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# Interactions with mycorrhizal fungi in two closely related hybridizing orchid species

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**Abstract** – The nuclear ribosomal DNA was used to identify the orchid mycorrhizal fungi found in roots of *Orchis xbivonae* and its parental species *Orchis anthropophora* and *Orchis italica*. Polymerase chain reaction products were sequenced and identified using the expanded database. We determined that closely related Tulasnellaceae are mycorrhizal in the three orchid taxa, suggesting that the mycorrhizal partner does not impair hybrid survival. This study demonstrates that *O. xbivonae* displays few differences in comparison with its two parental species in identity of its associated mycorrhizal fungi, it is a short--term by-product of the hybridizing behavior of common pollinators, and thus it will not easily origin descendents with potential new genetic combinations and/or ecological preferences.

**Keywords:** Mycorrhizal fungi, nrDNA, *Orchis anthropophora*, *Orchis italica*, *Orchis xbivonae*, Tulasnellaceae

## Introduction

The Orchidaceae is one of the largest plant families with close to 25,000 species and a circum-global distribution (DRESSLER 1993, CRIBB et al. 2003). It is also one of the most complex plant families with many adaptations. One adaptive mechanism is related to reproductive biology, indeed orchid flowers evolved a close relationship with insect pollinators such that sympatric species can be pollinated by different insect species (VAN DER PIJL and DODSON 1966), maintaining species isolation (SCOPECE et al. 2007). Interestingly, 30% of orchid species shows non-rewarding flowers (RENNER 2005), attracting pollinators by generalized food deception, mimicry or sexual deception (JERSAKOVA et al. 2006). Another example of adaptive mechanism is related to mycorrhizal interactions that have allowed orchids to fit in several habitat types and has led to colonize worldwide. The main group of fungi inhabiting orchid roots is Basidiomycetes (RASMUSSEN 2002), though Ascomycetes have been found (SELOSSE et al. 2004). Mycorrhizal fungi provide the basic

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organic carbon (SMITH 1966, CAMERON et al. 2006) and carbohydrates for seedling growth (LEAKE 1994, RASMUSSEN 1995). Also adult individuals of achlorophyllous and photosynthetic orchids obtain part of carbon heterotrophically by mycorrhizal fungi (BIDARTONDO et al. 2004, JULOU et al. 2005, SELOSSE and ROY 2009, JACQUEMYN et al. 2010). In addition, in an adult green-leaved orchid *Goodyera repens*, there is a flow of carbon from the plant to the fungus (CAMERON et al. 2006). This suggests that also the green-leaved adult orchids, after the initially mycoheterotrophic phase of development, establish the general symbiotic relationship with fungi in which carbon from the plant is exchanged for mineral nutrients gathered by the fungus.

In recent years, the application of modern molecular approaches has increased greatly the facility for identifying the mycobionts. From the beginning of 2000, a large number of orchid mycorrhizal has been identified directly from orchid roots, tubers and rhizomes through molecular biology techniques (SHEFFERSON et al. 2007, STOCKINGER et al. 2010, JACQUEMYN et al. 2011a). The main widespread molecular markers used to identify orchid mycobionts are the internal transcribed spacers (ITSs) of the nuclear ribosomal DNA using primer sets (WHITE et al. 1990, TAYLOR and MCCORMICK 2008) for PCR amplification.

The level of specificity between fungus and orchid is an important factor determining chances of successful seedling establishment (BIDARTONDO and READ 2008). Early molecular studies have revealed that terrestrial orchids interact with a wide range of mycorrhizal fungi. Some orchids are extreme specialists and are associated to a single fungal species (MCCORMICK et al. 2004, TAYLOR et al. 2004, DEARLANEY 2007, SHEFFERSON et al. 2008), while others are generalists, hosting many different fungi (MCCORMICK et al. 2006, BONNARDEAUX et al. 2007, IRWIN et al. 2007, ROY et al. 2009).

The requirement of terrestrial orchids to form a symbiosis with mycorrhizal fungi may act on orchid distribution and diversification (WATERMAN et al. 2008, PHILLIPS et al 2011). A large diversity and distribution of orchids is related to a broadly distributed fungi (OTERO et al. 2007), while rarity of orchids and narrow distribution is associated to a narrow mycorrhizal specificity (VALADARES et al. 2012).

Hybrid zones, in which hybrids live sympatrically with parental species, are highly suitable to verify mycorrhizal hybrid preferences, that means do hybrids form a symbiosis with the same fungi 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. Previous studies showed that hybrids of genus *Caladenia* had fungi genetically different from those associating with parental species (HOLLICK et al. 2005), while adult individuals of *Orchis simia* and *O. anthropophora* and their hybrid were associate to closely related Tulasnellaceae fungi (SCHATZ et al. 2010), such as three closely related hybridizing *Orchis* species showed common mycorrhizal associations in protocorms and adult stage (JACQUEMYN et al. 2011b).

In this study, we examined, with molecular analyses, a sympatric zone between *Orchis italica* Poir. and *O. anthropophora* L., which hybridize to form *O. xbivonae* Tod. Our main aim 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 study the role of mycorrhizal associations in allowing to the hybrid to exploit prospective new ecological niches different from parental habitat.

## Materials and methods

### Study area and orchid species

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 »Monte di Cassano« (39°47'N, 16°18'E, 512 m a.s.l.), one kilometre north-west of the city of »Cassano allo Ionio« (northern Calabria region, Italy). The whole area covers roughly 1,500 $m^2$ (25 m wide and 60 m long) of calcareous soil and is bounded on the west by a road and on the remainder by deep gorges.

In the studied site, *Orchis italica* and *O. anthropophora* overlap extensively in their spatial distribution and flowering time (flowering peak between the end of April and the middle of May) and grew together with *O. xbivonae* (PELLEGRINO et al. 2009).

*Orchis italica* and *O. anthropophora* are closely related (BATEMAN et al. 2003), have same chromosome number ($2n = 42$) (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). *Orchis italica* show a pendent lip, deeply tri-lobed and with a cylindrical spur, while, *O. anthropophora* has a narrow lip and, differently from all the others *Orchis* species, it lacks a spur (DELFORGE 2005). 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*.

### Molecular analysis

Molecular characterization of mycorrhizae involved: (i) extraction of DNA from orchid roots, (ii) amplification of nuclear ribosomal DNA regions useful in determining fungal identity, (iii) DNA sequencing for identification of mycorrhizal fungi and assessment of specificity levels.

Small parts of roots were cut from 15 randomly selected individuals of *Orchis anthropophora* and *O. italica* and for 8 individuals of *O.* x*bivonae* (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 (DOYLE 1991). DNA was resuspended in 50 μL of distilled water.

To discriminate among fungal taxa colonizing orchid roots, the internal transcribed spacers (ITSs) of the nuclear ribosomal DNA were amplified using fungal universal primer pairs ITS1-OF and ITS4-OF (TAYLOR and MCCORMICK 2008) chosen for their most constant and reliable amplification (JACQUEMYN et al. 2011b).

PCRs were performed on a PTC-100 Thermal Cycler (MJ Research Inc., Watertown, MA, USA) according to the following thermal cycling profile: 94 °C for 3 min (1 cycle), 94 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min (30 cycles); 72 °C for 5 min (1 cycle). 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; fluorescent dye sequencing was performed on a 310 Prism ABI DNA Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA).

### Data analysis

The ClustalW algorithm (THOMPSON et al. 1994) of the Molecular Evolutionary Genetics Analysis version 5 (MEGA5) program package 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 mycorrhizal sequences already available in DNA DATA BANK OF JAPAN (2013).

Sequence identity was determined using the blast algorithm available through the NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (2013). 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 mycobionts OTUs. The identification of OTUs was performed comparing the sequences obtained in this study with those of JACQUEMYN et al. (2011b) using the ClustalW algorithm of the MEGA 5 program package. Our fungi sequences were classified as OTUs when ITS sequence similarity exceeded 97%. Pairwise genetic distances between sequences were measured by the number of nucleotide substitutions occurring between them under the Tamura and Nei model (TAMURA and NEI 1993). A neighbour-joining tree (SAITOU and NEI 1987) was constructed using Clustal and displayed by TreeView v.1.6.6. Differences in mycorrhizal assemblages between taxa were analysed using a one-way ANOVA. When the F test was significant, means were compared using the Tukey test at 5% error probability.

## Results

The identification of orchids associated fungi by molecular techniques was successful for all the collected samples. On the basis of less than 5% of genetic distance (Tab. 1), out of 23 fungal OTUs identified previously (JACQUEMYN et al. 2011b) eight were observed (OTU 2, 4, 6, 7, 10, 11, 12 and 17) in our investigated plants; six were related to Tulasnellaceae, and two to Ceratobasidiaceae (Tab. 2). Six different fungal OTUs were found in *Orchis italica* (OTU 2, 4, 7, 10, 12 and 17) and *O. anthropophora* (OTU 2, 4, 6, 7, 10 and 11), and four (OTU 2, 4, 7 and 10) in *O. xbivonae* (Fig. 1). Parental species showed different frequently dominant OTUs (OTU 2 and 12 in *O. italica* and OTU 7 and 10 in *O. anthropophora*). OTU 2 and 4 were found in all three taxa, while *O. italica* and *O. anthropophora* showed two exclusive OTUs, OTU 12 and 17, OTU 6 and 11, respectively (Fig. 1). Phylogenetic analysis placed ITS sequences for *Orchis italica*, *O. anthropophora* and *O. xbivonae* in eight distinct groups closely associated with OTUs (Fig. 2).

ANOVA analysis showed no significant differences in mycorrhizal assemblage between parental species and hybrids, (*O. anthropophora* vs *O. xbivonae*: $F_{1,21} = 1.004$, $p = 0.35$, *O. italica* vs *O. xbivonae*: $F_{1,21} = 0.785$, $p = 0.38$), although the parental species have a slight different assemblages (*O. anthropophora* vs *O. italica*: $F_{1,28} = 3.411$, $p < 0.05$).

**Tab. 1.** Genetic distance between fungal operational taxonomic units (OTUs) identified in *Orchis italica* (I), *O. anthropophora* (A) and *O. xbivonae* (B). Less than 5% of genetic distance is marked in bold.

| *Orchis* species | OTU2 | OTU4 | OTU6 | OTU7 | OTU10 | OTU12 | OTU17 | OTU11 |
|---|---|---|---|---|---|---|---|---|
| I 33 | **0.004** | 0.327 | 0.317 | 0.321 | 0.334 | 0.121 | 0.420 | 0.438 |
| I 20 | **0.005** | 0.324 | 0.319 | 0.323 | 0.334 | 0.122 | 0.413 | 0.434 |
| I 28 | **0.004** | 0.324 | 0.313 | 0.315 | 0.331 | 0.118 | 0.420 | 0.434 |
| I 22 | **0.005** | 0.325 | 0.314 | 0.316 | 0.332 | 0.118 | 0.420 | 0.435 |
| I 30 | 0.327 | **0.004** | 0.133 | 0.097 | 0.200 | 0.329 | 0.339 | 0.354 |
| I 24 | 0.324 | **0.007** | 0.135 | 0.101 | 0.200 | 0.327 | 0.343 | 0.357 |
| I 29 | 0.320 | 0.107 | 0.113 | **0.009** | 0.228 | 0.351 | 0.380 | 0.376 |
| I 25 | 0.318 | 0.103 | 0.105 | **0.005** | 0.223 | 0.345 | 0.377 | 0.373 |
| I 27 | 0.342 | 0.204 | 0.231 | 0.226 | **0.015** | 0.349 | 0.412 | 0.407 |
| I 19 | 0.334 | 0.202 | 0.234 | 0.223 | **0.005** | 0.349 | 0.412 | 0.413 |
| I 15 | 0.118 | 0.327 | 0.317 | 0.345 | 0.348 | **0.005** | 0.388 | 0.400 |
| I 14 | 0.121 | 0.336 | 0.326 | 0.355 | 0.358 | **0.007** | 0.391 | 0.407 |
| I 12 | 0.121 | 0.336 | 0.326 | 0.355 | 0.358 | **0.007** | 0.391 | 0.407 |
| I 10 | 0.118 | 0.326 | 0.317 | 0.345 | 0.348 | **0.002** | 0.381 | 0.397 |
| I 4 | 0.413 | 0.336 | 0.390 | 0.377 | 0.402 | 0.382 | **0.005** | 0.091 |
| A 2 | **0.004** | 0.322 | 0.311 | 0.315 | 0.328 | 0.116 | 0.417 | 0.431 |
| A 1 | 0.330 | **0.007** | 0.137 | 0.101 | 0.199 | 0.332 | 0.345 | 0.359 |
| A 8 | 0.324 | **0.004** | 0.137 | 0.101 | 0.197 | 0.327 | 0.339 | 0.354 |
| A 9 | 0.313 | 0.139 | **0.005** | 0.113 | 0.238 | 0.317 | 0.396 | 0.402 |
| A 3 | 0.310 | 0.133 | **0.004** | 0.111 | 0.236 | 0.314 | 0.390 | 0.399 |
| A 15 | 0.316 | 0.137 | **0.004** | 0.111 | 0.236 | 0.320 | 0.396 | 0.402 |
| A 4 | 0.316 | 0.105 | 0.111 | **0.011** | 0.229 | 0.349 | 0.385 | 0.386 |
| A 14 | 0.312 | 0.101 | 0.107 | **0.005** | 0.221 | 0.343 | 0.371 | 0.368 |
| A 13 | 0.321 | 0.107 | 0.113 | **0.009** | 0.228 | 0.348 | 0.378 | 0.374 |
| A 5 | 0.312 | 0.099 | 0.105 | **0.002** | 0.223 | 0.343 | 0.374 | 0.370 |
| A 12 | 0.325 | 0.197 | 0.233 | 0.223 | **0.002** | 0.343 | 0.405 | 0.406 |
| A 6 | 0.325 | 0.197 | 0.233 | 0.223 | **0.002** | 0.343 | 0.405 | 0.406 |
| A 11 | 0.342 | 0.202 | 0.234 | 0.224 | **0.013** | 0.349 | 0.409 | 0.404 |
| A 7 | 0.342 | 0.202 | 0.234 | 0.224 | **0.013** | 0.349 | 0.409 | 0.404 |
| A 10 | 0.431 | 0.357 | 0.397 | 0.377 | 0.410 | 0.397 | 0.091 | **0.005** |
| B 1 | **0.002** | 0.324 | 0.313 | 0.318 | 0.331 | 0.118 | 0.416 | 0.433 |
| B 2 | 0.330 | **0.005** | 0.139 | 0.103 | 0.200 | 0.327 | 0.337 | 0.352 |
| B 4 | 0.330 | **0.005** | 0.130 | 0.099 | 0.202 | 0.333 | 0.343 | 0.357 |
| B 3 | 0.315 | 0.103 | 0.109 | **0.005** | 0.221 | 0.346 | 0.372 | 0.371 |
| B 5 | 0.318 | 0.105 | 0.111 | **0.007** | 0.226 | 0.349 | 0.378 | 0.374 |
| B 8 | 0.318 | 0.103 | 0.105 | **0.005** | 0.223 | 0.348 | 0.380 | 0.376 |
| B 7 | 0.325 | 0.197 | 0.233 | 0.223 | **0.002** | 0.343 | 0.405 | 0.406 |
| B 6 | 0.336 | 0.202 | 0.233 | 0.223 | **0.013** | 0.343 | 0.406 | 0.400 |

**Tab. 2.** Fungal operational taxonomic units (OTUs) identified in *Orchis anthropophora* (A), *O. italica* (I) and *O. xbivonae* (X)(marked by +). Fungi were grouped into OTUs defined by 97% internal transcribed spacer (ITS) sequence similarity.

| Target | Family | Closest match in GenBank | Presence in examined orchids | | |
|---|---|---|---|---|---|
| | | | A | I | B |
| OTU-2 | Tulasnellaceae | GQ907254 | + | + | + |
| OTU-4 | Tulasnellaceae | GQ907260 | + | + | + |
| OTU-6 | Tulasnellaceae | GQ907266 | | + | |
| OTU-7 | Tulasnellaceae | GQ907258 | + | + | + |
| OTU-10 | Tulasnellaceae | GU066935 | + | + | + |
| OTU-11 | Ceratobasidiaceae | GU066936 | | + | |
| OTU-12 | Tulasnellaceae | HQ330992 | + | | |
| OTU-17 | Ceratobasidiaceae | HQ331002 | + | | |

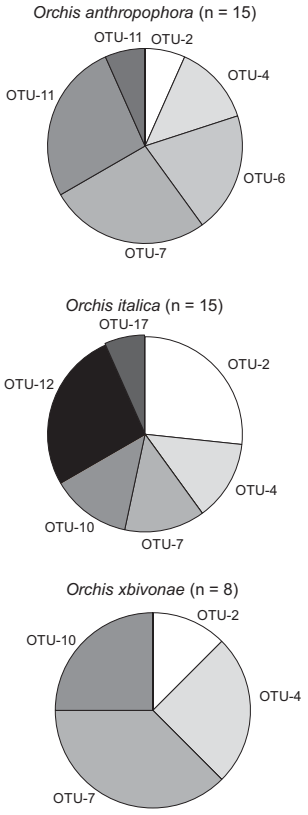


**Fig. 1.** Frequency distribution of identified operational taxonomic units (OTUs) in *Orchis anthropophora*, *O. italica* and *O. xbivonae*.

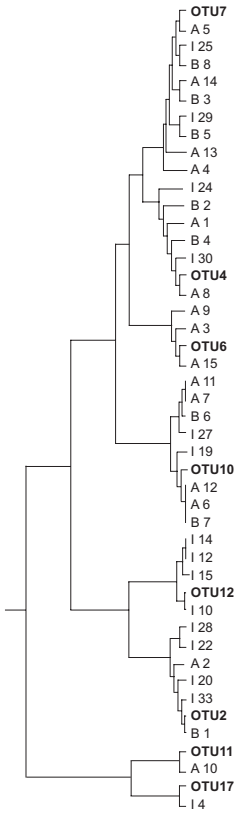


**Fig. 2.** Neighbour-joining phylogenetic tree showing the relationship between internal transcribed spacer (ITS) sequences for *Orchis anthropophora* (A), *O. italica* (I) and *O. xbivona* (B) mycobionts and operational taxonomic units (OTUs).

## Discussion

We have shown that adult plants 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* (JACQUEMYN et al. 2011b). Moreover, Tulasnellaceae have been recognized as the most important association with other Mediterranean orchids such as *Anacamptis* (GIRLANDA et al. 2011), *Cypripedium* (SHEFFERSON et al. 2007), *Dactylorhiza* (JACQUEMYN et al. 2012) and *Ophrys* (GIRLANDA et al. 2011). Previous literature showed that the number of fungal OTUs of *Orchis* sp. varied between one and nine; in detail *O. anthropophora*, *O. italica*, *O. simia* and *O. militaris* were associated with at least seven different OTUs, *O.s purpurea* with four, whereas *O. anatolica*, *O. mascula*, *O. olbiensis* and *O. troodi* with only one or two fungal OTUs (JACQUEMYN et al. 2010, JACQUEMYN et al. 2011a, b). In our case, in agreement with previous studies, *O. italica* and *O. anthopophora* were associated with six fungal OTUs, predominantly with OTU 2 and 12 (*O. italica*) and with OTU 7 and 10 (*O. anthopophora*) (Fig. 1). *Orchis italica* and *O. anthopophora* associated secondly with two different OTUs belonging to Ceratobasidiaceae confirming previous results (JACQUEMYN et al. 2011b).

*Orchis 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). Similarly, 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 parental species (SCHATZ et al. 2010).

Hybrid zones are notable for studying reproductive barriers 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, either the parental one or a totally new one, shows an own reproductive success and produces a sufficient number of seeds (ARNOLD 1997). Compared to parental species, previous study demonstrated that *O. xbivonae* showed low fruiting values in open-pollinated flowers and the absence of any form of postmating isolation (PELLEGRINO et al. 2009). The low levels of reproductive success, the lack of post-zygotic barriers and of $F_2$ (or later) generations (PELLEGRINO et al. 2009) suggest that the mycorrhizal symbiosis imposes no constraints on the survival 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 *Orchis xbivonae* with its parents suggests that this hybrid is a short--term by-product due to the behavior of shared pollinators (SCHATZ 2006). Mycorrhizal do not represent a limitation to *O. xbivonae* growth but at the same time do not offer an ecological opportunity to partially separate hybrid habitat from the parental ones. In our case the low specificity and divergent association pattern between hybrids and parental species do not represent an effective barrier to hybridization. Indeed, hybrids and parental species share most of their mycorrhizal fungi, and thus 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 orchids.

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, our results corroborate the idea that hybrid zone did not have a prominent role in speciation processes of Mediterranean orchids. Indeed, with the exclusion of one case (*Orchis xcolemanii*) in which the hybrids showed high levels of reproductive success and belonged to $F_2$ hybrid generation (LUCA et al. 2012), representing a potential reserve of adaptive variability (RIESEBERG 1995), natural hybridization do not show a high biological and evolutionary significance, but, rather, represent a strong postzygotic barriers actively maintain parental species boundaries from genome-wide introgression.

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