchid is thought to receive the essential nutrients and carbon needed to grow. Nutrient exchange may also, or instead, occur across intact cell membranes prior to “digested" as in other intracellular mycorrhizas.

The germinated seed develops into a mass of differentiated cells called protocorm, and remains in this form for a period that can extend up to several years, until leaves are produced. During this period of their life, many orchids are underground and rather than producing organic carbon through photosynthesis, they obtain all of their energy from fungal pelotons. Therefore, before the production of leaves, all orchids go through a stage of their life-cycle in which they are mycoheterotrophs, rather than autotrophs. Most adult orchids have a poorly developed root system, therefore they retain their mycorrhizal partnerships because they are still heavily reliant on mycorrhizal fungi for mineral nutrition (Waterman and Bidartondo, 2008). In contrast to other mycorrhizal symbioses, it has long been thought that orchid mycorrhizal fungi receive few benefits from the interaction. Indeed orchid mycorrhizae have historically been depicted as anomalous associations in which nutrient flux was plantoriented (Leake, 1994). However a recent study demonstrated bi-directional movement of carbon between adult Goodyeara repens and its fungal partner (Cameron et al., 2006, 2007). Orchidaceae have species with different levels of dependence on mycorrhizae, extending from fully auotrophic to fully-heterotrophyc associations. Generally all orchids need fungi to provide inorganic and organic nutrients for seed germination and early protocorm development. In addition in adult photosintetic orchids, N, P and water continue to flow from the fungal partner, but carbon exchange is essentially reversed with photosynthate providing incentive for continued fungal colonization (Dearnaley, 2007). Most orchid mycorrizal fungi belong to the Rhizoctonia group, a diverse polyphyletic group including plant pathogens, endophytes, saprophytes and

mycorrhizal fungi. There are also several exceptions as shown in both achlorophyllus and photosynthetically-active specimens of Epipactis which have a mycorrhizal interaction with fungi from the treeectomycorrhizal ascomycetes genus Tuber (Selosse et al. 2004) or Neottia nidus-avis which is specialized on fungi in the Sebacinaceae known to be ectomycorrhizal with trees (McKendrick et al., 2002; Selosse et al., 2002). Corallorhiza maculata and C.mertensiana, are instead specialized on ectomycorrhizal members of the Russulaceae (Taylor and Bruns, 1999; Taylor et al., 2004). In addition, other non-photosynthetic orchids specialize on free-living non-rhizoctonia fungi (Ogura-Tsujita and Yukawa, 2008). Some evidences indicate also that fungal partners may switch during the life of the orchid, so that the fungal-orchid association appears sensitive to enviromental stimuli and can possibly adjust to favor survival of the plant partner.

The identification of orchid mycorrhizal fungi is a critical step in exploring the biology of this symbiosis, considering that fungal isolation from orchids is not always easy, isolation success in many orchid varies with season and in some cases simbionts are difficult or impossible to isolate. Electron microscopy examination of septal ultrastructures can not allow to recognize fungal species, but molecular methods based on fungal-specific PCR amplification of the nuclear ribosomial internal transcribed spacer (ITS) are helpful to overcome the problems associated with limited morphological variation and inefficient culturing (Taylor and McCormick 2008).

PURPOSE

In the present PhD thesis, I present genetic and ecological data from three study cases of hybrid zones between food-deceptive Mediterranean orchid species in Italy. Our aim was to investigate the role of hybridization in orchid evolutionary processes.

The first study “1-*Orchis xcolemanii* hybridization: Molecular and morphological evidence, seed set success, and evolutionary importance” was focalizated on two food-deceptive species *Orchis mascula* and *Orchis pauciflora* and their hybrid, *O. xcolemanii*. Here, i have performed molecular analysis and hand pollination treatments to characterize a hybrid zone between these orchids.

The second study “2-Interactions with symbionts in a hybrid Mediterranean orchid.” addressed, with molecular analyses, a sympatric zone between *Orchis italica* Poir. and *O. anthropophora* L., and their hybrid *O. xbivonae* Tod. The main purpose was to compare the identity of mycorrhizal associates in two parental species and hybrids at the adult stage to determine if lack of appropriate fungal symbionts can be related to hybrid viability, and to verify if mycorrhizal fungi allow the hybrid to exploit new ecological niches different from parental habitat.

In the third one, “3-Pollen competition as a reproductive isolating mechanism between two sympatric *Orchis* species” I have examined whether conspecific pollen advantage (pollen competition) occurs in two interfertile species of *Orchis*, *Orchis italica* Poir. and *O. anthropophora* L. using different time of conspecific and heterospecific pollen arrival on stigma.

1-*Orchis xcolemanii* hybridization: Molecular and morphological evidence, seed set success, and evolutionary importance

INTRODUCTION

Hybridization is a major mechanism in plant evolution (Waser, 2001; Hegarty and Hiscock, 2005). A significant fraction of flowering plants are of hybrid origin (Ellstrand et al., 1996; Rieseberg et al., 1999), and at least a quarter of plant species are involved in hybridization and potential introgression with other species (Mallet, 2005). The most common mechanism of plant speciation through hybridization is allopolyploidy (Soltis and Soltis, 1999), however, there is strong empirical evidence that hybridization can also give rise to new species without a change in ploidy level (‘‘homoploid hybrid speciation’’) (Rieseberg et al., 1995; Arnold, 1997; Ungerer et al., 1998; Wolfe et al., 1998; Buerkle et al., 2000). This has strengthened the view that hybridization is not merely a kind of ‘‘evolutionary noise’’ with little evolutionary significance (Mayr, 1992), but may instead sometimes play a positive role in evolution, either through hybrid speciation, or through the origin and transfer of novel adaptations (Arnold, 1997; Paialek and Barton, 1997; Rieseberg and Carney, 1998). This creative nature of hybridization, stressed particularly by plant evolutionary biologists, contrasts sharply with the negative general perception of the role of hybridization in conservation biology (Wolf et al., 2001). Indeed, natural hybridization is typically considered deleterious for the conservation of biodiversity. Interspecific gene flow is often seen as a hazard in plant conservation genetics, especially when rare species come in contact and hybridize with more common and widespread related taxa as a consequence of habitat disturbance (Ellstrand and Schierenbeck, 2000; Ferdy and Austerlitz, 2002). In this scenario, hybridization may lead to the loss of rare taxa as a consequence of outbreeding depression and genetic assimilation (Allendorf et al., 2001; Arnold, 1997 and references therein). Accordingly, current conservation laws (such as the Endangered Species Act in the USA) tend to disregard hybrids, hybrid zones and hybridizing

species (Allendorf et al., 2001 and reference therein). In the light of this overemphasis on the negative consequences of hybridization, it is important to remember that hybridization does not always occur subsequent to human mediated habitat disturbance, and that it need not necessarily involve rare and threatened taxa. Hybridization may also result when previously isolated, allopatric taxa meet upon secondary contact in the course of natural range expansion (Millar, 1993; Hewitt, 2001). Under these circumstances, hybridization may also open entirely novel evolutionary trajectories, e.g. recombination of genetic material in hybrids may result in hybrid genotypes able to occupy novel environments (Barton, 2001; Lexer et al., 2003a,b; Rieseberg et al., 2003). As one of the most species-rich plant families, Orchidaceae display a large variety of pollination systems and extraordinarily high levels of interspecific diversity in associated floral traits. This phenotypic variability is thought to have arisen as a result of natural selection by pollinators – in orchids, pollinator specificity acts as the main ethological mechanism of premating reproductive isolation. Indeed, the observation that laboratory crosses are possible among many orchid species, including taxa with moderate degrees of phylogenetic relatedness, indicates a prominent role for pollinator specificity in maintaining species boundaries in the face of weak post-mating barriers (Darwin, 1862; Van der Pijl and Dodson, 1966). In contrast to tropical orchids, surprisingly high levels of natural hybridization have been documented among orchid species and genera of the Mediterranean region (over 200 records, see Willing and Willing, 1977; Willing and Willing, 1985). This exceptional number of reported natural hybrids (almost all species appear to be intercrossable with each other) contrasts sharply with the widespread perception of a highly specialized pollination biology in orchids. In recognition of the well-known role of plant hybridization in plant species formation (above), and in accordance with the large number of reported orchid hybrids, it has been suggested that these frequent hybridization events may also play a relevant role in Mediterranean orchid speciation and may provide clues to evolution within this orchid group (Ehrendorfer, 1980; Van der Pijl and Dodson, 1966). If this is true, it would imply that the significance of hybridization for conservation issues may be very different in orchids compared to many other taxa, and that hybridization may represent an advantage rather than a threat to biodiversity in

this particular case. Consequently, specific conservation strategies should be designed to protect hybrid populations and individuals in order to maintain them as invaluable sources of heritable variation for future evolution. On the contrary, if hybrid plants do not represent a first step in orchid speciation but simply a natural outcome of their peculiar pollination biology, then conservation priorities should focus mostly on the parental species instead of hybrids. Of course, assessing the conservation status of hybrid specimens should also take into account whether hybridization is a rare phenomenon, or whether it represents a renewable (or even repeatable) event that results naturally from sympatric co-occurrence.

Because of the peculiar eco-geographical heterogeneity of Mediterranean region, distribution areas of orchids are often overlapping or intermingled. In addition, most of deceptive orchids have similar ecological needs, so that several species may settle in the same habitat, bloom in the same period and share the same pollinator fauna (Dafni, 1984). For these reasons, hybrid zones of Mediterranean orchids are scattered across their overlapping ranges, and are usually narrow, with a variable number of both parental species and hybrid individuals.

At present, relatively few molecular studies have been carried out on hybrid zones of Mediterranean deceptive orchids. Recently, it has been assessed, with nrDNA sequences and AFLP markers, that a hybrid swarm of the food-deceptive species *Anacamptis morio* and *A. papilionacea* consisted of only F_1 hybrids, suggesting a their role as post-mating reproductive barrier (Moccia et al., 2007). Conversely, an extensive introgressive hybridization has been revealed by an AFLP analysis of a hybrid zone between *Ophrys lupercalis* and *O. iricolor*, suggesting a clear signal of low floral isolation (Stökl et al., 2008). These findings are consistent with those obtained by a large-scale experimental crosses, which has pointed out that speciation in Mediterranean food-deceptive orchids has been driven by the insurgence of post-mating barriers, whereas sexually deceptive species have evolved pre-mating barriers (Scopece et al., 2007). These molecular studies have revealed that the majority of hybrid individuals were F1's but did not address the question of the ecological impact of hybridization on parental taxa and its consequences for their conservation management. In fact, hybridization may represent a threat for parental taxa not

only by promoting genetic mixing but also by reducing their fitness through costly reproductive efforts (Arnold, 1997).

The goal of the present study was to characterize the genetic structure of a hybrid zone between *Orchis mascula* and *O. pauciflora* using a combination of nuclear ribosomal DNA (nrDNA) and chloroplast DNA (cpDNA) and to obtain information on reproductive biology of examined taxa evaluating fruit and seed production.

We used nrDNA analysis to identify and assign each presumed hybrid individuals to a specific hybrid class (F_1, F_2 or backcrosses) and cpDNA markers to assess maternal lineage of hybrids. Reproductive success was evaluated on plants left in natural conditions, whereas reproductive success of any possible bidirectional cross combinations between hybrid plants and both parental species was checked by hand pollination. Finally, levels of seed viability was established by measuring the percentage of seeds with embryo. Findings are discussed in light of the current hypothesis on the biological significance and/or evolutionary potential trajectories of plant hybrid zones.

MATERIALS AND METHODS

STUDY AREA AND ORCHID SPECIES STUDIED

The study site is a mixed settlement of several food-deceptive orchid species, occurring on poor calcareous soils at 1400 m above sea level, on the southern slope of Mount “Manfriana” (Pollino National Park, Calabria region, southern Italy). (Figure. 7)

Fig. 7 Mount “Manfriana”

In this site, there is a relatively large population of *O. mascula* L. (Figure. 8), *Orchis pauciflora* Tenore (Figure. 9), and their hybrid progeny, known as *O. xcolemanii* Cortesi, while other orchid species, *O. quadripunctata* Cyrillo ex Tenore and *Dactylorhiza sambucina* (L.) Soò, co-occur in a lower density.

Fig. 8 *Orchis mascula* (L.)

Orchis pauciflora and *O. mascula* have an identical chromosome number (2n=42) (D'Emerico et al., 2002) and resulted to be phylogenetically closely related, indeed *O. mascula* is included in the yellow-flowered *O. pauciflora* group separated from the other purple-flowered species (Bateman et al., 2003).

Fig. 9 *Orchis pauciflora* Ten.

Orchis pauciflora, O. mascula have similar flower morphology (convex trilobite lip, median lobe longer than lateral lobes, cylindrical, horizontal to ascendant spur without nectar) but the former has 2–8 (-15) yellow flowers and is 10–30 cm tall while the second has 15–50 red-purple flowers and is 20–60 cm tall (Delforge, 2005). They show a different distribution area, indeed *O. mascula* is a widespread species occurring on the European continent from the Canaries islands to Anatolia, from North Africa to Scandianavian peninsula, *Orchis pauciflora* is a narrow species with central and eastern Mediterranean distribution, along Apennine and balcanic peninsula up to Greek islands and Crete (Figure. 10).

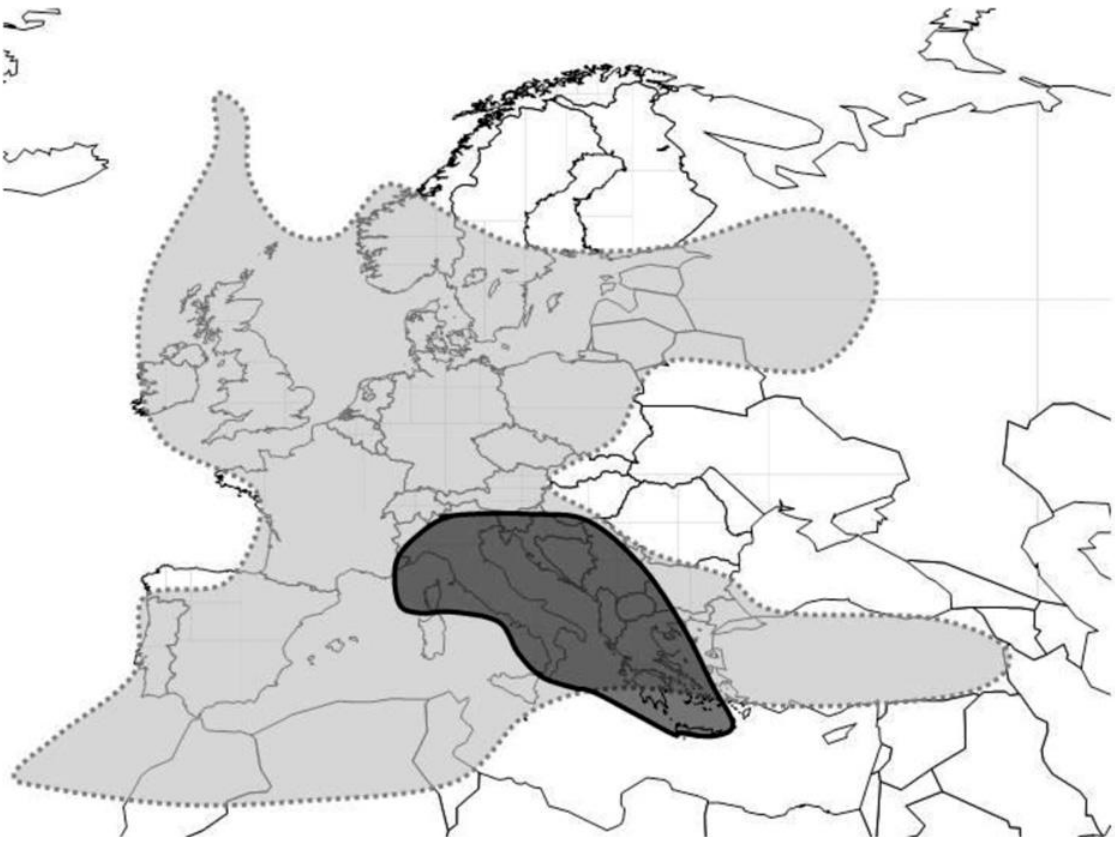

Fig. 10. Distribution area of Orchis mascula and O. pauciflora. Gray shadow and dotted circumference show the distribution area of widespread O. mascula, occurring from the Canaries islands to Anatolia, from North Africa to Scandinavian peninsula; dark shadow and black line indicate distribution area of narrowly distributed O. pauciflora, occurring along the Apennine and Balkan peninsula up to the Aegaean islands and Crete.

Their hybrid, *O. xcolemanii* (Figure. 11), can be morphologically variable, in term of habit, outer tepal shape, spur size and, in particular, flower color. *O. xcolemanii* specimens show flower color polymorphism, in fact its flower color ranges from yellowish to crimson-red to purplish (Del Prete and Miceli, 1981; Nazzaro et al., 1995).

Fig. 11 ***Orchis x colemanii*** **Cortesi**

These orchids are non-model mimic plants that exploit nectar-seeking bumblebee queens or solitary bees by providing general floral signals (Nilsson, 1983; Cozzolino et al., 2005), and by producing scent bouquets (Salzmann et al., 2007). The pollination biology of *O. mascula* has been extensively studied in the Sweden part of its distribution area by Nilsson (1983) who found that it was mainly pollinated by naïve *Bombus* queens, *Psithyrus* females and solitary bees of the genera *Eucera*, *Andrena* and *Osmia* searching for nectar during their first exploratory forays after hibernation. Recently, it has been reported that in Crete

island insects belonging to the genera *Apis* and *Bombus* were the most frequent pollinators and among *Bombus* only queens were observed to pollinate *O. pauciflora* (Valterová et al., 2007).

PHENOTYPIC TRAIT MEASUREMENTS

At the top of the blooming season we measured phenotypic traits on the second and third flowers from the bottom of the inflorescence of 15 individuals of each taxa, and used the average values from these two flowers in statistical analyses. Floral traits were measured to the nearest 1 mm using a digital caliper and were replicated on both collected flowers. Flower number was evaluated as the total number of opened flowers. Plant height was the distance from the ground to the top of the highest opened flower. Spur length was the distance between the spur mouth and the spur tip. Labellum length was the distance between the labellum tip and the spur mouth. Labellum width was the distance between the edges of the two lateral lobes.

Labellum anthocyanin concentration (purple pigment) was estimated extracting the anthocyanins with 0.5-ml methanol/0.1% HCl, and determining the absorbance at 510 nm. Labellum carotenoid concentration (yellow pigment) was estimated similarly, usingmethylene chloride for extraction and measuring absorbance at 450 nm (Bradshaw et al. 1998). The data matrix was analysed with Data Desk 6.3 (Velleman, 1997) and SPSS 14.0 (Norušis, 2005).

MOLECULAR ANALYSIS

In the last decade different molecular approaches have been applied in orchid hybrid studies in order to assess their taxonomic position, parental lineage and gene flow between parental species (Arduino et al., 1996; Pellegrino et al., 2005; Stökl et al., 2008).

To characterize the genetic structure of the hybrid zone, we have applied Internal Transcribed Spacers (ITSs) of nuclear ribosomal DNA (nrDNA), a powerful tool in investigating the occurrence and extent of hybridization and introgression (Rieseberg and Carney, 1998; Pellegrino et al., 2001), and

chloroplast DNA (cpDNA), since its strictly maternal inheritance in orchids (Cafasso et al., 2005).

One leaf of 46 plants of *O. xcolemanii*, 15 of *O. mascula*, 15 of *O. pauciflora* and three of the two other co-occurring orchid species were sampled and stored in silica gel. Genomic DNA was extracted using a slight modification of (cetyltrimethyl ammonium bromide) CTAB protocol of Doyle and Doyle (1987). Approx. 0.5 g of each leaf were separately pestled in a 2ml-eppendorf using 500 μl of standard CTAB buffer, incubated at 60°C for 30 min, extracted twice adding 500 μL chloroform-isoamyl alcohol (24:1), precipitated with isopropanol and washed with 250 μL of ethanol 70%. DNA was resuspended in 50 μL of distillated water.

The nuclear ribosomal internal transcribed spacers (ITS1 and ITS2) and the chloroplast non-coding spacer *psb*K-*psb*I were amplified by polymerase chain reaction (PCR) using universal pairs of primers as described in Pellegrino (2001) and in Chase et al. (2007) respectively.

PCRs were carried out in a total reaction volume of 100 μL, containing approx. 10-20 ng of DNA, 100 μL of reaction buffer1X, 2mM $MgCl_2$, 100 mM of each dNTP, and 2.5 Units of BioTaq^{TM} DNA Polymerase (Bioline Inc., Boston, MA, USA), and 0.2 mM of each primer (MWG-Biotech AG, Ebersberg, Germany), The thermocycling profile consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles with 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. PCRs were performed on a PTC-100 Thermal Cycler (MJ Research Inc., Watertown, MA, USA). PCR fragments were purified by QIAquick PCR purification kit (Qiagen S.p.A., Milan, Italy) to remove unincorporated primers and dNTPs. Amplification products were electrophoretically separated on a 2% agarose gel (Methaphore, FMS), compared to a 100 base pair (bp) ladder (Pharmacia Biotech) as the molecular weight marker, stained with ethidium bromide and photographed using a Kodak digital camera.

Plastidial and nuclear amplified fragments of three individuals for each parental species and the other two sympatric orchids were sequenced in both directions using a modification of the Sanger dideoxy method as implemented in a double stranded DNA cycle sequencing system with fluorescent dyes. Sequence reactions were then run on a 373A Applied Biosystems Automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Nuclear sequences were examined using GeneJockey to find a restriction site that would distinguish them using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). This approach allows the examination of a heterozygous individual (e.g., a hybrid) without the necessity of cloning and subsequently sequencing several ITS clones (heterozygous individuals give overlapping traces from direct sequencing that are often difficult to interpret).

Restriction enzyme *Taq*I, which cuts at 5'-TC/GA-3', differentiated the putative parental taxa due to the presence of a C/T substitution about 24 base (TCGA in *O. pauciflora*, CCGA in *O. mascula*) pairs into the ITS 2 sequence; while *Sma*I, which cuts at 5'-CCC/GGG-3', showed a nucleotide substitution A/G, about 195 base pairs (CCCGAG in *O. pauciflora*, CCCGGG in *O. mascula*) into the ITS 2 sequences. Sequences of other sympatric orchids, *O. quadripunctata and D. sambucina*, did not show these restriction sites.

Thus, the PCR fragments of all samples (100 ng) were digested in a final 20μL volume with the selected restriction endonuclease (1U/μg DNA), according to the manufacturer's instructions (Fermentas), in particular incubated for three hours at 30°C for *Sma*I and 65°C for *Taq*I. The fragments were electrophoretically separated on a 3% low melting agarose gel (Methaphore, FMS), compared to a 100 base pair (bp) ladder (Pharmacia Biotech) as the molecular weight marker, stained with ethidium bromide and photographed using a Kodak digital camera. The relative amounts of DNA were estimated on digital photos analyzing them with the Biomax 10 image analysis software (Kodak Digital Science, EDAS, USA).

One flowers from each individual were collected and photographed using a Kodak digital camera. Images (16 bit) of flowers were converted in grayscale with 32768 gray levels from 0 (black) to 32768 (white) using Adobe Photoshop CS4. The integrated density was calculated as the sum of the gray values of pixel for each labellum, equals the product of area (in pixels) and average value of gray .

POLLEN TRANSFER

To ascertain if fruit developed by hybrid plants could have been produced by pollen transferred by different donors, that is from hybrid plants and/or from parental species, we marked 5 individuals *O. xcolemanii* and left them in natural condition. The ITS-containing fragments profiles were assessed for each plant following to the protocol described above.

In June, capsule were collected and seeds of the central part were used for molecular analysis. Seeds were observed under a binocular microscope, and approx 50 viable seeds (my means seed with embryo) for each capsule were collected and transferred into single 2ml-eppendorf to extract DNA. Ribosomal DNA were amplified and the PCR fragments of all samples were digested using *Sma*I and *Taq*I, electrophoretically separated and photographed following to the protocol described above.

REPRODUCTIVE SUCCESS AND HAND POLLINATION

In accordance with our goals, we performed field experimental crosses to gain information on the existence of pre- and postzygotic barriers, as hybrid sterility or fertility selection (Wolf et al., 2001; Lau et al., 2005), and on the reproductive success of parental species and hybrids.

To test natural reproductive success we marked at the beginning of flowering period 20 plants of *O. xcolemanii* and 50 plants of each parental species and left them in natural condition.

Hand pollination treatments were conducted to evaluate the levels of reproductive fitness reached by any bi-directional possible mating between parental species and hybrids. To this end, 10 plants of *O. xcolemanii* and of each parental species with unopened flowers were bagged with a fine-meshed cloth to exclude pollinators. For hand-pollination, the cover was removed and five randomly selected flowers on each plant were marked with cotton threat and manually pollinated using a toothpick with the pollen of the same (intrataxon crosses) and of the other taxon (intertaxon crosses). After treatments, plants were bagged again to prevent any further natural pollination

or predation. In addition, two flowers on each plant were covered without manipulation to test for spontaneous autogamy.

In June, the number of produced fruits was counted for both spontaneous and experimental crosses, and the ratio between the number of fruit/flowers was determined. Ripe fruits were collected and stored in silica gel in order to prevent their degradation. Capsules were opened longitudinally with a razor blade. To ascertain the presence of viable embryos, at least 1000 seeds for each fruit were removed from the centre of the capsule and observed under an optical microscope with 100 enlargement. Seeds were assigned to two categories (viable and unviable seeds) due to presence or absence of viable embryos.

Fisher exact tests were used to compare the rate of fruit set between the different experiments. The statistical program package SPSS (version 10, SPSS Inc. Chicago, USA) was used.

RESULTS

Orchis xcolemanii SURVEY

The contribution from members of the “Italian Group for the Research on Wild Orchids (GIROS)” was crucial to this work, who signaled us many localities, not reported in the scientific literature, where are occurring *O. xcolemanii* zones. Reports were completed by details on the main features of the site and of orchid settlement. This information has allowed us to ascertain that many narrow hybrid zones are occurring across the entire distribution area of *O. pauciflora*. In general, it has been highlighted that most of hybrid zones are located on the calcareous slopes of the Apennine chain, usually above 1000 m elevation. Interestingly, the co-occurrence of parental species has been ever observed. Few exception have noticed that *O. pauciflora* is mixed with hybrid plants and *O. mascula* occur nearby, within few hundred meters.

PHENOTYPIC TRAIT MEASUREMENTS

Morphological analysis showed that hybrids exhibited phenotypic characters more or less intermediate between the two parental species (Figure. 12). As regards a structure of relevant diagnostic value, the labellum size (width and length) of the hybrid plants (14.58 mm $\pm$ 0.188; 13.34 mm $\pm$ 0.178) was intermediate between *O. mascula* (13.22 mm $\pm$ 0.208; 13.87 mm $\pm$ 0.198) and *O. pauciflora* (15.41 mm $\pm$ 0.235; 13.01 mm $\pm$ 0.169) (Figure. 12D, 12E), as spur lenght in *O. xcolemanii* (16.12 mm $\pm$ 0.342) was intermediate between parental species (13.48 mm $\pm$ 0.428 in *O. mascula* and 19.21 mm $\pm$ 0.318 in *O. pauciflora*) (Figure.12F).

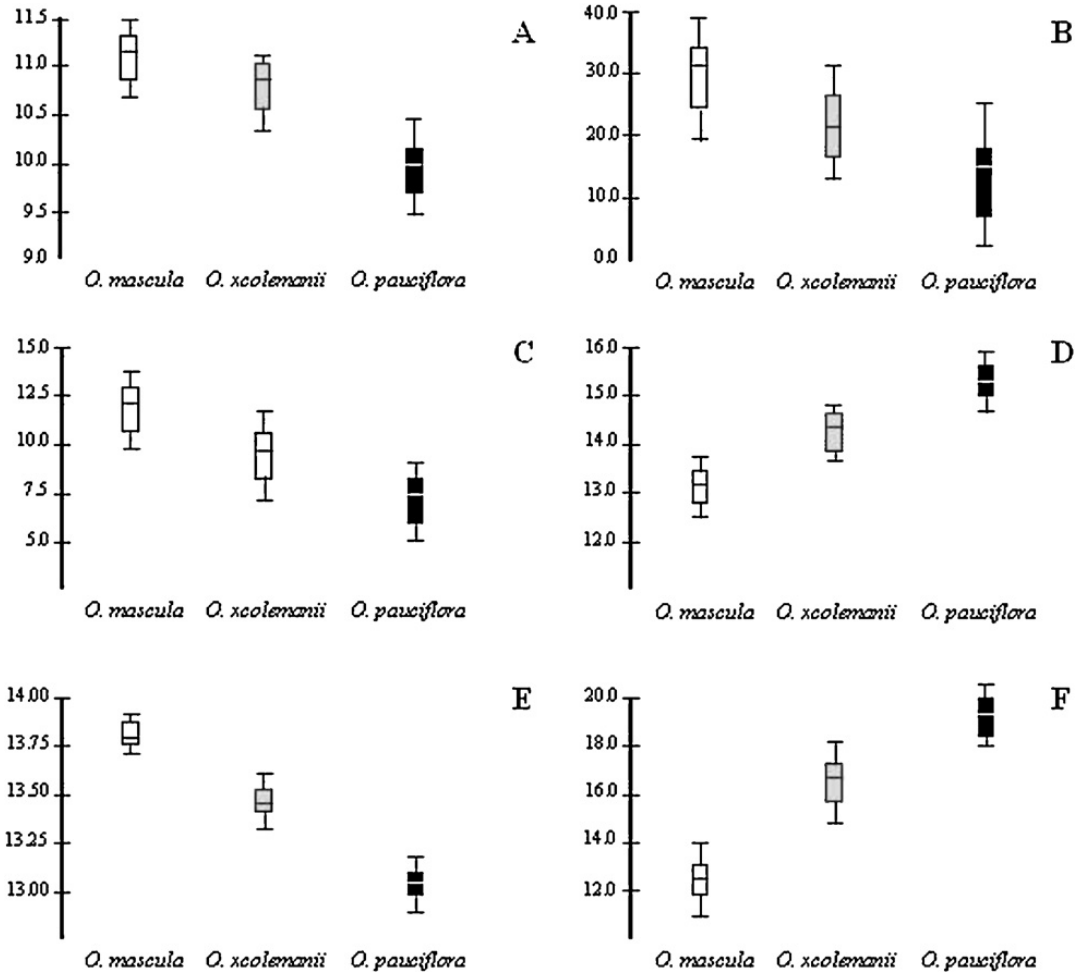

Fig. 12. Morphometric variation among Orchis mascula (white box), O. × colemanii (gray box) and O. pauciflora (black box). (A) flower number; (B) plant height (cm); (C) inflorescence height (cm); (D) labellum width (mm); (E) labellum length (mm) and (F) spur length (mm). The outlined central box depicts the middle 50% of the data extending from upper to lower quartile; the horizontal bar is at the median. Vertical bars indicate standard errors.

In addition *O.* x*colemanii* showed a continuous flower color variation (Figure. 13) ranging from red-purple flowers of *O. mascula* to yellow flowers of *O. pauciflora*. There was hybrids with high value of anthocyan and low value of carotenoid (more similar to *O. mascula* flowers) and hybrids with high value of carotenoid and low value of anthocyan (more similar to *O. pauciflora* flowers) and a lot of hybrids with intermediate concentrations of both pigments (Figure. 13.

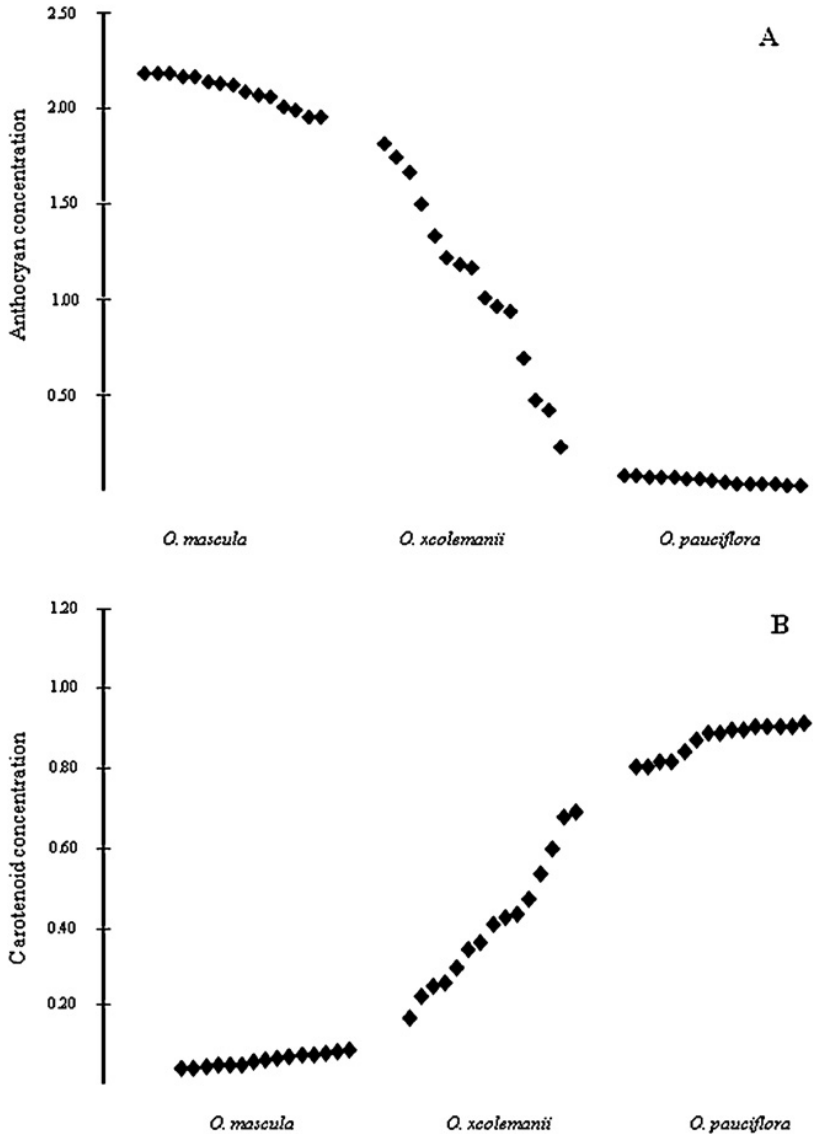

Fig. 13. Comparison of pigments, anthocyan (A) and carotenoid (B) extracted from labellums of *Orchis mascula*, *O. pauciflora* and *O. xcolemanii*.

MOLECULAR ANALYSIS

The ITS-containing fragments obtained from the parental species and hybrids were approximately 280 (ITS 1) and 300 (ITS 2) bp in length. As expected, ITS 2 of *O. pauciflora* and *O. mascula* differ in the presence of different recognition sites for the restriction enzymes *Taq*I and *Sma*I. Indeed, ITS 2-containing fragments digested with *Taq*I showed a single restriction site in *O. pauciflora* (with two fragments approx. 180 bp and 120 bp long) and no site in *O. mascula* (Figure. 14a). The ITS 2-containing fragments digested with *Sma*I showed a single restriction site in *O. mascula* (with two fragments approx. 160bp, and 140 bp long) and no site in *O. pauciflora* (Fig. 14b). All the 46 individuals of *O. xcolemanii* in study exhibited a direct additive inheritance of these profiles, their digested fragments produced the combination of diagnostic profiles obtained for both *O. pauciflora* and *O. mascula* (Figure. 14a,b).

However, the restriction profiles differ in parental band intensity among hybrids. About half of hybrids (24 individuals) displayed a balanced proportion (1:1) of ribosomal DNA of both parental species, while the remaining (22 individuals) showed a higher amount of ribosomal DNA from one parental species than from the other one. In detail, 6 and 11 specimens showed a preponderance (approximately 3:2 to 2:1) of *O. mascula* and *O. pauciflora*, respectively, 3 had a preponderance (approximately 3:2 to 2:1) of *O. mascula* and 2 of *O. pauciflora*. Chloroplast DNA amplification revealed a length polymorphism in the *psb*K. Indeed, the *psb*K amplified fragment of *O. mascula* was approximately 500-bp long while that of *O. pauciflora* was approximately 480-bp long. Thanks to this difference, we established that 19 out of 46 hybrids possessed the *O. pauciflora* plastidial DNA and the remaining 27 that of *O. mascula*. At the same time, we did not find evidence of introgression into the parental species. Also in this case there is no correlation between maternal inheritance and flower color.

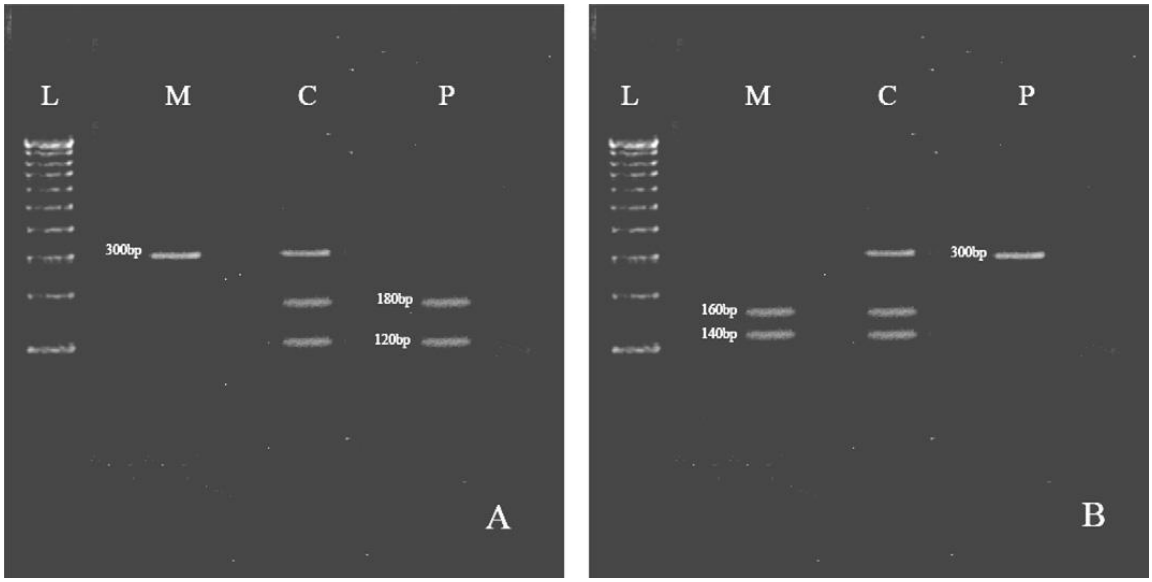

Fig. 14. Additive profile of nrDNA in Orchis ×colemanii and parental species. Gel electrophoresis of ITS 2 TaqI digestions (A) and ITS 2 SmaI digestions (B) showing the presence of characteristic fragments of ITS region of Orchis mascula (lane M), O. ×colemanii (lane C) and O. pauciflora (lane P). Molecular 100 bp ladder (line L).

POLLEN TRANSFER

Three out of five individuals of *O. xcolemanii* marked with plastic threads showed a preponderance of *O. mascula* nrDNA, while two had approximately equal proportion (1:1) of parental nrDNA.

From these five specimens were collected a total of 23 capsules (48.20% of flowers). PCR performed on DNA extracted from seeds gave amplification, and thus PCR fragments of all samples were digested with the selected restriction endonucleases.

Restriction analysis showed that all samples had the diagnostic profiles obtained for parental species. Four of the 10 capsules of two plants showing equal proportion of parental nrDNA had *O. mascula* nrDNA preponderance, 4 of *O. pauciflora* and two equal proportion; 9 of the 13 capsules of three plants having more *O. mascula* nrDNA showed *O. mascula* preponderance, while 4 had an equal proportion (Table. 2). Thus, 13 flowers received pollinia from *O. mascula*, 8 from *O. pauciflora* and two from F_1 hybrids.

Table 2 Ribosomal DNA amount in fruits of Orchis × colemanii. Restriction analysis of DNA extracted from seeds of 23 fruits collected from five specimens of O. xcolemanii showing different proportion of nrDNA.

			PARENTAL NRDNA IN FRUITS		
O.xcolemanii **specimens**	**parental nrDNA ratio**	**Fruits**	**preponderance of *O. mascula***	**preponderance of *O. pauciflora***	**1:1**
2	1:1	10	4	4	2
3	Preponderance of *O. mascula*	13	9	0	4

REPRODUCTIVE SUCCESS

Natural levels of fruit set in open-pollinated populations were 52.10% for *O. mascula*, 50.7% for *O. pauciflora* and 48.2% for *O. xcolemanii*. There was no significant difference in fruit set between species (χ^2 = 0.21, df =2, *P* = 0.84). Fruit set percentages derived from artificial hybridizations (80-86.7%) were slightly higher (Fisher exact test: χ^2 = 2.27, P<0.100) than those obtained from F_2 hybrid generations (62.5%), and from artificial backcrosses (75%) (Table. 3). If we consider directionality of artificial backcrosses, there was no significant differences (Fisher exact test: χ^2 = 0.27, P>0.99) between artificial hybridizations and artificial backcrosses (90%) in which *O. xcolemanii* give the pollinia (Table. 3). Finally, percentages of viable seeds observed were no statistically different among all hand manipulations ranging from 85.9 (*O. pauciflora* x *O. mascula*) to 97.9 (*O. xcolemanii* x *O. mascula*) (Table. 3). Hand manipulation experiments showed the absence of pre- and post-zygotic reproductive barriers, indeed pre-zygotic isolation index (RIpre-zygotic) was 0.19 indicating the absence of pre-zygotic barriers. Moreover *O. xcolemanii* was no affected by some sort of hybrid mortality or sterility, indeed values of hybrid mortality (0.24) and hybrid sterility (0.28) showed the absence of post-zygotic barriers.

Table 3 Fruit set in artificial hybridization (crosses between Orchis mascula and O. pauciflora), backcrosses (crosses between parental species and O. × colemanii), and F2 hybrid generations (crosses between O. × colemanii specimens). In the first column for each hand-pollination the species above is pollinia donor, the species below is pollinia receiver. (NP, number of plants observed; NF, number of flowers observed; FP, number of fruits produced; %F, percentage of fruits produced; %E, percentage of seeds with embryo.)

	NP	NF	FP	%F	%E
Artificial hybridization					
O. mascula* × *O. pauciflora	4	15	12	80.00	**86.30**
O. pauciflora* × *O. mascula	4	15	13	86.65	**85.90**
			mean	83.33	**86.10**
Artificial backcrosses					
O. mascula* × *O. xcolemanii	4	10	6	60.00	**94.10**
O. xcolemanii* × *O. mascula	4	10	9	90.00	**97.90**
			mean	75.00	**96.00**
O. pauciflora* × *O. xcolemanii	4	10	6	60.00	**96.90**
O.x×colemanii* × *O. pauciflora	4	10	9	90.00	**93.65**
			mean	75.00	**95.28**
F2 hybrid generations					
O. xcolemanii* × *O. xcolemanii	**3**	**8**	**5**	**62.50**	**95.35**

DISCUSSION

Molecular researches have shown that homoploid hybrid populations of Mediterranean deceptive orchids are composed of two main types of progenies. In particular, the hybrid swarms of food deceptive orchids (i.e: *Anacamptis* and *Orchis*) consist quite exclusively of F_1 individuals, which have usually an uniform, intermediate morphology. Because most of them are unfertile, F_1 hybrids function as a late post-zygotic barrier (Moccia et al., 2007, Bateman et al., 2008; Jacquemyn et al., 2012). Differently, sympatric species of *Serapias* have been proved to undergo introgressive hybridization (Stökl et al., 2008) and no introgressive hybridization (Xu et al., 2011).

In this scenario, we may affirm that the features of the hybrid zone of *O.* x*colemanii*, studied by us, are totally different from those of other Mediterranean orchids hybrid zones. Our molecular analysis have confirmed the hybrid origin of all the specimens reputed to be *O.* x*colemanii*, accounting once again for the clear morphological difference existing between the hybrid progenies and the parental plants. More relevantly, molecular approach have proved that the hybrids consist of several classes of hybrids, since hybrid specimens posses either a balanced amount of parental nrDNA either several unbalanced combination of both parental DNA (Table. 2). Another striking feature of *O.* x*colemanii* is the absence of effective pre- and post-zygotic reproductive barriers either between hybrids either between them and both parental species. In this respect, we have found that of three fruits ripen on the same plant, each contained a different seed set: one identical to the maternal rDNA combinations, others with changed combinations, clearly produced by pollen carried in any direction among all the co-occurring hybrid or parental plants (Table. 2).

The morphological distinctness and the continuous flower color variation of *O.* x*colemanii* are typical features observed by other authors (Nazzaro et al., 1995; Pellegrino et al., 2000; Cozzolino et al., 2006) and by Italian amateur orchidologists in almost the all known populations. Moreover, the hybrid zones regularly grow on arid, calcareous slopes upwards 1000 m above sea level and co-occur with parental species (Table. 4).

Table 4 Description of *Orchis xcolemanii* settlements. Word file showing population name, elevation (m above sea level), number of hybrid, presence of parental species, color and collector of known populations of *O. xcolemanii*.

REGION	POPULATIONS	ELEVATION m ASL	NUMBER OF HYBRIDS	PARENTAL SPECIES	COLOUR	COLLECTOR
Abruzzo	Prati Tivo (TE)	1200	10	Both	from yellowish to purplish	Romolini
Basilicata	Moliterno (PZ)	900	20	Both	from yellowish to purplish	Romolini
	Castelluccio (PZ)	1200	10-20	Both	from yellowish to purplish	Romano
Calabria	Monte Manfriana (CS)	1200	~ 200	Both	from yellowish to purplish	Gargano
Campania	Sassano (SA)	1200	~ 200	Both	from yellowish to purplish	Nazzaro
	Passo Padula (SA)	900	10	Both	from yellowish to purplish	Romolini
Latium	Monte Maio (FR)	900	10	Both	from yellowish to purplish	Romolini
	Prato di Campoli (FR)	1200	5	Both	from yellowish to purplish	Arrighi

	Monte Ode (RI)	900	3	*O. pauciflora* (*O. mascula* at 500m)	from yellowish to purplish	D'Elia
	Monte Flavio (RM)	800	10	Both	from yellowish to purplish	Romolini
Marche	Monte Pallone (AN)	700	6	More *O. pauciflora* than *O. mascula*	from yellowish to purplish	Klaver
	Monte Vermenone (MC)	1220	10	More *O. pauciflora* than *O. mascula*	purplish	Klaver
	Monte Nerone (PU)	1200	~ 400	Both	from yellowish to purplish	Klaver
	Monte Petrano (PU)	950	~ 50	Both	More purplish	Klaver
	Monte Catria (PU)	950	~ 500	Both	from yellowish to purplish	Klaver
	Monte Paganuccio (PU)	975	10-20	Both	purplish	Klaver
Tuscany	Campocecina (MS)	1100	~ 100	Both	from yellowish to purplish	Antonetti
	Sassalbo (MS)	850	~ 100	Both	from yellowish to purplish	Antonetti
	Monte Borla (MS)	1300	10	Both	from yellowish to purplish	Mazzoni

	Foce Pianza (MS)	1200	~ 20	Both	from yellowish to purplish	G.Pacifico
	Massa (MS)	1200	5	Both	from yellowish to purplish	G.Pacifico
	Carrara (MS)	850	~ 30	Both	from yellowish to purplish	G.Pacifico
	S. Maria Giudice (LU)	1000	5	Only *O. pauciflora*	purplish	Romolini
	Stazzema (LU)	950	~ 50	Both	from yellowish to purplish	Antonetti G.Pacifico
	Monte Piglione (LU)	1100	~ 100	Both	from yellowish to purplish	Antonetti
	Monte Matanna (LU)	1150	~ 50	Both	from yellowish to purplish	Antonetti
	Monte Nona (LU)	1000	~ 50	Both	from yellowish to purplish	Antonetti
	Monte Prana (LU)	1100	~ 50	Both	from yellowish to purplish	Antonetti
	Monti Pisani (LU)	900	10	Only *O. pauciflora*	yellowish	Antonetti
	Prato Fiorito (LU)	1100	~ 100	Both	from yellowish to purplish	Antonetti

	Monte Gabberi (LU)	1100	10	Both	from yellowish to purplish	Mazzoni
	Minucciano (LU)	800	1	Both	from yellowish to purplish	G.Pacifico
	Pescaglia (LU)	900	5	Both	from yellowish to purplish	G.Pacifico Viviani
Umbria	Monte Cucco (PG)	900	~ 200	Both	from yellowish to purplish	Antonetti Klaver
	Monte Macchialonga (PG)	1000	~ 200	Both	from yellowish to purplish	Bizzarri
	Monte il Monticello (PG)	1000	~ 800	Both	from yellowish to purplish	Bizzarri
	Monte di Campi (PG)	1200	~ 100	Both	from yellowish to purplish	Bizzarri
	Monte Lungo (PG)	1000	~ 150	Both	from yellowish to purplish	Bizzarri

So, it is reasonable to argue that these many hybrid zones are stable and of ancient, contemporaneous origin across the entire area of overlap of the parental species distribution ranges. In this perspective, we will consider two main evidences. Firstly, the greatly different distribution and ecological ranges of parental species (see Figure. 10) suggest a their longstanding divergence. Indeed, they occur separately in two large Mediterranean isles with different geo-climatic history, and precisely in Sardinia has been found only *O. mascula* and in Crete only *O. pauciflora* (Delforge, 2005). Secondly, it is not trivial that phylogenetic analysis of Orchidinae show an early divergence between *O. pauciflora* and the other members of the *O. mascula* group (Bateman et al., 2003). In accordance, evaluation of genetic distances based on ITS sequences has shown that these two species have a genetic distance higher than those of many other Mediterranean orchids species pairs (Scopece et al., 2007).

Overall, these evidences suggest that the origin of so many narrow hybrid zones of *O.* x*colemanii* could be ancient, much more respect to the first report of Cortesi (Cortesi, 1907). There is an old, large consensus on relevant role played by geo-climatic changes and human disturbance in the insurgence and establishment of hybrid zones (Stebbins, 1959; Comes and Kadereit, 1998). Moreover, it has been observed that current distribution of species and hybrid zones in both the Old and New continent may be originated during the Pleistocene glaciations (Barton and Hewitt, 1985). Noticeable, Apennines were interested by glaciations and glacier valleys and moraines are present until the southernmost Pollino massif (Acquafredda and Palmentola, 1986). In average, the ice border was at 1200-1300 m asl, the same altitude at which occur most of *O.* x*colemanii* populations. Thus, it is reasonably to hypothesize that these populations still live in the same places where they have originated by secondary contact occurred in the periglacial belt of Apennines.

The theory of adaptive speciation predict that hybrid zones could became larger or narrower under the influence of introgressive hybridization or reinforcement of reproductive isolation, respectively (Mayr, 1942). Thus, to explain the evident stability of many hybrid zones appropriate hypothesis have been proposed. In particular, the dynamic equilibrium hypothesis assumes the existence of an equilibrium between gene flow and selection against the hybrids, while the bounded superiority hybrid hypothesis retains that hybrids are more fit than

parental species in restricted regions where they occur (Moore, 1977). In this perspective, we think that features of *O.* x*colemanii* are apparently incompatible with equilibrium dynamic hypothesis, given the apparent lack of gene flow and hybrid selection. Conversely, their persistence could be accounted by hypothesis of bounded hybrid superiority. Indeed, the high comparable levels of reproductive success, found in all the experimental crosses, strongly suggest a relaxed selection against hybrids. Similarly, it has been proved that hybrids are regularly visited by pollinators independently from the emission of scent intermediate respect to parental taxa (Salzmann et al., 2007). On the other hand the existence of a hybrid superiority could be undetectable a long as ecological conditions are stable.

Even an accurate scrutiny of the literature, has confirmed the rarity of a plant hybrid zone with the overall features like that of *O.* x*colemanii*. Indeed, we are able to report only a narrow hybrid zone between two species of *Pitcarnia* (Bromeliacae), growing on the Pão de Açucar in Rio de Janeiro (Brazil), where hybrids have shown a fruit set, seed set and seed germination fitness equivalent to the parental taxa (Wendt et al., 2001). Since both taxa are two narrow endemics, the authors suggested that hybridization could have a positive role contributing to the expansion of individuals on the slope of the mountain.

An alternative hypothesis, worthy to be discussed, is the likelihood that *O.* x*colemanii* is a nucleus for a totally new species formation or even a yet incipient species. Previous authors have considered *O. xcolemanii* either as a quite stable taxon of hybrid origin (Del Prete and Miceli, 1981) and have listed it as an endemic form (Nazzaro et al., 1995). On the one hand, although underestimated, homoploid speciation was assumed to be the source of many plant speciation events (Gross and Rieseberg, 2005), in which a long time is needed to bring about appropriate reproductive barriers. We notice that although the high hybrid fertility could sustain such possibility, any ecological preference of *O. x*c*olemanii* has been documented and, also, information received by orchids amateur say that one parental species (*O. pauciflora*) is ever co-occurring and *O. mascula* is easily found nearby. A previous study (Salzmann et al., 2007) showed that *O. xcolemanii* exhibits a different odor bouquet from those of the two parent species, which could lead to a shift of

pollination. It seems that just a change in pollinators may play a key role, being the driving force of speciation by homoploid hybridization (Chase et al., 2010). It has recently been demonstrated, especially in orchids with nectar spur (Paun et al., 2007) or sexual deceptive orchids, as in the case of the genus *Ophrys* (Vereecken et al., 2010), that differences in the composition of floral odors have created differences in the attraction of different groups of insects, thus creating strong relationships between orchids and pollinators.

In conclusion, this study demonstrates that the hybrid zone of *O.* x*colemanii* might have a biological and evolutionary significance different from those attributed to hybrid zone of other Mediterranean deceptive orchids, although its own actual significance is very difficult to be envisaged. Certainly, it does not appear a dead end population and, so, could represent a potential reserve of adaptive variability, as seem to be typical of zones with several hybrid generations (Anderson, 1948; Rieseberg, 1995). In any case, *O.* x*colemanii* is an unusual case of frequently occurring fertile hybrids with a continuous phenotypic variation between the two parental species, an interesting step along the speciation process. As recently stressed by Mallet (2008) hybridization and introgression may often lead to a continuum of phenotypic and genotypic variation either over large geographical scale and locally in sympatry. The occurrence of this continuum is seen as the evidence that the original vision of Darwin (1859, 1877) on speciation might be reevaluated and that the speciation process is occurring all around us.

2-Interactions with symbionts in a hybrid Mediterranean orchid.

INTRODUCTION

Mycorrhizas are widespread intimate symbioses between members of three fungal phyla and the vast majority of plants. The symbiosis is generally a mutualistic one, with fungi providing the plant with soil nutrients in exchange for organic carbon assimilated by photosynthesis (Smith and Read, 1997). The most widespread of these symbioses are the ancestral arbuscular mycorrhizas involving members of the Glomeromycota and the more recent and repeatedly evolved ectomycorrhizas involving members of the Ascomycota, Basidiomycota, and many woody plants. In nature, the formation of a mycorrhizal symbiosis is typically an obligate step in the completion of the fungal and plant life cycles. Orchids have a unique mycorrhizal relationship that was first documented over a century ago (Bernard, 1902), but difficulties associated with studying the fungi forming orchid mycorrhizas hampered research over the subsequent decades. Orchid mycorrhizal fungi have generally been classified as belonging to rhizoctoniaforming fungi, a polyphyletic group of fungi from three basidiomycete families (Sebacinaceae, Ceratobasidaceae, and Tulasnellaceae) (Roberts, 1999). Fungal taxonomy is largely based on the morphology of sexual structures, but rhizoctonia-forming fungi rarely fruit in axenic culture and are difficult to identify using vegetative characteristics alone. Furthermore, most mycorrhizal fungi have proven unculturable in the absence of a plant host. Hence, questions relating to the identities of many orchid mycorrhizal fungi and the levels of specificity for fungal partners among orchids have long been controversial (Curtis, 1939; Hadley and Purves, 1974; Warcup, 1981; Clements, 1988; Masuhara and Katsuya, 1994). The advent of fungal molecular systematics and ecology revolutionized the study of mycorrhizas by allowing direct identification of fungi without axenic isolation. Rhizoctonia-forming fungi are often saprophytes or plant pathogens so, unlike the fungi that form arbuscular mycorrhizas and ectomycorrhizas, they are not obligately mycorrhizal. The distributions of orchid mycorrhizal fungi have been shown to be independent of orchids (Brundrett et al., 2003; Feuerherdt et al., 2005); the ability to utilize otherwise free-living fungi in mycorrhizal symbioses appears to

be a unique characteristic of orchids. Another characteristic trait of the orchid family is the exceptionally prolific production of tiny dust-like seeds (microspermy) that can engage in mycorrhizal interactions during the earliest stages of germination. Recruitment limitation is of paramount importance in orchid biology; in his book on orchid pollination, Charles Darwin (1877) estimated that if the germination of viable seeds went unchecked an orchid plant could 'clothe with one uniform green carpet the entire surface of the land throughout the globe' in only three generations. Each orchid seed is miniscule, lengths as small as 0.05 mm in Anoectochilus imitans (Arditti and Ghani, 2000) and has minimal nutritional reserves; it is essential for the germinating seed to undergo mycorrhization with an appropriate fungal partner in order to grow. Otherwise, dormancy periods of up to several years can occur in some species (Whigham et al., 2006). Upon germination, fungal hyphae penetrate the cell walls of the orchid and form characteristic coils, called pelotons, within the cells. Growth of the fungus is restricted to cortical cells (Peterson et al., 1998), probably by the deposition of phenolics (Beyrle et al., 1995) and the production of anti-fungal compounds (Shimura et al., 2007). Pelotons are subsequently 'digested', and through this process the orchid is thought to receive the essential nutrients and carbon that it needs to grow. Nutrient exchange may also, or instead, occur across intact cell membranes prior to 'digestion' as in other intracellular mycorrhizas. The germinated seed grows into a mass of differentiated cells called a protocorm, and remains in this form for a period that can extend up to several years, until leaves are produced. During this period of their life, many orchids are underground and rather than producing carbon through photosynthesis like most autotrophic plants, they obtain all of their energy from fungal pelotons. Therefore, before the production of leaves, all orchids go through a stage of their life-cycle in which they are mycoheterotrophs (Leake, 1994), rather than autotrophs. Most adult orchids retain their mycorrhizal partnerships, and due to their characteristically poorly developed root systems, they are thought still to be heavily reliant on mycorrhizal fungi for mineral nutrition (Smith and Read, 1997). In contrast to other mycorrhizal symbioses, it has long been thought that orchid mycorrhizal fungi receive few benefits from the interaction (Hadley and Purves, 1974; Alexander and Hadley, 1985; Smith and Read, 1997). However, a recent study demonstrated bi-

directional movement of carbon between adult Goodyera repens and its fungal partner (Cameron et al., 2006). This study conducted on orchids growing in agar microcosms and the relevance of these results to orchids growing in natural conditions remains to be determined. The presence of fungi in albino variants of green orchids (Selosse et al., 2004; Julou et al., 2005) and the fact that some orchids undergo prolonged periods of underground dormancy (Shefferson et al., 2007) suggests that the fungi are not reliant on carbon derived from orchid photosynthesis. The overall benefits and costs to fungi of associating with orchids remains debatable due to difficulties in quantifying fungal fitness in natural conditions. Some orchid species have lost the ability to photosynthesize and remain entirely myco-heterotrophic as adults. This mode of nutrition has evolved in several plant families but is most common in Orchidaceae, with over 100 such species known, probably due to the obligate myco-heterotrophy of all orchid seedlings (Leake, 1994). Phylogenetic analyses have revealed that the loss of photosynthesis in adult plants may have occurred independently at least 20 times in the family (Molvray et al., 2000). The understanding of these intriguing plants has increased greatly in recent years due to the application of molecular techniques for identifying the fungi involved. Since the review by Rasmussen (2002), a large number of additional orchid mycobionts have been identified directly from orchid protocorms, roots, tubers and rhizomes through molecular biology approaches (Shefferson et al. 2007; Stockinger et al. 2010; Jacqemyn et al. 2011). Sequencing of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA after PCR amplification using a variety of primer combinations (White et al. 1990; Taylor and Mccormick 2008) has been the method of choice for identifying orchid mycobionts.

This has revealed that the degree of specificity between fungus and orchid is an important factor determining chances of successful seedling establishment (Bidartondo and Read 2008). Mycorrhizal specificity is often high in orchids, ranging from a few genera to a single fungal species (Mccormick et al. 2004; Taylor et al. 2004; Dearlaney 2007; Shefferson et al. 2008); or photosynthetic orchids are associated with a narrow range of fungi over large geographic areas, indicating narrow specificity (Shefferson et al. 2005, 2007; Mccormick et al. 2006; Bonnardeaux et al. 2007; Irwin et al. 2007; Roy et al. 2009).

Hybrid zones, where parental species and hybrids co-occur, are highly suitable to verify if hybrids display the symbionts of one or both parents or have totally different partners.

There are only few studies that have investigated mycorrhizal associations in hybrid compare to parental species. Hybridizing species of genus Caladenia showed fungi genetically different from those associating with the parents (Hollick et al. 2005). Schatz et al. (2010) determined that adult individuals of *Orchis simia* and *O. anthropophora* and their hybrid were associate to closely related Tulasnellales fungi. Investigation on mycorrhizal associations in three closely related hybridizing Orchis species showed common mycobionts in protocorms and adults suggesting that mycorrhizal associations play a small role in reproductive isolation (Jacquemyn et al. 2010).

In this study, has been examined, with molecular analyses, a sympatric zone between *Orchis italica* Poir. and *O. anthropophora* L., which hybridize to form *O. xbivonae* Tod. The main purpose was to compare the identity of mycorrhizal associates in two parental species *O. italica*, *O. anthropophora*, and their hybrid *O. xbivonae* at the adult stage to determine if lack of appropriate fungal symbionts can be related to hybrid viability, and to verify if mycorrhizal fungi allow the hybrid to exploit new ecological niches different from parental habitat. All authorities agree that the correct name for this hybrid is *Orchis bivonae* Tod. or *Orchis x bivonae* Tod. The recent change of accepted name of Man Orchid (LHS above) from *Aceras anthropophorum* to *Orchis anthropophora* means that the existence of this hybrid orchid is perhaps less surprising as it is now intrageneric (within the *Orchis* genus) rather than intergeneric (a hybrid between species in different genera).

MATERIALS AND METHODS

STUDY AREA AND ORCHID SPECIES STUDIED

The study on *Orchis italica* Poir., *O. anthropophora* (L.) All., and their hybrid *Orchis xbivonae* Tod. was conducted in a natural population located onto the "Mount of Cassano" (39°47'N 16°18'E, 512m a.s.l.), Calabria region, Southern Italy. (Figure. 15)

Fig. 15 "Mount of Cassano"

The whole area covers roughly 1500 m^2 (25 m wide and 60 m long) of a calcareous soil and is bounded on the west by a road and by deep gorges for the rest. In the studied site *O. italica* and *O. anthropophora* overlap extensively in their spatial distribution and grew together with 8 individuals of *O. xbivonae* (Figure. 16).

Fig. 16 *O. anthropophora* (L.) All., *O. xbivonae* Tod. and *Orchis italica* Poir.

O. italica and *O. anthropophora* are closely related (Bateman et al. 2003), have same chromosome number (2*n*=42) (Queiros 1985; Cauwet-Marc and Balayer 1986; Bianco et al. 1987; Costantinidis et al. 1997) and have been both included in the *O. militaris* (Delforge 2005) or “anthropomorphic” group (Bateman et al. 2003). *O. italica* (Figure. 17) show a pendent lip, deeply tri-lobed and with a cylindrical spur,

Fig. 17 *Orchis italica* Poir.

while, *O. anthropophora* (Figure. 18) has a narrow lip and, differently from all the others *Orchis* species, it lacks a spur (Delforge, 2005).

Fig. 18 *O. anthropophora* (L.) All.

The detected specimens of *O. xbivonae* are 20-40 cm high, with oblong leaves and cylindrical inflorescences. The pendent labellum is trilobate with median lobule reduced to a minuscule dent. Spur is very short, saccate and pointing downwards, with a length of about the half of that of *O. italica*. Recent molecular studies have supported that the majority of Orchis mycorrhizal fungi belong to Tulasnellaceae, and in few plants were also found members of Ceratobasidiaceae, Telephoraceae and Cortinariaceae (Jacquemyn et al. 2010; Schatz et al. 2010; Jacquemyn et al. 2011). In particular roots of adult individuals of *Orchis italica* and *O. anthropophora* were colonized by eight and nine different fungal OTUs (operational taxonomic units), respectively (Jacquemyn et al. 2011).

MOLECULAR ANALYSIS

Characterization of mycorrhizae involved: (i) extraction of DNA from mycorrhizal plant tissue, (ii) amplification of fungal genomic regions useful in determining fungal identity, (iii) DNA sequencing for identification of mycorrhizal fungi and assessment of specificity.

Small parts of roots were cut from 15 randomly selected individuals of O. anthropophora and O. italica and for 8 individuals of O. xbivonae (all hybrids found) for molecular analysis. All roots were surface sterilized using 1% hypochlorite (30 s) followed by three rinses in distilled water (30 s). Total DNA was extracted from 1–2 cm length of root pieces per plant using the cetyltrimethyl ammonium bromide (CTAB) method (Henrion et al. 1992). Each root were separately pulverized in a 2ml-eppendorf using 500 µL of CTAB buffer, incubated at 65°C for 20 min, extracted twice adding 500 µL chloroform-isoamyl alcohol (24:1), precipitated with isopropanol and washed with 250 µL of ethanol 70%. DNA was resuspended in 50 µL of distillated water.

To discriminate among fungal taxa colonizing orchid roots, the internal transcribed spacers (ITSs) of the nuclear ribosomal DNA were amplified using broad-spectrum basidiomycete primers ITS1-OF and ITS4-OF (Taylor and McCormick, 2008). These primers are the most efficient primer pair because gave the most consistent amplification (Jacquemyn et al. 2011).

All PCR reactions of 100 µL final volume contained 2 µL DNA template, 10 µL reaction buffer 10x, 100 µM of each dNTP, 0.3 µM of each primer, 2 units Taq polymerase, 2 µM $MgCl_2$ and 2.5 Units of BioTaq^{TM} DNA Polymerase (Bioline Inc., Boston, MA, USA). The thermocycling profile consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles with 45 s at 94°C, 45 s at 58°C, and 45 s at 72°C, with final elongation step at 72°C for 7 min. PCRs were performed on a PTC-100 Thermal Cycler (MJ Research Inc., Watertown, MA, USA). Amplification products were electrophoretically separated on a 1.8% agarose gel (Methaphore, FMS), photographed after ethidium bromide staining and purified with the QIAEX II Gel Extraction Kit (QIAGEN) to remove unincorporated primers and dNTPs following the manufacturer's instructions.

The purified PCR fragments were sequenced directly in forward and reverse directions using each primer used for amplification; fluorescent dye sequencing

was performed on a 310 ABI DNA Sequencer (Applied Biosystems) using the Sanger dideoxy method.

The ClustalW algorithm (Thomson et al. 1994) of the MEGA 5 program package (http://www.megasoftware.net) was applied for the exact alignment of sequences (Tamura et al. 2011). Ambiguous sites were checked manually and corrected by comparing electropherograms from both strands. Consensus sequences were obtained for each specimen (5' and 3' borders were identified using mychorizzal sequences already available in GenBank [http://www.ddbj.nig.ac.jp]) and used for neighbour-joining analyses. Based on the final alignment, a distance matrix was constructed using MEGA 5 software.

Sequence identity of all obtained sequences was determined using the blast algorithm available through the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/index.html). In addition, since none of the ITS sequence types obtained had 100% identity with GenBank sequences of identified Rhizoctonia group, OTUs were identified comparing our ITS sequences and previously developed fungal OTUs (Jacquemyn et al. 2011).

RESULT

Successful double-stranded amplifications and complete sequences were obtained using the primer set ITS1-OF and ITS4-OF for all the orchid roots examined. The DNA sequences were deposited in DDBJ/EMBL/GenBank nucleotide databases. On the basis of at least 97% DNA sequence similarity, out of 23 fungal OTUs identified previously (Jacquemyn et al. 2011), eight were observed (OUT 2, 4, 6, 7, 10, 11, 12 and 17) in our investigated plants; six were related to Tulasnellaceae, and two to Ceratobasidiaceae (Table. 5).

Table 5. List of fungal operational taxonomic units (OTUs) identified in *Orchis anthropophora* (A), *O. italica* (I) and *O. xbivonae* (X). Fungi were grouped into OTUs defined by 97% internal transcribed spacer (ITS) sequence similarity.

			PRESENCE IN EXAMINED ORCHIDS		
TARGET	FAMILY	CLOSEST MATCH IN GENBANK	A	I	X
OTU-2	Tulasnellaceae	GQ907254	X	X	X
OTU-4	Tulasnellaceae	GQ907260	X	X	X
OTU-6	Tulasnellaceae	GQ907266		X	
OTU-7	Tulasnellaceae	GQ907258	X	X	X
OTU-10	Tulasnellaceae	GU066935	X	X	X
OTU-11	Ceratobasidiaceae	GU066936		X	
OTU-12	Tulasnellaceae	HQ330992	X		
OTU-17	Ceratobasidiaceae	HQ331002	X		

Six different fungal OTUs were found in *O. anthropophora* (OUT 2, 4, 7, 10, 12 and 17) and *Orchis italica* (OUT 2, 4, 6, 7, 10 and 11), and four (OUT 2, 4, 7 and 10) in *O. xbivonae* (Figure. 20). Parent species showed different frequently dominant OTUs (OTU 2 and 12 in O. italica and OTU 7 and 10 in *O. anthropophora*). OUT 2 and 4 were present in all three taxa, while *O. italica* and *O. anthropophora* showed two exclusive OTUs, OTU 12 and 17, OTU 6 and 11, respectively (Figure. 19)

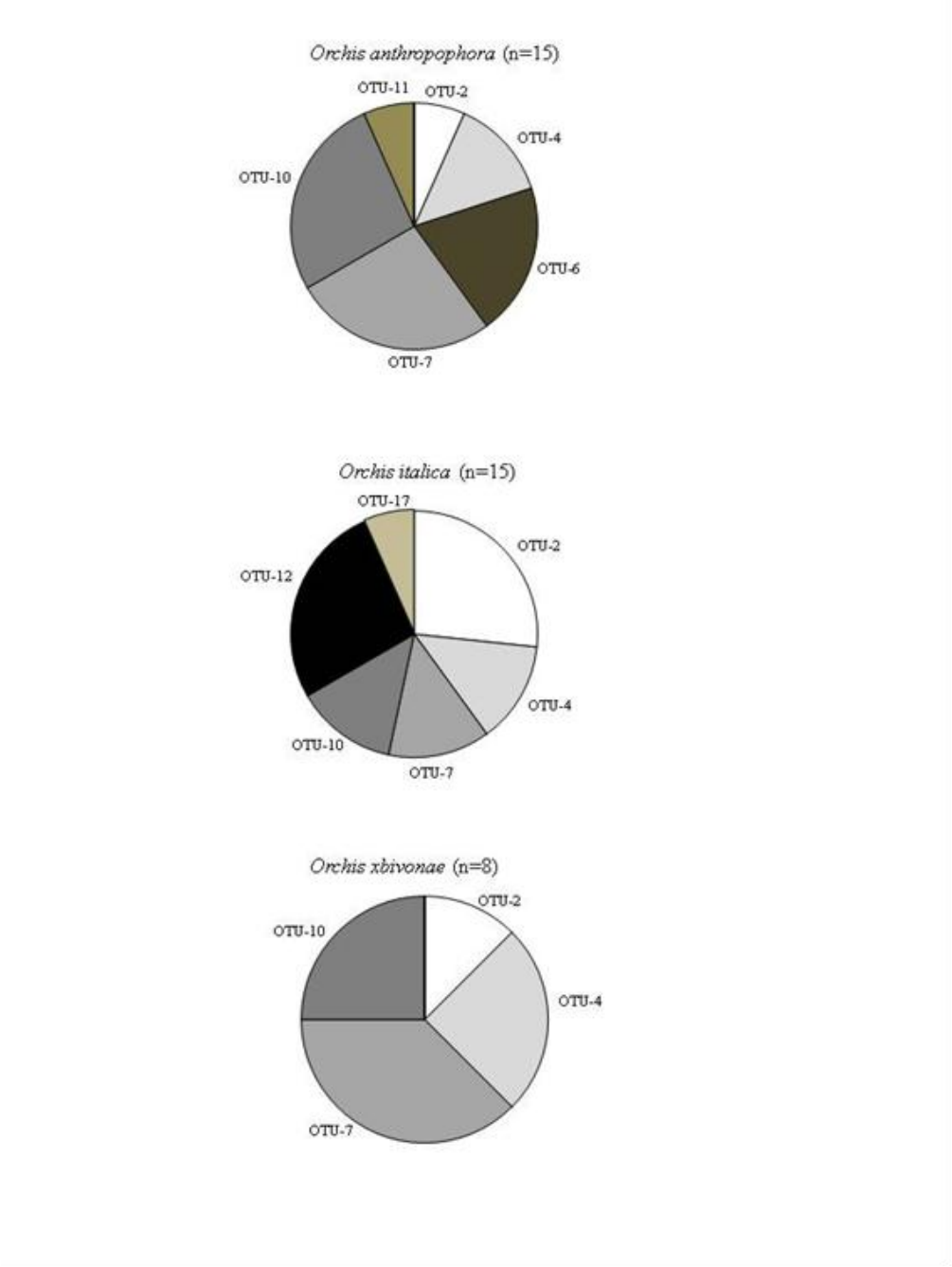

Figure 19 Frequency distribution of identified operational taxonomic units (OTUs) in *Orchis anthropophora*, *O. italica* and *O. xbivonae*.

The mycorrhizal assemblages are not significantly different between each parent species and the hybrids (Fisher test), *O. anthropophora* vs *O. xbivonae*: P = 0.2569, *O. italica* vs *O. xbivonae*: P = 0.1221), although the parent species have different assemblages (*O. anthropophora* vs *O. italica*: P = 0.005247).

DISCUSSION

Molecular analysis have shown that adult plant of the two parental species and their hybrid associated with several frequently different fungal OTUs. First, 75% of mycorrhizal fungi identified belong to Tulasnellaceae, a large, common group of orchid mycorrhizal fungi that have already been recorded in *O. anthropophora* and *O. italica* (Schatz et al. 2010; Jacquemyn et al. 2010; Jacquemyn et al. 2011). Consistent with previous studies, *Orchis italica* predominantly associated with OTU 2 and 12, while *O. anthopophora* with OTU 7 and 10 (Figure. 19). *Orchid xbivonae* associated with fewer mycorrhizal fungi in comparison with its two parental species. Similar fungi occurred in the two parents, perhaps due to the close phylogenetic positions of the two parental species (Bateman et al. 2003), and hybrids too, suggesting that mycorrhizal interaction did not constrain hybrid survival. Similarly, Schatz et al. (2010) reported that micorrhizal fungi of *Orchis xbergonii* mostly belonged to Tulasnellales associated with the two parental species (*O. simia* and *O. anthopophora*) and that the fungi associated with hybrids had less-diverse sequences than those associated with the parents.

Hybrid zones are natural laboratories for studying reproductive isolation mechanisms among closely related species, role of selection in maintaining or eroding species differences, and role of hybridization in plant evolution (Rieseberg and Buerkle 2002; Lexer et al. 2005).

Essential conditions for speciation by hybridization are that the hybrid exploits an ecological niche (Arnold, 1997), either the parental one or a totally new one, and produces a sufficient number of seeds for its ecological maintenance (as much as parental species in the case of sympatry). Compared to parental species, previous study demonstrated that *O. xbivonae* showed low fruiting values in open-pollinated flowers (Pellegrino et al. 2009). While experimental crosses proved the absence of any form of postmating isolation, and the use of SNPs allowed to accurately classify individuals to F1 generation (Pellegrino et al. 2009). The low levels of reproductive success, the lack of post-zygotic barriers and of F2 (or later) generations suggest that the mycorrhizal symbiosis imposes no constraints on the fate of hybrids, and that the lack of pollinators appears to strongly limit hybrid fitness, as has previously been reported in

parental species (Pellegrino et al. 2010) and other deceptive orchids (Mattila and Kuitunen 2000; Pellegrino et al. 2005; Smithson 2006).

The coexistence of *O. xbivonae* with its parents suggests that this hybrid is a short-term by-product of the hybridizing behavior of common pollinators (Schatz 2006). Micorrhizal do not represent a limitation to hybrid grow and do not offer an ecological opportunities to partially separate hybrid habitat from the parental ones. It can be hypothesized that high specificity and divergent association patterns between species could lead to an effective barrier to hybridization due to incompatibilities between orchid and mycorrhizal fungi. If, on the other hand, orchid species share most of their mycorrhizal fungi, no such incompatibilities are to be expected, and post-mating barriers at the seed germination stage should be weak, implying that mycorrhizal associations only play a minor role in affecting hybridization between species.

Overall, these results indicate that our hybrid zone represents a phenomenon of little evolutionary meaning and that the few hybrid plants will not easily origin descendents with potential new genetic combinations and/or ecological preferences.

In conclusion, these results alongside with other similar studies support that natural hybridization does not seem to play a prominent role in speciation processes of Mediterranean food deceptive orchid, but that, rather, strong postzygotic barriers actively maintain parental species boundaries from genome-wide introgression.

3-Pollen competition as a reproductive isolating mechanism between two sympatric *Orchis* species

INTRODUCTION

Natural hybridization results when two species meet, mate and produce offspring (Harrison, 1990). One of the best-studied plant groups in terms of hybridization is the orchids. Indeed, frequent hybridization has been documented among the Mediterranean orchids, (over 300 records, see http://www.guenther-blaich.de/engl/hybrid.htm) and attributed to their unspecific pollination system (van der Cingel, 1995) and the evolution of deceptive pollination mechanism (Jersakova et al. 2006). In the Mediterranean region food- and sexually-deceptive orchids often are sympatric, have overlapping flowering periods, and share pollinators, and thus hybridization might occur frequently (Cozzolino et al., 2005, etc.). The extent of hybridization can vary widely among orchids: some consist primarily of F1 individuals, this is the case of a hybrid zone between two food-deceptive species *Anacamptis morio* and *A. papilionacea* (Moccia et al., 2007); others contain only a very small proportion of F1s but many backcross individuals, ad example between *Ophrys lupercalis* and *O. iricolor* (Stökl et al., 2008) or *Serapias vomeracea* and *S. cordigera* (Bellusci et al., 2010) or between *Orchis mascula* and *O. pauciflora* (Luca et al. 2012)**.** Interfertile orchid species found sympatrically or parapatrically in the wild may be reproductively isolated by the action of one or more isolating mechanisms. Fidelity of pollinators represents the primary pre-pollination barrier among the specialized sexually-deceptive *Ophrys* species (Moccia et al. 2007, Scopece et al. 2007) or differences in peak flowering periods (Steiner et al., 1994). On the contrary, for food-deceptive orchids, the likelihood of receiving pollen loads comprising a mixture of conspecific (i.e., same species) and heterospecific (i.e., another species) pollen is expected to be high. Many closely related plant species form hybrids after pollination with pure loads of interspecific pollen (Klips et al. 1998). After pollination but before zygote formation, a reproductive barrier can arise from a reduced siring ability of heterospecific pollen as compared with conspecific pollen (Hauser et al., 1997;

Brown and Mitchell, 2001; Wolf et al., 2001b). This differential fertilization success often is stronger or exclusively observed when pollen of both species compete for fertilization (pollen competition: Darwin, 1859; Howard, 1999; Aldridge and Campbell, 2006).

The pollen competition was observed for the first time in 1859 by Darwin when he led evolutionary studies on plants: “It is well known that if pollen of a distinct species be placed on the stigmas of a flower, and its own pollen be afterward, even after a considerable interval of time, placed on the same stigma, its action is so strongly prepotent that it generally annihilates the effect of the foreign pollen”. The concept of “pollen competion” and “conspecific pollen advantage” were born in those years, and “pollen competion” was recognized as a major and frequent reproductive barrier (Riesemberg et al 1995; Carney et al 1996; Howard, 1999).

The formation of hybrid offspring can be greatly reduced if conspecific is more advantaged when compared to heterospecific in term of ovule fertilization. This has been termed Conspecific Pollen Advantage (CPA) (Alarcòn and Campbell, 2000) and is believed to be a common isolating mechanism in the plant kingdom (Stace, 1989). Heterospecific pollen may have reduction of germination on the stigma, reduction of pollen tube growth or decrease fertilization of ovules than conspecific pollen. Conspecific pollen advantage at any of these stages reduces the frequency of hybrid seed formation following mixed pollinations as in *Helianthus* (Rieseberg et al., 1995) and *Iris* (Arnold et al., 1993; Carney et al., 1994; Carney and Arnold, 1997).

In order for pollen competition to take place between a given pair of flowering plant species, a set of conditions should be simultaneously met. First, species should occur in the same locality or area. Second, sympatric species should overlap—totally or partially—in flowering times. Third, co-flowering sympatric species should share one or more pollinator species. Fourth, shared pollinators of co-flowering sympatric species should switch between flowers of different species during single foraging bouts or flights.

Pollen competition have examined whether CPA occurs in interfertile pair species that form a hybrid zone using pollen mixtures comprising different pollen ratios of conspecific and heterospecific pollen.

In this study, was examined, using a combination of molecular analyses and experimental crosses, a contact zone between *Orchis italica* (Figure. 17) and *O. anthropophora* (Figure. 18), in which was found some individuals of their hybrid, *O. xbivonae* (Figure. 20). The main aim was to elucidate the potential role of *O. xbivonae* as a genetic bridge between its parental species.

Fig. 20 *Orchis xbivonae* Tod

In particular, the purpose of the present study was to determine whether pollen competition potentially acts as a reproductive barrier, by assessing the degree to which hybridization is prevented in mixed pollinations.

MATERIALS AND METHODS

STUDY AREA AND ORCHID SPECIES STUDIED

The effect of pollination with heterospecific-pollen loads on seed set in orchids was tested with *Orchis italica* Poir., *O. anthropophora* (L.) All., plants which grow in a natural population located on Monte di Cassano (39°47'N 16°18'E, 512m a.s.l.), Calabria, southern Italy. The whole area covers roughly 1500 m^2 (25 m wide and 60 m long) on a calcareous soil and is bounded to the west by a road and by deep gorges in other directions.

In the studied site, *O. italica* and *O. anthropophora* overlap extensively in their spatial distribution and grow together with eight individuals of *O. xbivonae* Tod. *Orchis italica* and *O. anthropophora* are closely related (Bateman et al. 2003), have the same chromosome number (2n = 42) (Queiros 1985; Cauwet-Marc and Balayer 1986; Bianco et al. 1987; Costantinidis et al. 1997) and have both been included in *O. militaris* (Delforge, 2005) or an anthropomorphic group (Bateman et al. 2003). *O. italica* has a pendant lip, is deeply tri-lobed with a cylindrical spur, while *O. anthropophora* has a narrow lip and, unlike all other Orchis species, lacks a spur (Delforge 2005). The detected specimens of *O. xbivonae* are 20–40- cm high, with oblong leaves and cylindrical inflorescences. The pendant labellum is trilobate, with the median lobule reduced to a minuscule dent. The spur is very short, saccate and points downwards, being half the length of that in *O. italica*. We have little information on pollinators of the two parental species. *O. anthropophora* has been reported to be pollinated by two species of sawfly (Hymenoptera) and three species of beetle (Coleoptera) (Reinhard et al. 1991; Schatz 2006). No information is available for pollinators of *O. italica*, however, according to van der Cingel (1995), its pollinators might belong to the insect assemblage that visits taxa in the *Orchis militaris* group.

POLLINATION EXPERIMENTS

Hand pollination experiments were conducted during spring 2011 in laboratory of Plant Biosystematic at the University of Calabria. 20 plants that had about 75% unopened flowers from each selected species were excavated, potted and transferred to the laboratory, so that we could choose flowers with specific and comparable stigma receptivity. No more than four flowers for each plant were used in crossing experiments, to avoid energetic deficit in fruit and seed formation. We produced 84 bi-directional crosses (Figure. 21) between *O. italica* and *O. anthropophora*; 42 crossing with *O. italica* as pollen receiving (mother plant) and other 42 crossing with *O. anthropophora* as pollen receiving (mother plant). The hand pollination experiments were divided in two type: in the first round of hand pollinations the mother plant received before the homospecific pollen, and heterospecific pollen was deposited on stigma after different time (from 1 to 48 hours); in the second case mother plant received before the heterospecific pollen, and conspecific pollen was deposited on stigma after different time (from 1 to 48 hours). In addition, 6 flowers for each taxa were pollinated only with conspecific or heterospecific pollen

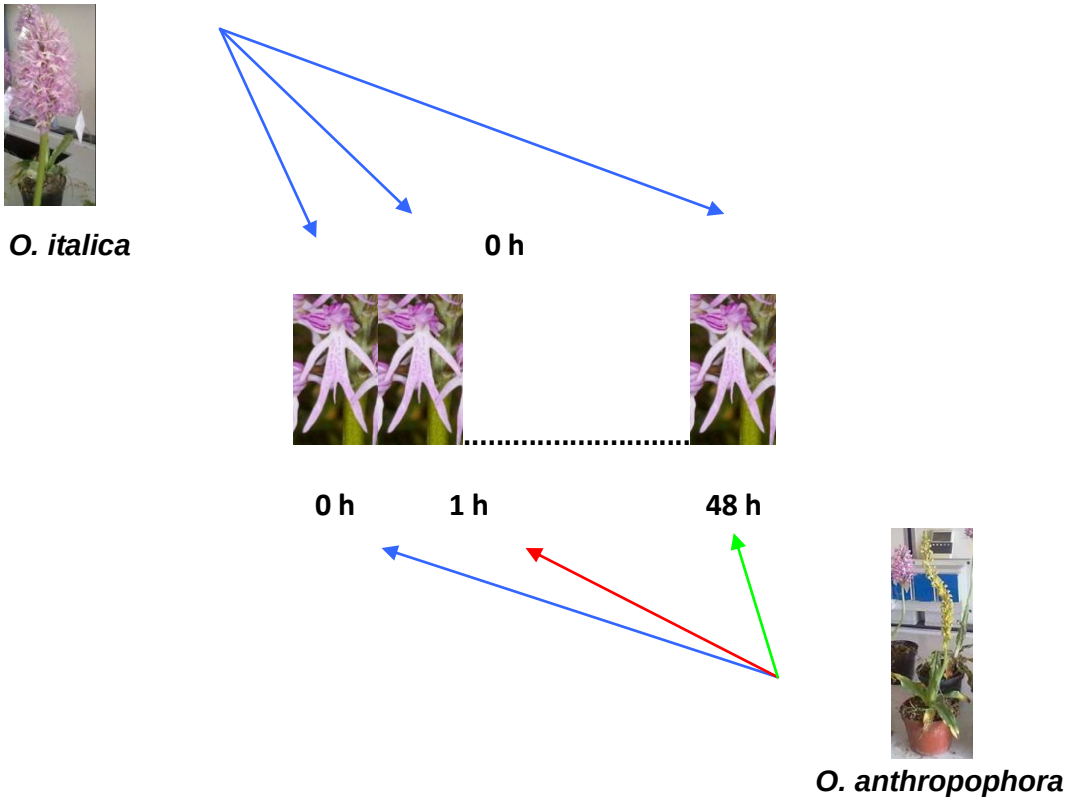

Fig. 21 Homo- and heterospecific crosses with *O. italica*' s mather line

Fruit production was signified by the development of swollen ovaries, and the percentage of fruit set was calculated as the ratio of swollen ovaries to the number of treated flowers. It is important to note that, in orchids, fruit production is a direct consequence of pollination and is independent of subsequent fertilization because pollen deposition triggers swelling of the ovary (O'Neill et al. 1993). As a consequence, estimates of fruit production represent indirect estimates of plant pollination success. Ripe fruits, when produced, were collected and stored in silica gel in order to prevent degradation. To ascertain the presence of viable embryos, at least 1000 seeds for each fruit were removed from the centre of the capsule and observed under an optical microscope with 100x magnification. Seeds were assigned to two categories (viable and unviable seeds) according to presence or absence of viable embryos. Fisher exact tests were used to compare the rate of fruit set between the different experiments. The statistical package SPSS (version 10, SPSS Inc. Chicago, USA) was used for analysis.

MOLECULAR ANALYSIS

For analysis of nuclear and chloroplast markers, genomic DNA was extracted using a slight modification of the (cetyltrimethyl ammonium bromide) CTAB protocol of Doyle and Doyle (1987). Several seeds, about 0.1g was separately pulverised in a 1.5-ml Eppendorf tube using 400 µl of standard CTAB buffer, incubated at 60° C for 30 min, extracted twice by adding 500 µl chloroform:isoamyl alcohol (24:1), precipitated with isopropanol and washed with 250 µl 70% ethanol. DNA was resuspended in 70 µl distilled water.

The nuclear ribosomal internal transcribed spacers (ITS1 and ITS2) were amplified by PCR using pairs of universal primers as described in Pellegrino et al. (2001) and in Taberlet et al. (1991), respectively (Figure. 22). All PCR reactions of 50 µl final volume contained 1 µl DNA template, 5 µm of each dNTP, 0.3 µl of each primer, 2 µl Taq polymerase, 2 µl $MgCl_2$ and 5 µl reaction buffer. PCR reactions were conducted in a thermal cycle (BIOMETRA) for 30 cycles. Initial conditions were as follows: 3' denaturation at 94°C, followed by 30 cycle of 30'' at 94°C, annealing at 55°C for 30'', extension at 72°C for 2'; extension time was increased to 3 s per cycle; extension was further prolonged

for 7 min at the end of the last cycle. Amplification products were electrophoretically separated on a 2% agarose gel (Methaphore, FMS) and photographed after ethidium bromide staining.

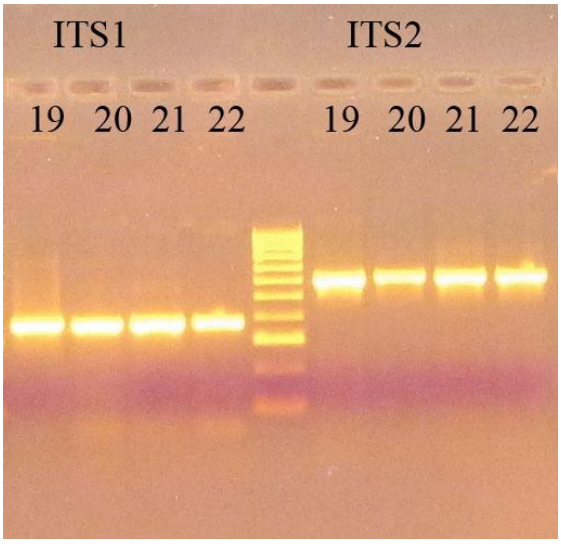

Fig. 22 Amplification products

Amplified fragments were sequenced in both directions using a modification of the Sanger dideoxy method as implemented in a double stranded DNA cycle sequencing system with fluorescent dyes. Sequence reactions were then run on a 373A Applied Biosystems Automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were examined using GeneJockey to find a restriction site that would distinguish them using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

Restriction enzyme *Sma*I, which cuts at 5'-CCC/GGG-3' differentiated the putative parental taxa due to the presence of a C/G substitution about 196 base pairs into the ITS2 sequence (C in *O. italica*, G in *O.anthropophora*), while *Pvu*II which cuts at 5'-CAG/CTG-3' showed a nucleotide substitution C/A about 60 base pairs into the ITS1 sequance (C in *O. italica*, A in *O.anthropophora*) (Figure. 23).

Thus, the PCR fragments of all samples (100 ng) were digested in a final 25μL volume with the selected restriction endonuclease (10μl DNA), according to the

manufacturer's instructions (Fermentas), in particular incubated for three hours at 30°C for *Sma*I and 37°C for PvuII. The fragments were electrophoretically separated on a 2% low melting agarose gel (Methaphore, FMS), compared to a 100 base pair (bp) ladder (Pharmacia Biotech) as the molecular weight marker (Figure. 24), stained with ethidium bromide and photographed using a Kodak digital camera. The relative amounts of DNA were estimated on digital photos analyzing them with the Biomax 10 image analysis software (Kodak Digital Science, EDAS, USA).

5' … C C C ↓ G G G … 3'
3' … G G G ↑ C C C … 5'
Sma I

5' … C A G ↓ C T G … 3'
3' … G T C ↑ G A C … 5'
Pvu II

Fig. 23 Restriction enzyme *SmaI* and *Pvu II*

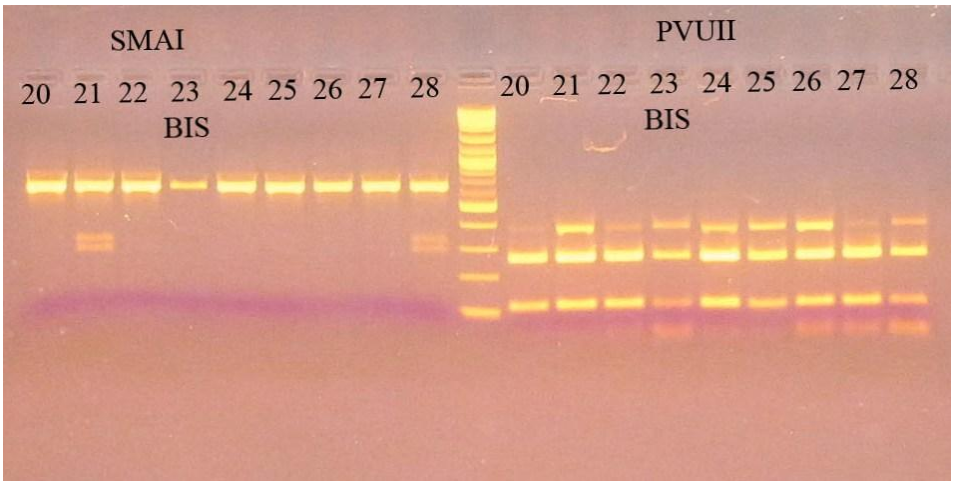

Fig. 24 Enzymatic digestion products

RESULTS

All of the manual crosses performed on plants of *O. italica* and *O. anthropophora* using homospecific and eterospecific pollen triggered the development of fruits. Indeed, the fruit set percentage from 96 interspecific (12 only with heterospecific pollen and 84 with both pollen) and 12 intraspecific crosses was 100% for both orchids.

Whereas the situation is different for percentage of seeds with embryo. We observed seeds from intraspecific crosses using an optical microscope and determined that 82% (*O. italica*) and 84% (*O. anthropophora*) of them contained viable embryos. In addition, for both species higher percentage of seeds with embryo was present in intraspecific than interspecific crosses. (Figure. 25 and 27). Indeed, fruits from interspecific crosses contained 55% of viable seeds for both taxa.

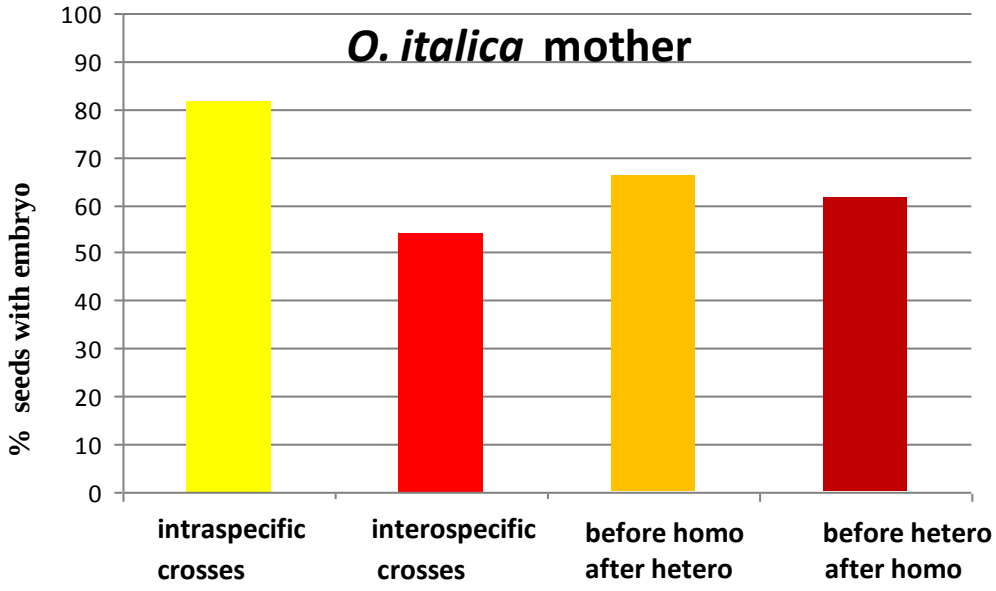

Fig. 25 Percentage of seeds with embryos in homo- and heterospecific crosses, considering *O. italica* mather.

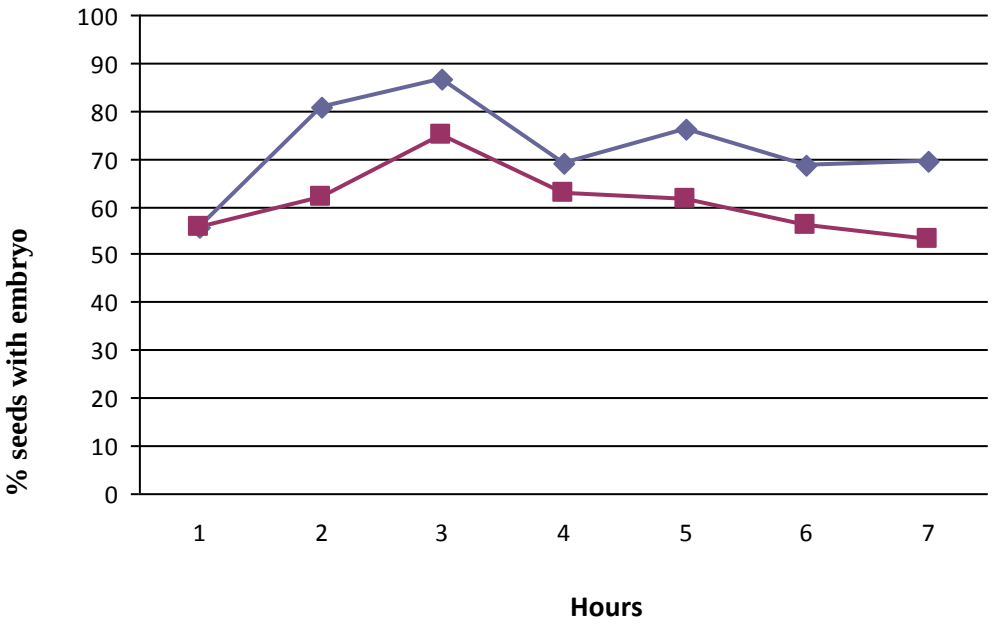

Fig. 26 The blue line shows the values of seeds with embryos when the homospecific pollen to come first hetero; the red line indicates the arrival of heterospecific pollen than homo, considering as *Orchis italica* as receiver.

Furthemore in the crosses in which flowers were pollinated with pollen from both species, we have the higher percentage of viable seeds when homospecific pollen arrives on stigma before than heterospecific one (Figure. 26 and 28) for both species.

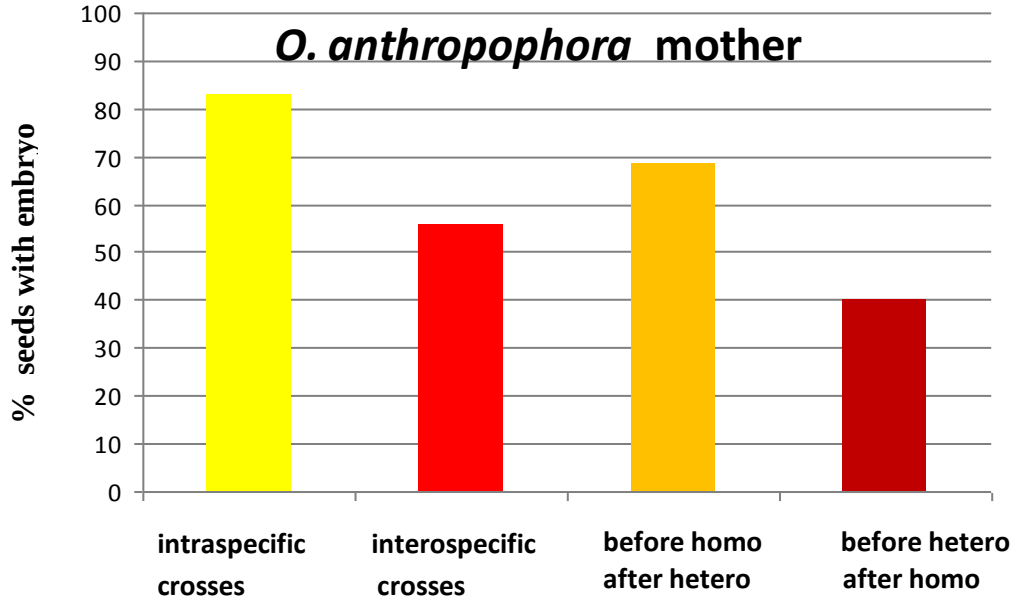

Fig. 27 Percentage of seeds with embryos in homo- and heterospecific crosses, considering *O. anthopophora* mather.

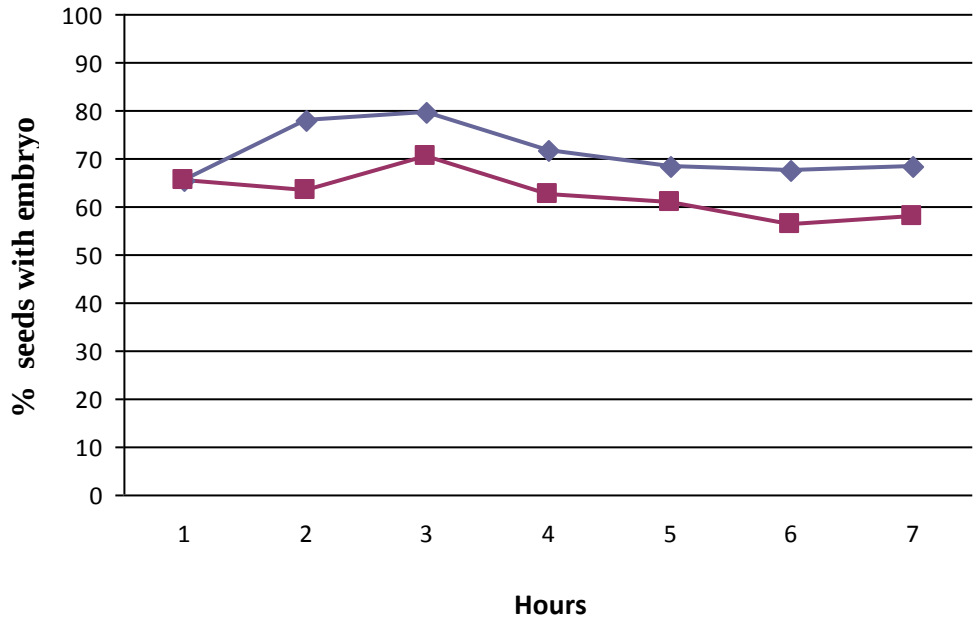

Fig. 28 The blue line shows the values of seeds with embryos when the homospecific pollen to come first hetero; the red line indicates the arrival of heterospecific pollen than homo, considering as *O. anthopophora* as receiver.

Molecular analysis has enabled to emphasize that none of seeds due to crosses in which the homospecific pollen preceded heterospecific one, is a hybrid seed, highlighting that the homospecific pollen in these cases has a total advantage on the other pollen (Table. 6)

Tab. 6 Results of manual crosses after the addition of homospecific pollen before the hetero at different times

O. anthropoprora WITH HOMOSPECIFIC POLLEN AT TIME "0"								
TIME ADDITION HETERO POLLEN	0h	1h	2h	4h	8h	16h	24h	48h
HYBRID FRUITS PRODUCTION	0	0	0	0	0	0	0	0

O. italica WITH HOMOSPECIFIC POLLEN AT TIME "0"								
TIME ADDITION HETERO POLLEN	0h	1h	2h	4h	8h	16h	24h	48h
HYBRID FRUITS PRODUCTION	0	0	0	0	0	0	0	0

The situation change when seeds comes from crosses in which heterospecific pollen has anticipated coming of homospecific pollen. Indeed just in 6 cases we have obtained hybrid seeds, three in the crosses in which *O. italica* has received pollen and three in the cases in which *O. anthopophora* acting as maternal line (Table. 7). In detail in the crosses in which *O. italica* has received the pollen we had two hybrid seeds when conspecific pollen was added after one hour and another hybrid seed when the pollen was added after 24 hours; in the crosses in which *O. anthropophora* received the pollen we obtained two capsules with hybrid seeds when homospecific pollen was added after two hours and one fruit with hybrid seeds h omospecific pollen was added after 4 hours (Table. 7).

Tab.7 Results of manual crosses after the addition of heterospecific pollen before the homo at different times

O. anthropoprora WITH HETEROSPECIFIC POLLEN AT TIME "0"								
TIME ADDITION HOMO POLLEN	0h	1h	2h	4h	8h	16h	24h	48h
HYBRID FRUITS PRODUCTION	0	0	2	1	0	0	0	0

O. italica WITH HETEROSPECIFIC POLLEN AT TIME "0"								
TIME ADDITION HOMO POLLEN	0h	1h	2h	4h	8h	16h	24h	48h
HYBRID FRUITS PRODUCTION	0	2	0	0	0	0	1	0

It should be emphasized, except this 6 cases, the remaining 78 fruits (92.80% of crosses) are all the result of fertilization due to the homospecific pollen when it was added before or that it was added after the heterospecific pollen.

DISCUSSION

Hybrid zones are natural laboratories for studying reproductive isolation mechanisms among closely related species, the role of selection in maintaining or eroding species differences, and the role of hybridisation in plant evolution (Rieseberg and Buerkle 2002; Lexer et al. 2005).

For most plant species, several mechanism act in concert to prevent or reduce hybridization with other species (Ramsey et al., 2003). One such mechanism is conspecific pollen advantage (CPA), which refers to the advantage of conspecific over heterospecific pollen in fertilization and production of offspring.

This study has shown that in a pair of Mediterranean deceptive orchids there is always an advantage of homospecific pollen to fruit formation, whether it comes before or after heterospecific pollen. The stigma of orchids studied, thus remains viable for at least two days before the commencement of the stages of fertilization of ovules. Therefore, even if the heterospecific pollen is deposited 48 hours prior to the homospecific the latter “overtakes” the heterospecific ensuring the formation of homospecific seeds. In our case, there is no asymmetrical CPA as described in other studies (Diaz and McNair, 1999; Chapman et al., 2005).

In many plant species, various mechanisms are put in place to prevent or reduce the formation of hybrids. In our case, the mechanism of the “advantage of conspecific pollen” understood as greater capacity for fertilization of homospecific pollen than to the heterospecific, it prevents the formation of hybrid individuals. Noticeably, in such zones, a high frequency of hybrids could be expected because *O. italica* and *O. anthropophora* lack any form of postmating pre-zygotic isolation mechanism (Pellegrino et al. 2009). However, hybrids were few and backcross generations were absent (Pellegrino et al. 2009). Indeed, *O. italica* and *O. anthropophora* frequently hybridize but in all hybrid zone the number of hybrids is low. Ad example, in our examined population we have found just 8 individuals. Given proximity sufficient to allow individual pollinators to visit flowers of the two species a large amount of hybridization is likely to occur in nature. Because of the conspecific pollen advantage detected here, if flower receives both homospecific and

heterospecific pollen we have high probability to obtain homospecific seeds, while to find a hybrid it needs that flower receive only heterospecific pollen.

Our data are in agreement with other observations on hybrid zones between *Orchis* species, which have regularly found a small number of hybrids (max. 40), derived by bi-directional interbreeding and mostly belonging to the F1 generation (Aceto et al. 1999; Pellegrino et al. 2000, 2005, 2009; Cozzolino et al. 2006).

Previous studies have measured CPA between interfertile species and have addressed the problem of conflict between homospecific and heterospecific pollen load demonstrating the presence of advantage of conspecific pollen (Alarcon & Campbell, 2000; Campbell et al. 2003) or the absence of advantage (Chapman et al 2005).

This is the first work that evalues the effects of fertilization of different pollens in relation to the time of arrival of pollen on the stigma.

The CPA exhibited by *O. italica* and *O. anthropophora* is likely to result from reduced germination of heterospecific pollen or above all retarded growth of heterospecific pollen tubes in the stigma and ovary.

This study suggest that, in the absence of ethological or mechanical barriers between sympatric hybridizing species, the observed low inter-specific gene flow and lack of backcross generations could be explained by a strong post-zygotic barrier such as hybrid sterility (Pellegrino et al. 2009), but the low number of hybrids could be related to conspecific pollen advantage that reduce the probability of formation of hybrid seeds. Overall, the results indicate that our hybrid zone represents a phenomenon of little evolutionary consequence, and that the few hybrid plants will not easily produce descendants with potential new genetic combinations and/or ecological preferences. Moreover, the genetic integrity of *O. italica* and *O. anthropophora* species is not eroded when in contact thanks to effects of conspecific pollen advantage.

Our results show that in a pair of Mediterranean food-deceptive orchids act different kind of reproductive barrier: pre-zygotic barriers (Cozzolino and Widmer 2005), and late post-zygotic mechanisms (Scopece et al. 2007). Indeed, to reduce loss of ovules, orchid wait for long time the arrival of conspecific pollen also if flower has yet received heterospecific pollen. For

these species pollen competition appears to function as a early post-pollination pre-zygotic barrier to hybridization.

REFERENCES

Aceto, S., Caputo, P., Cozzolino, S., Gaudio, L., and Moretti, A., 1999. Phylogeny and evolution of *Orchis* and allied genera based on ITS DNA variation: Morphological gaps and molecular continuity. Molecular Phylogenetics and Evolution, 13: 67–76.

Ackerman, J.D., 1986. Mechanisms and evolution of food deceptive pollination systems in orchids. Lindleyana, 1: 108–113.

Acquafredda, P., Palmentola, G., 1986. Il glacialismo quaternario nell'Italia meridionale. Biogeografia, 10: 13–8.

Alarcòn, R., and Campbell, D.R., 2000. Absence of conspecific pollen advantage in the dynamics of an *Ipomopsis* (Polemoniaceae) hybrid zone. American Journal of Botany, 87: 819–824.

Aldridge, G., and Campbell, D.R., 2006. Asymmetrical pollen success in *Ipomopsis* (Polemoniaceae) contact sites. American Journal of Botany, 93: 903-909.

Alexander, C., Hadley, G., 1985. Carbon movement between host and mycorrhizal endophyte during the development of the orchid *Goodyera repens* Br. New Phytologist, 101: 657–665.

Allendorf, F.W., Leary, R.F., Spruell, P., Wenburg, J.K., 2001. The problems with hybrids: setting conservation guidelines. Trends Ecology Evolution, 16: 613–622.

Ames, O., 1937. Pollination of orchids through pseudocopulation. Botanical Museum Leaflets 5: 1–28.

Anderson, E., 1948. Hybridization of the habitat. Evolution, 2: 1-9.

Angiosperm Phylogeny Group 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. Botanical Journal of the Linnean Society ,161(2): 105–121.

Arditti, J., and Ghani, A., 2000. Tansley review No. 110; Numerical and physical properties of orchid seeds and their biological implications. New Phytologist, 145: 367-421.

Arduino, P., Verra, F., Cianchi, R., Rossi, W., Corrias, B., Bullini, L., 1996. Genetic variation and natural hybridization between *Orchis laxiflora* and *Orchis palustris* (Orchidaceae). Plant Systematic Evololution, 202: 87–109.

Arnold, M.L., 1993. *Iris nelsonii* (Iridaceae): origin and genetic composition of a homoploid hybrid species. American Journal of Botany, 80: 577– 583.

Arnold, M.L., 1997. Natural hybridization and evolution. Oxford University Press, New York, New York, USA.

Ayasse, M., Schiestl, F.P., Paulus, H.F, Lofstedt, C., Hanson, B.S., Ibarra, F. and Francke, W., 2000. Evolution of reproductive strategies in the sexually deceptive orchid *Ophrys sphegodes* : how does flower-specific variation of odor signals influence reproductive success? Evolution, 54: 1995–2006.

Ayasse, M., Schiestl, F.P., Paulus, H.F., Ibarra, F., and Francke, W., 2003. Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270: 517–522.

Barton, N.H., 2001. The role of hybridization in evolution. Molecular Ecology, 10: 551–568.

Barton, N.H., Hewitt, G.M., 1985. Analysis of hybrid zones., Annual Review of Ecology, evolution and Systematics, 16: 113-148.

Bateman, R.M., Hollingsworth, P.M., Preston, J., Yi-Bo, L., Pridgeon, A.M., Chase, M.W., 2003. Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). Botanical Journal of the Linnean Society, 142: 1–40.

Bateman, R.M, Smith, R.J, Fay M.F., 2008 Morphometric and population genetic analyses elucidate the origin, evolutionary significance and conservation implications of *Orchis* ×*angusticruris* (*O. purpurea* × *O. simia*), a hybrid orchid new to Britain. Botanical Journal of the Linnean Society, 157: 687–711.

Bellusci, F., Pellegrino, G., Palermo, AM., Musacchio, A., 2010. Crossing barriers between the unrewarding Mediterranean orchids *Serapias vomeracea* and *S. cordigera*. Plant Species Biology, 25: 68-76.

Bergström, G., 1978. Role of volatile chemicals in *Ophrys* pollinator interactions. In Biochemical aspects of plant and animal co-evolution (ed. J. B. Harborne), pp. 207–232.

Bernard, N., 1902. E´ tudes sur la tubérization. Revue Générale de Botanique, 14: 1–92.

Beyrle, H.F., Smith, S.E., Peterson, R.L., Franco, C.M.M., 1995. Colonization of *Orchis morio* protocorms by a mycorrhizal fungus: effects of nitrogen nutrition and glyphosate in modifying the responses. Canadian Journal of Botany, 73: 1128–1140.

Bianco, P., Medagli, P., D' Emerico, S., Ruggiero, L., 1987. Numeri cromosomici per la flora Italiana. Inf Botanica Italiana, 19: 322-332.

Bidartondo, M.I, Read, D.J., 2008. Fungal specificity bottlenecks during orchid germination and development. Molecular Ecology, 17: 3707-3716

Bino, R.J., Dafni, A. and Meeuse, A.D.J., 1982. The pollination ecology of *Orchis galilaea* (Bronm. et Schulze) Schltr. (Orchidaceae). New Phytologist, 90: 315–319.

Blanco, M.A., and Barboza, G., 2001. Polinizaciònen Lepanthes : un Nuevo caso de pseudocopulaciòn en las orquìdeas. Abstract of the Conference, 2do Seminario Mesoamericano de Orquideologìa y Conservaciòn. San Josè, Costa Rica, 23 al 26 de mayo, 2001.

Blanco, M.A., and Barboza, G., 2005. Pseudocopulatory pollination in *Lepanthes* (Orchidaceae : Pleurothallidinae) by fungus gnats. Annals of Botany 95 : 763–772.

Bonnardeaux, Y., Brundrett, M., Batty, A., Dixon, K., Koch, J., Sivasithamparam, K., 2007. Diversity of mycorrhizal fungi of terrestrial orchids: Compatibility webs, brief encounters, lasting relationships and alien invasions. Mycological Research, 111: 51-61.

Borg-Karlson, A. K., 1990. Chemical and ethological studies of pollination in the genus *Ophrys* (Orchidaceae). Phytochemistry, 29:1359–1387.

Bower, C.C., 1996. Demonstration of pollinator-mediated reproductive isolation in sexually deceptive species of *Chiloglottis* (Orchidaceae: Caladeniinae). Australian Journal of Botany, 44: 15–33.

Bradshaw, H.D., Otto, K.G., Frewen, B.E., McKay, J.K., Schemske, D.W., 1998. Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*). Genetics, 149: 367–382.

Bremer, K., 1994. Asteraceae: Cladistics and Classification. Timber Press, Portland OR.

Brown, B. J., Mitchell R. J., 2001. Competition for pollination: effects of pollen of an invasive plant on seed set of a native congener. Oecologia, 129: 43-49.

Brown, P., 2002. Wild Orchids of Florida. University of Florida Press, Florida.

Brundrett, M., 2002. Coevolution of roots and mycorrhizas of land plants. New Phytologist, 154: 275-304.

Brundrett, M.C, Scade, A., Batty, A,L., Dixon, K,W., Sivasithamparam K. 2003. Development of in situ and ex situ seed baiting techniques to detect mycorrhizal fungi from terrestrial orchid habitats. Mycological Research, 107: 1210–1220.

Buerkle, C.A., Morris, R.J., Asmussen, M.A., Rieseberg, L.H., 2000. The likelihood of homoploid hybrid speciation. Heredity, 84: 441–451.

Cafasso, D., Widmer, A., Cozzolino, S., 2005. Chloroplast DNA inheritance in the orchid *Anacamptis palustris* using single-seed polymerase chain reaction. Journal of Heredity, 96: 66-70.

Cameron, D.D., Johnson, I., Leake, J.R., and Read, D.J., 2007. Mycorrhizal acquisition of inorganic phosphorus by the green-leaved terrestrial orchid *Goodyera repens*. Annual of Botany, 99: 831-834.

Cameron, D.D., Leake, J.R., and Read, D.J., 2006. Mutualistic mycorrhiza in orchids: evidence from plant–fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. New Phytology, 171: 405- 416.

Cameron, K.M., 2004. Utility of plastid psaB gene sequences for investigating intrafamilial relationships within Orchidaceae. Molecular Phylogenetics and Evolution, 31: 1157–1180.

Cameron, K.M., Chase, M.W., Whitten, W.M., Kores, P.J., Jarrell, D.C., Albert, V.A., Yukawa, T., Hills, H.G., and Goldman, D.H., 1999. A phylogenetic analysis

of the Orchidaceae: evidence from rbcL nucleotide sequences. American Journal of Botany, 86: 208–224.

Campbell, D.R.R., Alarcón, C.A., Wu, 2003. Reproductive isolation and hybrid pollen disadvantage in *Ipomopsis*. Journal of Evolutionary Biology, 16:536-540.

Carney, S.E., and Arnold, M.L., 1997. Differences in pollen-tube growth rate and reproductive isolation between *Louisiana irises*. Journal of Heredity, 88: 545–549.

Carney, S.E., Cruzan, M.B., and Arnold, M.L., 1994. Reproductive interactions between hybridising *irises*: analysis of pollen-tube growth and fertilization success. American Journal of Botany, 81: 1169–1175.

Carney, S.E., Hodges, S.A., and Arnold, M.L., 1996. Effects of differential pollen-tube growth on hybridization in the *Louisiana irises*. Evolution, 50: 1871–1878.

Castellanos, M.C., Wilson, P., and Thomson , J. D., 2004. ‘ Antibee ’ and ‘ pro-bird ’ changes during the evolution of hummingbird pollination in *Penstemon* flowers. Journal of Evolutionary Biology, 17: 876 – 885.

Cauwet-Marc, A.M, Balayer, M., 1986. Les Orchidees du bassin Mediterraneen. Contribution a l'etude caryolgique des especes des Pyrenees-orientales (France) et contrees limitrophes. II - tribu des Ophrydae Lindl. pro parte. Bulletin de la Societe Botanique de France, Lettres Botanique, 133: 265-277.

Chapman, M.A., Forbes, D.G., and Abbott, R.J., 2005. Pollen competition among two species of *Senecio* (Asteraceae) that form a hybrid zone on Mt. Etna, Sicily. American Journal of Botany, 92: 730-735.

Chase, M.W., Barrett, R.L., Cameron, K.M., and Freudenstein, J.V., 2003. DNA data and Orchidaceae systematics: a new phylogenetic classification. In ixon, K.M. (Ed.): Orchid conservation, pp 69–89.

Chase, M.W., Cameron, K.M., Hills, H.G., and Jarrell, D., 1994. Molecular systematics of the Orchidaceae and other lilioid monocots. In Pridgeon, A. (Ed.): Proceedings of the 14th World Orchid Conference, 61–73.

Chase, M.W., Cowan, R.S., Hollingsworth, P.M., Van Den Berg, C., Petersen, G., Seberg Orgsensen, T., Cameron, K.M., Carine, M., Pedersen, N., Hedderson, T.A.J., Conrad, F., Salazar, G.A., Richardson, J.E., Hollingsworth, M.L., Barraclough, T.G., Kelly, L., Wilkinson, M., 2007. A proposal for a standardised protocol to barcode all land plants. Taxon, 56: 295-299.

Chase, M.W., Paun, O., Fay, M.F., 2010. Hybridization and speciation in angiosperms: a role for pollinator shifts? BMC Biology, 8: 45-47.

Clayton, S., and Aizen, M. 1996. Effects of pollinia removal and insertion on flower longevity in *Chlorea alpina* (Orchidaceae). Evolutionary Ecology, 10: 653-660.

Clements, M.A., 1988. Orchid mycorrhizal associations. Lindleyana, 3: 73–86.

Coleman, E., 1927. Pollination of the orchid *Cryptostylis leptochila*. Victorian Naturalist 44 C.

Coleman, E., 1928. Pollination of *Cryptostylis leptochila*. Victorian Naturalist 44: 333–340.

Comes, P.T., Kadereit, J.W., 1998. The effect of quaternary climatic changes on plant distribution and evolution. Trends in Plant Science, 3: 432-438.

Correvon, H., Pouyanne, M., 1916. Un curieux cas demimetisme chez lez Ophryde es. Journal de la Societè Nationale d'Horticulture de France, 17: 29–31.

Cortesi, F., 1907. New or rare orchids. Annual of Botany, 5: 539-545.

Costantinidis, T., Kamari, G., Phitos, D., 1997. A cytological study of 28 phanerogams from the mountains of SE Sterea Ellas, Greece. Willdenowia, 27: 121-142.

Cozzolino, S., D' Emerico, S., and Widmer, A., 2004 . Evidence for reproductive isolate selection in Mediterranean orchids: Karyotype differences compensate for the lack of pollinator specifi city. Proceedings of the Royal Society of London, B, Biological Sciences, 271 : 259 – 262 .

Cozzolino, S., Nardella, A.M., Impagliazzo, S., Widmer, A., and Lexer, C., 2006. Hybridization and conservation of Mediterranean orchids: Should we protect the orchid hybrids or the orchid hybrid zones? Biological Conservation, 129 : 1.

Cozzolino, S., and Widmer, A., 2005. Orchid diversity: An evolutionary consequence of deception? Trends in Ecology & Evolution, 20: 487 – 494.

Curtis, J.T., 1939. The relation of specificity of orchid mycorrhizal fungi to the problem of symbiosis. American Journal of Botany, 26: 390–399.

Dafni, A., 1984. Mimicry and deception in pollination. Annual Review of Ecology, evolution and Systematics, 15: 259–278.

Dafni, A., 1987. Pollination in *Orchis* and related genera: evolution from reward to deception. In Orchid Biology: Reviews and Perspectives 4 (ed. J. Arditti), pp. 79–104. Cornell University Press, Ithaca

Dafni,, A., and Bernhardt, P., 1990. Pollination of terrestrial orchids of Southern Australia and the Mediterranean region. Systematics, ecological, and evolutionary implications. In Evolutionary Biology, Vol. 24 (eds. M. K. Hecht, B. Wallace and R. J. Macintyre), pp. 193–252. Plenum Publishing Corporation, New York.

Darwin, C., 1859. On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. J. Murray Press, London.

Darwin, C., 1862. On the Various Contrivances by which British and Foreign Orchids are Fertilised by Insects. John Murray, London.

Darwin, C., 1877. On the various contrivances by which orchids are fertilized by insects. London: John Murray.

Dearnaley, J. D.W., 2007. Further advences in orchid mycorrhizal research. Mycorrhiza, 17: 475-486.

Delforge P., 2005. Orchids of Europe, North Africa and the Middle East.

Del Prete, P., Miceli, C., 1981. *Orchis colemanii* Cortesi natural hybrid between *Orchis mascula* (L.) L. and *Orchis pauciflora* Ten. Giornale Botanico Italiano. 115: 396-397.

D'Emerico, S., Cozzolino, S., Pellegrino, G., Pignone, D., Scrugli, A., 2002. Heterochromatin distribution in selected taxa of the 42-chromosomes *Orchis* s.l. (Orchidaceae). Caryologia, 55: 55-62.

Diaz, A., MacNair, M.R., 1999. Pollen tube competition as a mechanism of prezygotic reproductive isolation between *Mimulus nasutus* and its presumed progenitor *M. guttatus*. New phytologist, 144: 471-478.

Dixon, K., 1991. Seeder/ clonal concepts in Western Australian orchids. In Wells, T., and Willems, J (eds). Population Ecology of Terrestrial Orchids. SPB Academic Publishing, Netherlands, 111-123.

Dixon, K., and Barret, R. 2003. Defining the role of fire in south-west Western Australian plants. In (eds.) Abbott, I., and Burrowx, N. Fire in Ecosystems of South-West Western Australia: Impacts and Management. Backhuys Publishers, Leiden, The Netherlands, 205-223.

Dixon, K., Kell, S., Barret, R., and Cribb, P. 2003. Orchid conservation: a global perspective. In, Dixon, K., Kell, S., Barret, R., and Cribb, P. (eds.) Orchid Conservation. Natural History Publications, Borneo.

Dobson, H.E.M., 1994. Floral volatiles in insect biology. In Insect-Plant Interactions (Bernays, E. A., ed.), Boca Rato: CRC Press, pp.47–81.

Dod, D.D., 1976. *Oncidium henekenii* – bee orchid pollinated by bee. American Orchid Society Bulletin 45: 792–794.

Dodson, C.H., 1975. Coevolution of orchids and bees. In Coevolution of Animals and Plants (eds. L. E. Gilbert and P. H. Raven), pp. 91–99. Univ. of Texas Press, Austin.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bulletin, 19: 11-15.

Dressler, R., 1968. Pollination by euglossine bees. Evolution, 22: 202–210.

Dressler, R., 1981. Orchids – natural history and classification. 1st Edn. Hardvard University Press, Cambridge, Massachusetts.

Dressler, R.L., 1993. Phylogeny and classification of the orchid family. Cambridge University Press, Cambridge.

Ehrendorfer, F., 1980. Hybridisierung, Polyploidie und Evolution bei europ¨aisch-mediterranen Orchideen. Die Orchidee Sonderheft, 15–34.

Ellstrand, N.C., Schierenbeck, K.A., 2000. Hybridization as a stimulus for the evolution of invasiveness in plants. Proceedings of the National Academy of Sciences, 97: 7043– 7050.

Ellstrand, N.C., Whitkus, R., and Rieseberg, L.H., 1996. Distribution of spontaneous plant hybrids. Proceedings of the National Academy of Sciences, 93: 5090 – 5093.

Ferdy, J.B., Austerlitz, F., 2002. Extinction and introgression in a community of partially cross-fertile plant species. The American Naturalist, 160: 74–86.

Feuerherdt, L., Petit, S., Jusaitis, M.. 2005. Distribution of mycorrhizal fungus associated with the endangered pink-lipped spider orchid [Arachnorchis (syn. Caladenia) behrii] at Warren Conservation Park in South Australia. New Zealand Journal of Botany, 43: 367–371.

Franklin-Tong, V.E., 1999. Signaling and the modulation of pollen tube growth. Plant Cell, 11:727–738.

Freudenstein, J.V. and Rasmussen, F., 1999. What does morphology tell us about orchid relationships? – a cladistic analysis. American Journal of Botany, 86: 225–248.

Freudenstein, J.V., van den Berg, C., Goldman, D.H., Kores, P.J., Molvray, M. and Chase, M.W., 2004. An expanded plastid DNA phylogenetic analysis of Orchidaceae and analysis of jackknife clade support strategy. American Journal of Botany, 91: 149–157.

Grant, V., 1994. Modes and origins of mechanical and ethological isolation in angiosperms. Proceedings of the National Academy of Sciences of the United States of America, 1: 3-10.

Gross, B.L., Rieseberg, L.H., 2005. The ecological genetics of homoploid hybrid speciation. Journal of Heredity, 96: 241-252.

Hadley, G., Purves, S., 1974. Movement of 14carbon from host to fungus in orchid mycorrhiza. New Phytologist, 73: 475–482.

Harrison, R.G., 1990. Hybrid zones: windows on evolutionary process. Oxford Surveys in Evolutionary Biology (D. J. Futuyma and J. Antonovics, eds.), 7: 69–128.

Hauser, T., Jorgensen, R., and Ostergard, H., 1997. Preferential exclusion of hybrids in mixed pollinations between oilseed rape (*Brassica napus*) and weedy *B. campestris* (Brassicaceae). American Journal of Botany.

Hegarty, M.J. and Hiscock, S.J., 2005. Hybrid speciation in plants: new insights from molecular studies. New Phytologist, 165: 411–423.

Henrion, B., Le Tacon, F., Martin, F., 1992. Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. New Phytologist, 122: 289-298.

Hewitt, G.M., 2001. Speciation, hybrid zones and phylogeography – or seeing genes in space and time. Molecular Ecology, 10: 537–549.

Hodges, S.A., Burke, J.M., and Arnold, M.L., 1996. Natural formation of *Iris* hybrids: Experimental evidence on the establishment of hybrid zones. Evolution, 50: 2504 – 2509.

Hollick, P.S., Taylor, R.J., McComb, J.A., Dixon, K.W., 2005. If orchid mycorrhizal fungi are so specific, how do natural hybrids cope? Selbyana, 26: 159–170.

Howard, D.J., 1999. Conspecific sperm and pollen precedence and speciation. Annual Review of Ecology, Evolution and Systematics, 30: 109–132.

Irwin, M.J,, Bougoure, J.J., Dearnaley, J.D.W., 2007. *Pterostylis nutans* (Orchidaceae) has a specific association with two Ceratobasidium root associated fungi across its range in Eastern Australia. Mycoscience, 48: 231-239.

Jacquemyn, H., Brys, R., Cammue, B.P.A., Honnay, O., Lievens, B., 2011. Mycorrhizal associations and reproductive isolation in three closely related *Orchis* species. Annals of Botany, 107: 347-356.

Jacquemyn, H., Brys, R., Honnay, O., Roldàn-Ruiz, I., Lievens, B., Wiegand, T., 2012. Nonrandom spatial structuring of orchids in a hybrid zone of three *Orchis* species. New Phytologist, 193, 454–464.

Jacquemyn, H., Honnay, O., Cammue, B.P.A., Brys, R., Lievens, B., 2010. Low specificity and nested s,ubset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis.* Molecular Ecology, 19: 4086-4095.

Jersáková, J., Johnson, S.D., Kindlmann, P., 2006. Mechanisms and evolution of deceptive pollination in orchids. Biological Reviews, 81: 219–235.

Johnson, S.D., and Dafni, A., 1998. Response of bee-flies tothe shape and pattern of model flowers: implications for floral evolution in a Mediterranean herb. Functional Ecology ,12: 289–297.

Johnson, S.D., and Midgley, J., 1997. Fly pollination of *Gorteria diffusa* (Asteraceae), and a possible mimetic function for dark spots on the capitulum. American Journal of Botany.

Julou, T., Burghardt, B., Gebauer, G., Berveiller, D., Damesin, C., Selosse, M.A., 2005. Mixotrophy in orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of Cephalanthera damasonium. New Phytologist, 166: 639–653.

Klips, R.A., 1998. Pollen competition as a reproductive isolating mechanism between two sympatric *Hibiscus* species (Malvaceae). American Journal of Botany.

Koopowitz, H., Lavarack, P., and Dixon, K., 2003. The nature of Threats to orchid conservation. In: Dixon, K., Kell, S., Barrett, R., and Cribb, P. (eds). Orchid Conservation. Kota Kinabalu, Sabah: Natural History Publications, 25-42.

Kullenberg, B., 1961. Studies in *Ophrys* pollination. Zoologiska Bidrag Fran Uppsala, 34:1–340.

Kullenberg, B., and Bergoström, M,G., 1973. The pollination of *Ophrys* orchids. In Chemistry in Botanical Classification (eds. G. Bendz and J. Santesson), pp. 253–258. Proceedings Nobel Symposium 25, Stockholm.

Kullenberg, B., and Bergoström, G., 1976b. Pollination of *Ophrys* orchids. Botaniska Notiser, 129:11–19.

Kullenberg, B., Borg-Karlson, A.K., and Kullenberg, A.Z., 1984. Field studies on the behaviour of the *Eucera nigrilabris* male in the odour flow from flower labellum extract of *Ophrys tenthredinifera*. Nova Acta Regiae Societatis Scientiarum Upsaliensis Ser. V, 3: 79–110.

Lang, D., 1980. Orchids of Britain. Oxford University Press, Oxford, UK.

Lau, CPY., Ramsden, L., Saunders, RMK., 2005. Hybrid origin of *Bauhinia blakeana* (Leguminosae: Caesalpinioideae), inferred using morphological, reproductive, and molecular data. American Journal of Botany, 92: 525–533.

Leake, J.R., 1994. The biology of myco-heterotrophic (saprophytic) plants. New Phytologist,127:171-216.

Lexer, C., Fay, M.F., Joseph, J.A., Nica, M.S., Heinze, B., 2005. Barrier to gene flow between two ecologically divergent Populus species, *P. alba* (white poplar) and *P. tremula* (European aspen): the role of ecology and life history in gene introgression. Molecular Ecology, 14: 1045-1057.

Lexer, C., Randell, R.A., Rieseberg, L.H., 2003a. Experimental hybridization as a tool for studying selection in the wild. Ecology, 84: 1688–1699.

Lexer, C., Welch, M.E., Raymond, O., Rieseberg, L.H., 2003b. The origin of ecological divergence in *Helianthus paradoxus* (Asteraceae): selection on transgressive characters in a novel hybrid habitat. Evolution, 57: 1989–2000.

Luca, A., Bellusci, F., Menale, B., Musacchio, A., and Pellegrino, G., 2012. *Orchis* × *colemanii* hybridization: Molecular and morphological evidence, seed set success, and evolutionary importance. Flora, 10: 753-761.

Mallet, J., 2005. Hybridization as an invasion of the genome. Trends in Ecology & Evolution, 20: 229 – 237.

Mallet, J., 2008. Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. Phil. Trans. R. Soc. Lond. B: Biological Sciences, 363: 2971-2986.

Mant, J., C. Br¨andli, N. J. Vereecken, C. M. Schulz, W. Francke, and Schiestl, F.P., 2005a. Cuticular hydrocarbons as sex pheromone of the bee *Colletes cunicularius* and the key to its mimicry by the sexually deceptive orchid, *Ophrys exaltata*. Journal of Chemical Ecology, 31:1765–1787.

Mant, J., Peakall, R., and Schiestl, F.P.,2005b. Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus *Ophrys*? Evolution, 59:1449–1463.

Marques, I., Rossello´ -Graell, A., Draper, D. and Iriondo, J.M., 2007. Pollination patterns limit hybridization between two sympatric species of *Narcissus* (Amaryllidaceae). American Journal of Botany, 94: 1352– 1359.

Masuhara, G., Katsuya, K., 1994. In situ and in vitro specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon) Ames var Amoena (M. Bieberstein) Hara (Orchidaceae). New Phytologist, 127: 711–718.

Mattila, E., Kuitunen, M.T., 2000. Nutrient versus pollination limitation in *Platanthera bifolia* and *Dactylorhiza incarnata*. Oikos, 89: 360-366.

Mayr, E., 1942. Systematics and the origin of species. New York: Columbia University Press.

Mayr, E., 1992. A local flora and the biological species concept. American Journal of Botany, 72: 222–238.

McCormick, M.K., Whigham, D.F., O' Neil, J., 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. New Phytologist, 163: 425-438.

McCormick, M.K., Whigham, D.F., Sloan, D., O' Malley, K., Hodkinson, B., 2006. Orchid – fungus fidelity: A marriage meant to last? Ecology, 87: 903-911.

McKendrick, S.L., Leake, J.R., Taylo,r D.L., and Read, D.J., 2002. Symbiotic germination and development of the mycoheterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed Sebacina spp. New Phytologist, 154:233-247.

Millar, C.I., 1993. Impact of the eocene on the evolution of *Pinus* L. Annals Missouri Botanical Garden, 37: 311–319.

Moccia, M.D., Widmer, A., and Cozzolino, S., 2007. The strength of reproductive isolation in two hybridizing food-deceptive orchid species. Molecular Ecology, 16 : 2855 – 2866.

Molvray, M., Kores, P., Chase, M.W., 2000. Polyphyly of mycoheterotrophic orchids and functional influences on floral and molecular characters. In: Wilson KL, Morrison DA, eds. Monocots: systematics and evolution. Melbourne: CSIRO.

Moore, W.S., 1977. An evaluation of narrow hybrid zones in vertebrates. Quarterly Review of Biology, 52: 263-267.

Nazzaro, R., Menale, B., Di Novella, N., 1995. Le Orchidaceae della zona occidentale del Vallo di Diano (Salerno). Webbia, 50: 25-35.

Nilsson, L.A., 1983. Anthecology of *Orchis mascula* (Orch.). Nordic Journal of Botany, 3: 157-179.

Norušis, M.J., 2005. SPSS 14.0 Advanced Statistical Procedures Companion. Upper Saddle, Prentice Hall, New York.

Ogura-Tsujita, Y., Yukawa, T., 2008. High mycorrhizal specificity in a widespread mycoheterotrophic plant, *Eulophia zollingeri* (Orchidaceae). American Journal of Botany, 95: 93-97.

O'Neill, S.D., Nadeau, J.A., Zhang, X.S., Bui, A.Q., Halevy, A.H., 1993. Interorgan regulation of ethylene biosynthetic genes by pollination. Plant Cell, 5: 419–432.

Pacini, E., 1997. Tapetum character states: analytical keys for tapetum types and activity. Canadian Journal of Botany, 75: 1448-1459.

Pacini, E., and Franchi, G.G., 1998. Pollen dispersal unit, gynoecium and pollination. In Reproductive biology (eds. S. J. Owens and P. I. Rudall), pp. 183–195. Royal Botanic Gardens, Kew.

Pacini, E., and Franchi, G.G., 2000. Types of pollen dispersal units in Monocots. In:Wilson KL, Morrison DA, eds. Monocots: systematics and evolution. Melbourne: CSIRO, 295-300.

Pacini, E., and Hesse, M., 2002. Types of pollen dispersal units in orchids, and their consequences for germination and fertilization. Annals of Botany, 89: 653–664.

Paialek, J., Barton, N.H., 1997. The spread of an advantageous allele across a barrier: the effects of random drift and selection against heterozygotes. Genetics, 145: 493–504.

Parsons, R., and Hopper, S., 2003. Monocotyledenous geophytes: comparison of south-western Australia with other areas of Mediterranean climate. Australian Journal of Botany, 51: 129-133.

Paulus, H.F., and Gack, C., 1990. Pollinators as prepollinating isolation factors: evolution and speciation in *Ophrys* (Orchidaceae). Israel Journal of Botany 39: 43–79.

Paulus, H.F., and Gack, C., 1990a. Pollination of *Ophrys* (Orchidaceae) in Cyprus. Plant Systematics and Evolution, 169: 177–207.

Paulus, H.F., and Gack, C., 1990b. Pollinators as prepollinating isolation factors—evolution and speciation in *Ophrys* (Orchidaceae). Israel Journal of Botany, 39: 43–79.

Paun, O., Fay, MF., Soltis, DE., Chase, MW., 2007. Genetic and epigenetic alterations after hybridization and genome doubling. Taxon, 56: 649-656.

Peakall, R., 1990. Responses of male *Zaspilothynnus trilobatus* Turner wasps to females and the sexually deceptive orchid it pollinates. Functional Ecology, 4: 159–167.

Peakall, R., and Beattie, A. J., and James, S. H., 1987. Pseudocopulation of an orchid by male ants: a test of two hypotheses accounting for the rarity of ant pollination. Oecologia, 73: 522–524.

Peakall, R., and Beattie, A. J., 1996. Ecological and genetic consequences of pollination by sexual deception in the orchid *Caladenia tentaculata*. Evolution, 50: 2207–2220.

Peakall, R., Ebert, D., Poldy, J., Barrow, R. A., Francke, W., Bower, C.C., and Schiestl, F.P., 2010. Pollinator specificity, floral odour chemistry and the hylogeny of Australian sexually deceptive *Chiloglottis* orchids: implications for pollinator-driven speciation. New Phytologist, 188: 437–450.

Pellegrino, G., Bellusci, F., Musacchio, A., 2009. Genetic integrity of sympatric hybridizing plant species. The case of *O. italica* and *O. anthropophora.* Plant Biology, 11: 434-441.

Pellegrino, G., Bellusci, F., Musacchio, A., 2010. The effects of inflorescence size and flower position on female reproductive success in three deceptive orchids. Botanical Studies, 51: 351-356.

Pellegrino, G., Caputo, P., Cozzolino, S., Menale, B., Musacchio, A., 2000. Molecular characterization of a hybrid zone between *O. mascula* and *O. pauciflora* in Southern Italy. Biologia Plantarum, 43: 13–18.

Pellegrino, G., Cozzolino, S., Grünanger, P., Musacchio, A., 2001. Ribosomal DNA (ITS) as a molecular tool in the study of orchid hybridization. Journal European of Orchid, 3: 369–376.

Pellegrino, G., D'Emerico, S., Scrugli, A., Musacchio, A., Cozzolino, S., 2005. Confirmation of hybridisation among sympatric insular populations of *Orchis mascula* and *Orchis provincialis*. Plant Systematics of Evololution, 251: 131–142.

Peterson, R.L., Uetake, Y., Zelmer, C., 1998. Fungal symbioses with orchid protocorms. Symbiosis, 25: 29–55.

Pouyanne, M., 1917. La fecondation des *Ophrys* par les insectes. Bulletin de la Socie´tè d'Histoire Naturelle l'Afrique du Nord, 8: 6–7.

Priesner, E., 1973. Reaktionen von Riechrezeptoren mannlicher Solita rbienen (Hymenoptera, Apoidea) auf Inhaltsstoffe von *Ophrys*-Blu ten. Zoon/Uppsala Supplement, 1: 43–54.

Primack, R., 1985. Longevity of individual flowers. Annual Review of Ecology and Systematics, 16: 15-37.

Queiros, M., 1985. Números cromosómicos para a flora Portuguesa. Boletim da Sociedade Broteriana, 58: 85-96.

Raguso, R.A., 2008. Wake up and smell the roses: The ecology and evolution of fl oral scent. Annual Review of Ecology, Evolution, and Systematics, 39: 549 – 569.

Ramsay, R., Dixon, K., and Sivasithamparam, K., 1986. Patterns of infections and endophytes associated with Western Australian orchids. Lindleyana, 1: 203-214.

Ramsey, J., Bradshaw, H.D., and Schemske, D.W., 2003. Components of reproductive isolation between the monkeyfl owers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57: 1520 – 1534.

Rasmussen, H., 1995. Terrestrial Orchids From Seed to Mycotrophic Plant. Cambridge University Press, New York.

Rasmussen, H.N., 2002. Recent developments in the study of orchid mycorrhiza. Plant Soil, 244: 149-163

Reinhard, H.R., Go¨lz, P., Peter, R., Wildermuth, H. 1991. Die Orchideen der Schweiz und angrenzender Gebiete, Fotorotar AG, Egg. Switzerland, p. 348.

Ren, Z.-X., Li, D.-Z., Bernhardt, P., and Wang, H., 2011. Flowers of *Cypripedium fargesii* (Orchidaceae) fool flat-footed flies (Platypezidae) by faking fungus-infected foliage. National Academy of Sciences of the United States of America, 18: 7478-7480.

Rieseberg, L.H., Buerkle, C.A., 2002. Genetic mapping in hybrid zones. American Naturalist , 159: S36–S50.

Rieseberg, L.H., Carney, S.E., 1998. Plant hybridization. New Phytologist ,140: 599–624.

Rieseberg, L.H., Raymond, O., Rosenthal, D.M., Lai, Z., Livingstone, K., Nakazato, T., Durphy, J.L., Schwarzbach, A.E., Donovan, L.A., Lexer, C., 2003. Major ecological transition in wild sunflowers facilitated by hybridization. Science, 301:1211–1216.

Rieseberg, L.H., Vanfossen, C., Desrochers, A.M., 1995. Hybrid speciation accompanied by genomic reorganization in wild sunflowers. Nature, 375: 313–316.

Rieseberg, L.H., Whitton, J., and Gardner, K.,. 1999. Hybrid zones and the genetic architecture of a barrier to gene fl ow between two sunfl ower species. Genetics, 152: 713 – 727.

Rieseberg, L.H. and Willis, J.H., 2007. Plant speciation. Science, 317: 910–914.

Roberts, P., 1999. Rhizoctonia-forming fungi. A taxanomic guide. Royal Botanic Gardens, Kew.

Roy, B.A., and Widmer, A., 1999. Floral mimicry: a fascinating yet poorly understood phenomenon. Trends in Plant Science, 4: 325–330.

Roy, M., Whatthana, S., Richard, F., Vessabutr, S., Selosse, M., 2009. Two mycoheterotrophic orchids from Thailand tropical dipterocarpacean forests associate with a broad diversity of ectomycorrhizal fungi. BMC Biology, 7, 51.

Salzmann, C.C., Cozzolino, S., Schiestl, F.P., 2007. Floral scent in food-deceptive orchids: species specificity and sources of variability. Plant Biology, 9: 720–729.

Schatz, B., 2006. Fine scale distribution of pollinator explains the occurrence of the natural orchid hybrid × *Orchis bergonii*. Ecoscience, 13 : 111 – 118 .

Schatz, B., Geoffroy, A., Dainat, B., Bessière, J., Buatois, B., Hossaert-McKey, M., Selosse, M., 2010. A case study of mofified interactions with symbionts in a hybrid Mediterranean orchid. American Journal of Botany, 97: 1278-1288.

Schiestl, F.P. 2005. On the success of a swindle: pollination by deception in orchids. Naturwissenschaften.

Schiestl, F.P., Ayasse, M., Paulus, H.F., L¨ofstedt, C.,. Hansson, B. S., Ibarra, F., and Francke, W., 1999. Orchid pollination by sexual swindle. Nature, 399: 421–422.

Schiestl, F.P., Ayasse, M., Paulus, H.F., L¨ofstedt, C., Hansson B.S., barra, F., and Francke, W., 2000. Sex pheromone mimicry in the early spider orchid (*Ophrys sphegodes*): patterns of hydrocarbons as the key mechanism for pollination by sexual deception. Journal of Comparative Physiology, 186: 567–574.

Schiestl, F.P., and Ayasse, M., 2002. Do changes in floral odor cause peciation in sexually deceptive orchids? Plant Systematics and Evolution, 234: 111–119.

Schiestl, F.P., Peakall, R., Mant, J.G., Ibarra, F., Schulz, C., Franke, S., and Francke, W., 2003. The chemistry of sexual deception in an orchid-wasp pollination system. Science, 302: 437–438.

Schiestl, F.P., Peakall, R., Mant, J.G., 2004. Chemical communication in the sexually deceptive orchid genus *Cryptostylis*. Botanical Journal of the Linnean Society, 144: 199–205.

Schiestl, F.P., and Schlüter, P.M., 2009. Floral isolation, specialized pollination, and pollinator behavior in orchids. Annual Review Entomology, 54: 425– 446.

Scopece, G.,A. Musacchio, A., Widmer, and S. Cozzolino. 2007. Patterns of reproductive isolation in Mediterranean deceptive orchids. Evolution, 61: 2623–2642.

Selosse, M.A., Faccio, A., Scappaticci, G., and Bonfante, P., 2004. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microbial Ecology, 47: 416-426.

Selosse, M.A., Weiss, M., Jany, J.L., and Tillier, A., 2002. Communities and populations of sebacinoid basidiomycetes associated with the chlorophyllous orchid *Neottia nidus-avis* (L.) LCM Rich. And neighbouring tree ectomycorrhizae. Molecular Ecology,11: 1831-1844.

Shefferson, R.P., Kull, T., Tali, K., 2008. Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills. American Journal of Botany, 95: 156-164.

Shefferson, R.P., Taylor, D.L., Weiß, M., Garnica, S., McCormick, M.K., Adams, S., Gray, H.M., McFarland, J.W., Kull, T., Tali, K., Yukawa, T., Kawahara, T., Miyoshi, K., Lee, Y-I., 2007. The evolutionary history of mycorrhizal specificity among lady ' s slipper orchids. Evolution, 61: 1380-1390.

Shefferson, R.P., Weiß, M., Kull, T., Taylor, D.L., 2005. High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. Molecular Ecology 14: 613-626.

Shimura, H., Matsuura, M., Takada, N., and Koda, Y., 2007. An antifungal compound involved in symbiotic germination of *Cypripedium macranthos* var. *rebunense* (Orchidaceae). Phytochemistry, 68: 1442-1447.

Singer, R.B., 2002. The pollination mechanism in *Trigonidium obtusum* Lindl (Orchidaceae: Maxillariinae): sexual mimicry and trap-flowers. Annals of Botany, 89: 157–163.

Singer, R.B., Flach, A., Koehler, S., Marsaiolo, A.J., and Amaral, M.E., 2004. Sexual Mimicry in *Mormolyca ringens* (Lindl.) Schltr. (Orchidaceae: Maxillariinae). Annals of Botany, 93: 755–762.

Smithson, A., 2006. Pollinator limitation and inbreeding depression in orchid species with and without nectar rewards. New Phytologist, 169: 419-430.

Smith, S.E., Read D.J., 1997. Mycorrhizal symbiosis. New York: Academic Press.

Smithson, A., 2006. Pollinator limitation and inbreeding depression in orchid species with and without nectar rewards. New Phytologist, 169: 419-430.

Soltis, D.E., Soltis, P.S., 1999. Polyploidy: recurrent formation and genome evolution. Trends in Ecolology & Evolution, 14: 348–352.

Stace, C.A., 1989. Plant taxonomy and biosystematics. Cambridge University Press, Cambridge, UK.

Stebbins, G.L., 1959. The role of hybridization in evolution. Philadelphia American Philosophical Society, 103: 231-251.

Steiner, K.E., Whitehead, V.B., and Johnson, S.D., 1994. Floral and Pollinator Divergence in Two Sexually Deceptive South African Orchids. American Journal of Botany.

Stockinger, H., Krüger, M., Schüßler, A., 2010. DNA barcoding of arbuscular mycorrhizal fungi. New Phytologist, 187: 461-474

Stökl, J.P., Schlüter, M., Stuessy, T.F., Paulus, H.F., Assum, G., and Ayasse, M., 2008. Scent variation and hybridization cause the displacement of a sexually deceptive orchid species. American Journal of Botany, 95: 472 – 481.

Stoutamire, W.P., 1975. Pseudocopulation in Australian terrestrial orchids. American Orchid Society Bulletin, 44: 226–233.

Stoutamire, W.P., 1983. Wasp-pollinated species of Caladenia (Orchidaceae) in south-western Australia. Australian Journal of Botany, 31: 383–394.

Swarts, N., and Dixon, K., 2009. Terrestrial orchid conservation in the age of extinction. Annals of Botany, 104(3): 543-556.

Swofford, D.L., 2004. paup* – Phylogenetic Analysis Using Parsimony* and Other Methods, version 4. Sunderland, MA: Sinauer Associates.

Szentesi, A´., 2002. Insect-plant relationships – chance and necessity. Acta Zoologica Academiae Scientiarum Hungaricae, 48: 55–71.

Taberlet, P., Gielly, L., Pautou, G., Bouvet, J., 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology, 17: 1105–1109.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution.

Taylor, D.L., Bruns, T.D., 1999. Population, habitat and genetic correlates of mycorrhizal specialization in the „cheating orchids C*orallorhiza maculate* and *C. mertensiana.* Molecular Ecololy, 8: 1719-1732.

Taylor, D.L., Bruns, T.D., and Hodges, S.A., 2004. Evidence for mycorrhizal races in a cheating orchid. Proceedings of the Royal Society of London Series B-Biological Sciences, 271: 35-43.

Taylor, D.L., and McCormick, M.K., 2008. Internal transcribed spaser primers and sequences for improve characterization of basidiomycetous orchid mycorrhizas. New Phytologist, 177: 1020-1033.

Templeton, A.R., 1989. The meaning of species and speciation: a genetic perspective. In: Speciation and its Consequences (D. Otte & J.A. Endler, eds), 3–27.

Thompson, J.,D., Desmond, G., Higgins, and Toby, J., 1994. Gibson CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Oxford Journals.

Ungerer, M.C., Baird, S.J.E., Pan, J., Rieseberg, L.H., 1998. Rapid hybrid speciation in wild sunflowers. Proceedings of the National Academy of Sciences, USA 95: 11757–11762.

Valterová, I., Kunze, J., Gumbert, A., Luxová, A., Liblikas, I., Kalinová, B., Borg-Karlson, A., 2007. Male bumble bee pheromonal components in the scent of deceit pollinated orchids; unrecognized pollinator cues? Arthropod-Plant Interactions, 1: 137–145.

van der Cingel, N.A., 1995. An atlas of orchid pollination — European orchids. Balkema, Rotterdam, Netherlands.

van der Pijl, L., Dodson, C.H., 1966. Orchid Flowers: Their Pollination and Evolution. University of Miami Press, Coral Gables.

Velleman, P.F., 1997. DataDesk Ver. 6.0 Statistic Guide. Data Description, Inc., Ithaca.

Vereecken, NJ., Cozzolino, S., Schiestl, FP., 2010. Hybrid floral novelty drives pollinator shift in sexually deceptive orchids. BMC Evolutionary Biology, 10: 103-105.

Vogel, S., 1976. Zur Ophrys – Bestaubung auf Kreta. Jahresberichte des Naturwissenschaftlichen Vereins Wuppertal, 29: 131–139.

Vöth, W., 1984. Besta¨ubungsbiologische Beobachtungen an griechischen Ophrys-Arten. AHO Mitteilungsblatt Baden-Wu¨rttemberg, 16: 1–20.

Warcup, J.H., 1981. The mycorrhizal relationships of Australian orchids. New Phytologist, 87: 371–381.

Waser, N.M., 2001 . Pollinator behaviour and plant speciation: Looking beyond the " ethological isolation " paradigm. In L. Chittka and J. D. Thomson [eds.], Cognitive ecology of pollination, 318 – 335.

Waterman, R.J., and Bidartondo, M.I., 2008 . Deception above, deception below: Linking pollination and mycorrhizal biology of orchids. Journal of Experimental Botany, 59: 1085 – 1096.

Wendt, T., Ferreira Canela, M.B., Gelli De Faria, A.P., Iglesias Rios, R., 2001. Reproductive biology and natural hybridization between two endemic species of *Pitcairnia* (Bromeliaceae). American Journal of Botany, 88: 1760-1767.

Whigham, D.F., O'Neill, J.P., Rasmussen, H.N., Caldwell, B.A., McCormick, M.K., 2006. Seed longevity in terrestrial orchids: potential for persistent in situ seed banks. Biological Conservation, 129: 24–30.

White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR-protocols and applications – A laboratory manual. Innis MA, Gelfard H, Sninsky JS, White TJ (eds). Academic Press, New York, pp. 315-322.

Widmer, A., Lexer, C., and Cozzolino, S., 2009. Evolution of reproductive isolation in plants. Heredity, 102: 31–38.

Williams, N.H., 1982. The biology of orchids and euglossine bees. In Orchid Biology. Reviews and Perspectives. II (ed. J. Arditti), pp. 119–171. Cornell University Press, Ithaca, NY.

Willing, B., Willing, E., 1977. Bibliographie u¨ ber die Orchideen Europas und der Mittelmeerla¨nder 1744–1976. Willdenowia, 11: 1–325.

Willing, B., Willing, E., 1985. Bibliographie u¨ ber die Orchideen Europas und der Mittelmeerla¨nder. Englera, 5: 1–280.

Wolf, D.E., Takebayashi, N., Rieseberg, L.H., 2001. Predicting the risk of extinction through hybridization. Conservation Bioogy, 15: 1039–1053.

Wolf, P.G., Campbell, D.R., Waser, N.M., Sipes, S.D., Toler, T.R. & Archibald, J.K., 2001b. Tests of pre- and postpollination barriers to hybridization between sympatric species of *Ipomopsis* (Polemoniaceae). American Journal of Botany, 88: 213–219.

Wolfe, A.D., Xiang, Q.Y., Kephart, S.R., 1998. Diploid hybrid speciation in *Penstemon* (Scrophulariaceae). Proceedings of the National Academy of Sciences, USA 95: 5112–5115.

Wong, B.B.M., and Schiestl, F. P., 2002. How an orchid harms its pollinator. Proceedings of the Royal Society of London Series B-Biological Sciences 269: 1529–1532.

Wong, B.B.M., Salzmann, C.H., and Schiestl, F.P., 2004. Pollinator attractiveness increases with distance from flowering orchids. Proceedings of the Royal Society of London Series B-Biological Sciences (Suppl.), 271: 212–214.

Xu, S., Schlüter, P.M., Scopece, G., Breitkopf, H., Gross, K., Cozzolino, S., Schiestl, F.P., 2011. Floral isolation is the main reproductive barrier among closely related sexually deceptive orchids. Evolution, 65: 2606–2620.

RINGRAZIAMENTI

Ringrazio affettuosamente il Dott. Giuseppe Pellegrino per la pazienza e la professionalità dimostratami ancora una volta durante questo percorso, insegnandomi un metodo scientifico e inculcandomi la passione per la ricerca.

Sono onorata di essere stata la sua "ultima dottoranda", quindi ringrazio con ammirazione ed affetto il caro Prof. Aldo Musacchio, che con i suoi giudizi critici ha contribuito alla mia formazione.

Ringrazio la Dott.ssa Francesca Bellusci e la Dott.ssa Anna Maria Palermo sempre disponibili nei miei confronti, sono contenta di aver trascorso questi anni in stretta collaborazione con loro.