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Genetic relatedness of insular segregates of mediterranean orchid species as inferred from ITS sequences analysis

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Abstract

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A sequence analysis has been carried out on the ribosomal DNA Internal Transcribed Spacers (ITS) of Sardinian and Sicilian *Orchis*, *Anacamptis* and *Neotinea* endemics, aiming at the understanding of genetic relatedness of these geographically isolated entities. Resulting sequences were compared with those of strictly related species with broader distribution ranges. No sequence difference has been detected between *O. mascula* ssp. *ichnusae* (endemic of Sardinia) and *O. mascula* ssp. *mascula* accessions from north-eastern Italy and southern France. *Orchis brancifortii* (endemic of Sicily and Sardinia) displays the same genetic distance both with *O. quadripunctata* accessions from mainland Italy and with the eastern mediterranean *O. anatolica*. The accessions of *A. longicornu* from Sicily and Sardinia have identical sequences, which in turn clearly differ from those of *A. morio* ssp. *morio* and *A. morio* ssp. *picta*, thus suggesting an ancient separation event. No sequence difference occurs between *N. commutata* from Sicily and *N. tridentata* from southern Italy, in spite of the presence of some heterozygous positions in some accessions of *N. commutata*. These results seem to indicate that different levels of genetic distance may be observed in insular mediterranean orchids.

Key words: rDNA analysis, ITS, insular segregation, genetic relatedness.

Introduction

Orchids are renowned for their extraordinary floral diversity and for their intricate plant-pollinator relationships. Euro-asiatic Orchidinae, in particular, represent an interesting group, in which different processes (vicariance, hybridization, polyploidization etc.) seem to be actively driving speciation.

In the last years, molecular techniques have given new insights on the relationships in european Orchidinae (Aceto et al. 1999; Cozzolino et al. 1998; Pridgeon et al. 1997). In particular, the approaches based on the sequences of the Internal Transcribed Spacers (ITS1 and ITS2) of the nuclear ribosomal DNA disclosed a new pattern of relationship within Orchidinae (Aceto et al. 1999; Pridgeon et al. 1997) and made available a large dataset of nuclear sequences. These sequences may be used to predict genetic relatedness at genus and species level when sufficient divergence is observed (Coleman and Mai 1997). Analysis of ITS sequence datasets of the genera *Orchis* L., *Anacamptis* L.C.M. Richard and *Neotinea* Reichenb. fil, as recently circumscribed by Bateman et al. (1997), revealed that sequence divergence among species is far higher than within other plurispecific european genera of Orchidinae (namely *Serapias* L., *Ophrys* L. and *Dactylorhiza* Necker ex Nevski). In fact complete identity of ITS sequences has been never detected, not even between species that are very similar from a morphological point of view, and the minimum distance between sister taxa, for example *O. provincialis* Balbis ex Lam. et DC. and *O. mascula* (L.) L., is 97%, corresponding to a differences of 13 bases.

Sequence divergence, if found also at intraspecific level, may also be employed as a biogeographic marker. In this regard, insular endemisms may represent an ideal opportunity to estimate the degree of genetic divergence with continental populations and/or with sister species and to reveal the geographical isolation.

In Sardinia, the geographical isolation begun during eocene (Chiappini 1985), caused the presence of a consistent proportion (about 10%) of endemic entities or geographical ecotypes of species with a wider range in the continental flora.

Differently, Sicilian flora, as a consequence of the position of the island, shows recent influences from various floristic regions (Camarda 1992).

Within *Orchis*, *Anacamptis* and *Neotinea*, various narrowly endemic entities of uncertain taxonomic status, not present in mainland Europe, have been reported for these two islands: *N. commutata* (Tod.) R. M. Bateman, Pridgeon et M. W. Chase, probably present only in Sicily, (Del Prete and Tosi 1988) can be distinguished from *N. tridentata* (Scop.) R. M. Bateman, Pridgeon et M. W. Chase (with a widespread mediterranean range) for its longer and crenate labellum and for the tetraploid chromosome number (Mazzola 1984). Similarly, the Sardinian endemic *O. mascula* (L.) L. ssp. *ichnusae* Corrias is reported as different from the widespread *O. mascula* (L.) L. ssp. *mascula* mainly for its smaller pink-liliac flowers.

Two entities, *A. longicornu* (Poiret) R. M. Bateman, Pridgeon et M. W. Chase and *O. brancifortii* Biv. are present in both islands and absent from the rest of Europe (Corrias 1980; Corrias et al. 1991). *Anacamptis longicornu*, reported for Sicily, Sardinia and northern Africa (Camarda 1992), has been described as a close relative of the widespread *A. morio* (L.) R. M. Bateman, Pridgeon et M. W. Chase, distributed from Portugal to Anatolia, with different local subspecies. *Orchis brancifortii* is one of the five endemic entities exclusively present in the two islands (Camarda 1992) and it is closely related *O. quadripunctata* Cyr. ex Ten., distributed in the central and eastern mediterranean area.

Aiming at the understanding of the genetic relatedness of the above mentioned endemisms a sequence analysis has been carried out on the ribosomal DNA Internal Transcribed Spacers (ITS). The resulting sequences have been compared with those of continental populations and/or of strictly related species with broader ranges. In the present paper, we adopt the new nomenclature combinations as defined by Bateman et al. 1997.

Material and methods

Total DNA was extracted from 1 g of silica gel dried leaves of the following taxa from accessions of different collection areas (Table 1) according to the procedure described in Doyle and Doyle (1987): *Orchis brancifortii*, *O. quadripunctata*, *N. tridentata*, *O. mascula*, *N. commutata*, *O. mascula* ssp. *ichnusae*, *A. morio*, *A. morio* L. ssp. *picta* R. M. Bateman, Pridgeon et M. W. Chase, *A. longicornu*. When available, more than one individual from the same locality has been examined (Table 1). In addition, DNA was also extracted from specimens of *N. tridentata* and *O. anatolica* Boissier growing in Israel. Some accession had already been investigated in Aceto et al. (1999).

All specimens were field collected by the authors or obtained through the courtesy of the colleagues acknowledged below. Voucher specimens of the examined plants are deposited at NAP, CAG or CAT. ITS1 and ITS2 were amplified by using two pairs of primers which anneal in the 3' region of the 18S and in the 5' region of the 5.8S and in the 3' region of the 5.8S and in the 5' region of the 25S respectively. Primer sequences, PCR conditions and fragment purification techniques are reported in Aceto et al. (1999) and in Widmer and Baltisberger (1999). PCR fragments were then double-strand sequenced in both directions by using a modification of the Sanger dideoxy method (Sanger et al. 1977) as implemented in a double strand DNA cycle sequencing system with fluorescent dyes. Sequence reactions were then loaded into a 373A Applied Biosystems Automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.). The Fractura software (Applied Biosystem-Perkin Elmer, Foster City, CA, U.S.), with a 25% base peak height setting, has been used to detect heterozygous positions. Raw sequences were then inspected to visually confirm the heterozygous positions. In one case, *N. commutata* (accession NCOMSI-9) from Arcia (ME), the purified ITS2 PCR product was ligated into pUC 18 vector and used to transform *E. coli* strains DH5a. Recombinant clones were selected as white colonies on ampicillin plates containing X-gal (5-bromo-4-chloro-3-indolyl-D-galactoside) and IPTG (isopropyl-D-thiogalactopyranoside). Plasmid DNA was isolated using the Quiagen Plasmid Purification Kit (Quiagen, Germany) and sequenced in both directions with universal forward and reverse M13 primers.

Sequences were then reduced to only ITS1 and ITS2 by aligning them with the 3' termini of 18S and 5.8S and with the 5' termini of 5.8S and 25S of other Orchidinae sequences already available in the databank and aligned using the Sequence Navigator software.

Results

All the examined accessions of *A. morio* ssp. *morio* and *A. morio* ssp. *picta* (AMORIT and AMORSI) have identical ITS sequences. The accessions of *A. longi-*

Table 1. Accessions of examined specimens with collection areas and abbreviations.

Species	Locality		Abbreviation
Anacamptis longicornu (Poiret) R.M. Bateman, Pridgeon et M.W. Chase	Troina (EN)	Sicily	ALONSI-1,2,3,4
A. longicornu	Arcia (ME)	Sicily	ALONSI-5,6,7
A. longicornu	Piana dei Buccheri (PA)	Sicily	ALONSI-8,9
A. longicornu	Mt. Ciurma Laghi (PA)	Sicily	ALONSI-10,11,12
A. longicornu	Pennina di Lupo (CT)	Sicily	ALONSI-13,14
A. longicornu	Mt. Rossi (CT)	Sicily	ALONSI-15,
A. longicornu	Campu Ornu (CA)	Sardinia	ALONSA-1,2,3
A. longicornu	Laconi (CA)	Sardinia	ALONSA-4,5,6
A. longicornu	Sorgono (NU)	Sardinia	ALONSA-7,8
Anacamptis morio ssp. *morio* (Poiret) R.M. Bateman, Pridgeon et M.W. Chase	Sassano (SA)	Campania	AMORIT-1,2,3
A. morio ssp. *morio*	Brescia (BS)	Lombardia	AMORIT-4,5
A. morio ssp. *morio*	Lecce (LE)	Apulia	AMORIT-6,7
A. morio ssp. *morio*	Cesarò (ME)	Sicily	AMORSI-1,2,3
A. morio ssp. *morio*	Mt. Rossi (CT)	Sicily	AMORSI-7,8
A. morio ssp. *picta* (Poiret) R.M. Bateman, Pridgeon et M.W. Chase	S. Nicola (ME)	Sicily	AMORSI-9,10,11
A. morio ssp. *picta*	Chiappazzo (ME)	Sicily	AMORSI-12,13
A. morio ssp. *picta*	Cesarò (ME)	Sicily	AMORSI-14,15,16
Neotinea commutata (Tod.) R.M. Bateman, Pridgeon et M.W. Chase	Monreale (PA)	Sicily	NCOMSI-1
N. commutata	Mt. Cuccio (PA)	Sicily	NCOMSI-2,3,4
N. commutata	Mt. Longo di Carini (PA)	Sicily	NCOMSI-5,6
N. commutata	Portella Marvitti (ME)	Sicily	NCOMSI-7,8
N. commutata	Arcia (ME)	Sicily	NCOMSI-9,10,11
N. commutata	Martello river (ME)	Sicily	NCOMSI-12,13
Neotinea tridentata (Scop.) R.M. Bateman, Pridgeon et M.W. Chase	Sassano (SA)	Campania	NTRIIT-1,2,3
N. tridentata	Miralago Matese (CE)	Campania	NTRIIT-4,5,6

Table 1. continued

Species	Locality		Abbreviation
N. tridentata	Brescia (BS)	Lombardia	NTRIIT-7,8
N. tridentata	Gargano (FG)	Apulia	NTRIIT-9,10
N. tridentata	Laconi (CA)	Sardinia	NTRISA-1,2
N. tridentata	Mt. Carmel	Israel	NTRIIS-1,2,3
N. tridentata	Mt. Maron	Israel	NTRIIS-4,5,6
Orchis anatolica Boissier.	Mt. Carmel	Israel	OANAIS-1,2,3
O. anatolica	North Galilea	Israel	OANAIS-4,5
Orchis brancifortii Biv.	Cologone (NU)	Sardinia	OBRASA-1,2,3
O. brancifortii	Mt. Tuttavista (NU)	Sardinia	OBRASA-4,5
O. brancifortii	Perdasdefogu (NU)	Sardinia	OBRASA-6,7
O. brancifortii	Castelmola (ME)	Sicily	OBRASI-1,2,3
O. brancifortii	Sciacca (AG)	Sicily	OBRASI-4,5
O. brancifortii	Piana degli Albanesi (PA)	Sicily	OBRASI-6,7
O. brancifortii	Pennina d i Lupo (CT)	Sicily	OBRASI-8
Orchis mascula (L.) ssp. *mascula* L.	Mt. S. Giacomo (SA)	Campania	OMASIT-1,2,3
O. mascula ssp. *mascula*	Miralago (CE)	Campania	OMASIT-4,5
O. mascula ssp. *mascula*	S. Giulia (GE)	Liguria	OMASIT-6,7
O. mascula ssp. *mascula*	Mt. Baldo (VR)	Veneto	OMASIT-8
O. mascula ssp. *mascula*	Antibes	France	OMASFR-1
O. mascula (L.) L. ssp. *ichnusae* Corrias	Domus Novas (CA)	Sardinia	OMASSA-1,2,3
O. mascula ssp. *ichnusae*	Campu Ornu (CA)	Sardinia	OMASSA-4,5,6
O. mascula ssp. *ichnusae*	S'astaria (NU)	Sardinia	OMASSA-7,8
Orchis quadripunctata Cyr. Ex Ten.	Sassano (SA)	Campania	OQUAIT-1,2,3
O. quadripunctata	Grosseto (GR)	Tuscany	OQUAIT-4,5
O. quadripunctata	Gargano (FG)	Apulia	OQUAIT-6

cornu from Sicily (ALONSI) and Sardinia (ALONSA) have identical sequence and clearly differ from *A. morio* ssp. *morio* and spp. *picta*, for 3 deletions/insertions and 9 base substitutions (positions 21, 41, 42, 55 and 113 of ITS1 and 31, 44, 217 and 218 of ITS2).

Sequence differences also occur between the accessions of *N. tridentata* from southern Italy (NTRIIT) and from Israel (NTRIIS). These two sequences differ for 8 bases substitution (in position 45, 70, 80, 102, 122, 187 and 213 of ITS1 and 196 of ITS2) and 2 deletions/insertions. The sequences of some *N. commutata* accessions (NCOMSI-1,2,3,4,5,6,7,8,12,13) are identical to those of *N. tridentata* from southern Italy and Sardinia (NTRIIT and NTRISA) but specimens of *N. commutata* from Arcia (ME) (NCOMSI-9,10,11) heterozygously display different bases in some positions in which *N. tridentata* from Italy and Israel differ (Fig. 2, see discussion for details). Cloning experiment of a *N. commutata* specimen from Arcia (ME) (NCOMSI-9) confirmed the presence of heterozygous positions in the ITS sequence.

The sequences of *O. brancifortii* accessions from Sardinia (OBRASA) and from Sicily (OBRASI) differ for a single base (position 41 of ITS2) from *O. quadripunctata* accessions from Italy (OQUAIT). No sequence difference has been detected between *O. mascula* ssp. *ichnusae* accessions from Sardinia (OMASSA) and *O. mascula* ssp. *mascula* accessions from southern France and Liguria (northern Italy) (namely OMASIT-6,7 and OMASFR-1), and both differ from *O. mascula* ssp. *mascula* accessions from southern Italy (OMASIT-1,2,3,4,5,8) for a single point mutation (position 45 of ITS1).

Discussion

The orchids object of this paper have in common both the characteristic of being mainly insular endemics and the fact that they are related to a taxon with a wider range in the mainland mediterranean region. In this circumstance, sequence divergence, if occurring, may be the consequence of the progressive onset of isolating mechanisms that hinder or prevent gene flow among taxa.

Even in the limit of present sampling, with the exclusion of *N. commutata*, extremely low sequence variation has been found within specimens of the same taxon collected in different localities. The few differences among specimens of the same taxon, when occurring, are related to presence of heterozygous positions. These differences, when exclusive of a single sample, have not been taken in account in the present analysis.

A. morio ssp. *morio* and *A. morio* ssp. *picta* share identical ITS sequences and show conspicuous differences with *A. longicornu* (Fig. 1); this comparatively large number of differences (comparable to that occurring, for example, between *O. mascula* and *O. provincialis*) may indicate a rather ancient splitting event. *Anacamptis longicornu* probably originated as a vicariant species of *A. morio* either in north Africa or in one of the two islands and then may have spread to the other two regions of its range, but not to mainland Italy. In Sicily, a contact with the vicariant species *A. morio* sometimes occurs, so producing few hybridization events (near Mount Etna, for example) that indicate a not complete ecological or genetic differentiation between the two taxa (Grasso and Grillo 1996).

The tetraploid status of *N. commutata* (Mazzola 1984) would indicate that this species originated through an event of autopoliploidy. This condition, i.e., speciation via autopolyploidy, even if less common in nature than allopolyploidy (Soltis and Riese-

A

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A. morio          TCGAGACCCTAAAGAGAGAACGATTTGATAACCTGTGAATTATTTCAGCA   50
A. longicornu     ..................T..................AT........   50

A. morio          GCTTACTAAAGTTGTTGCGCACCCGTTCATCTGYCGCATGATGACCTTAC  100
A. longicornu     ....G............................C.............   100

A. morio          GGAAACATGCTGCAGGYGGAGGGGAGATCAATTCGGCGCGGCTCTGCGCC  150
A. longicornu     .........Y..T...T................................   150

A. morio          AAGGTAAAATGCATCATGAGCATTCTCRACCACATCCCCAAAGCATTTTG  200
A. longicornu     ......................Y..G......................   200

A. morio          TTTTGCGGAGTTGTTGT--TTTGCTCCCAATTAGAGTTGTATGGCTC     245
A. longicornu     ................CG..............--...........     245
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B

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A. morio          CATTGTGTCGCTCCATAGGACCTTCGCGGCCACGCGGCTGTCTCATCATG   50
A. longicornu     ............................T............T......   50

A. morio          GATGCGGAGAATGGCCTGTCATGCGCTTATGTGTGGCTGGCTGAAGAGCG  100
A. longicornu     ........................Y.....................   100

A. morio          GGATGATACTCTCTTGGCAATGGCCGATTAATGGGTGGGATGGAAGCCCC  150
A. longicornu     ................................................   150

A. morio          GTTGATTCATCGTCCGGTTGCTCTGAGAAATTATTGGATATTCCAGCT--  198
A. longicornu     ..............K..............................AT   200

A. morio          AACCCAATACAGTTGTCATCGCAAGACAATTGACAT                234
A. longicornu     ................CT..............                   236
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Fig. 1. Alignment of ITS1 (A) and ITS2 (B) sequences of *A. morio* and *A. longicornu*. Dots indicate sequence matches to the first line.

berg 1986) seems to be frequently occurring in the closely related orchid genus *Dactylorhiza* (Hedren 1996). However, as far as *N. commutata* is concerned, no definite difference has been found between its sequence and those of the *N. tridentata* accessions from southern Italy and Sardinia. On the contrary, the israelian accessions of N. tridentata display some differences from the two former ones (Fig. 2), thus indicating that the ITS region for this taxon may also be a useful source of data to infer relationship at the population level (Vargas et al. 1999; Vilgalys and Sun 1994). At this regard, the presence of heterozygous positions in the sequences of some *N. commutata* accessions, as confirmed also by cloning procedures, may indicate a possible occurrence of gene flow in Sicily among different *N. tridentata* genotypes from the rest of the mediterranean area. However, a more detailed investigation on *N. commutata* populations, with an extensive sampling throughout the entire range, as well as further studies on mediterranean populations of *N. tridentata* is needed to quantify the occurrence of this gene flow in Sicily, and may help to understand whether the above mentioned heterozygous positions depend upon sequence differences among the actual, not yet examined, diploid genotypes involved, to repeated backcrosses with local genotypes or to ongoing gene conversion (Sang et al. 1995).

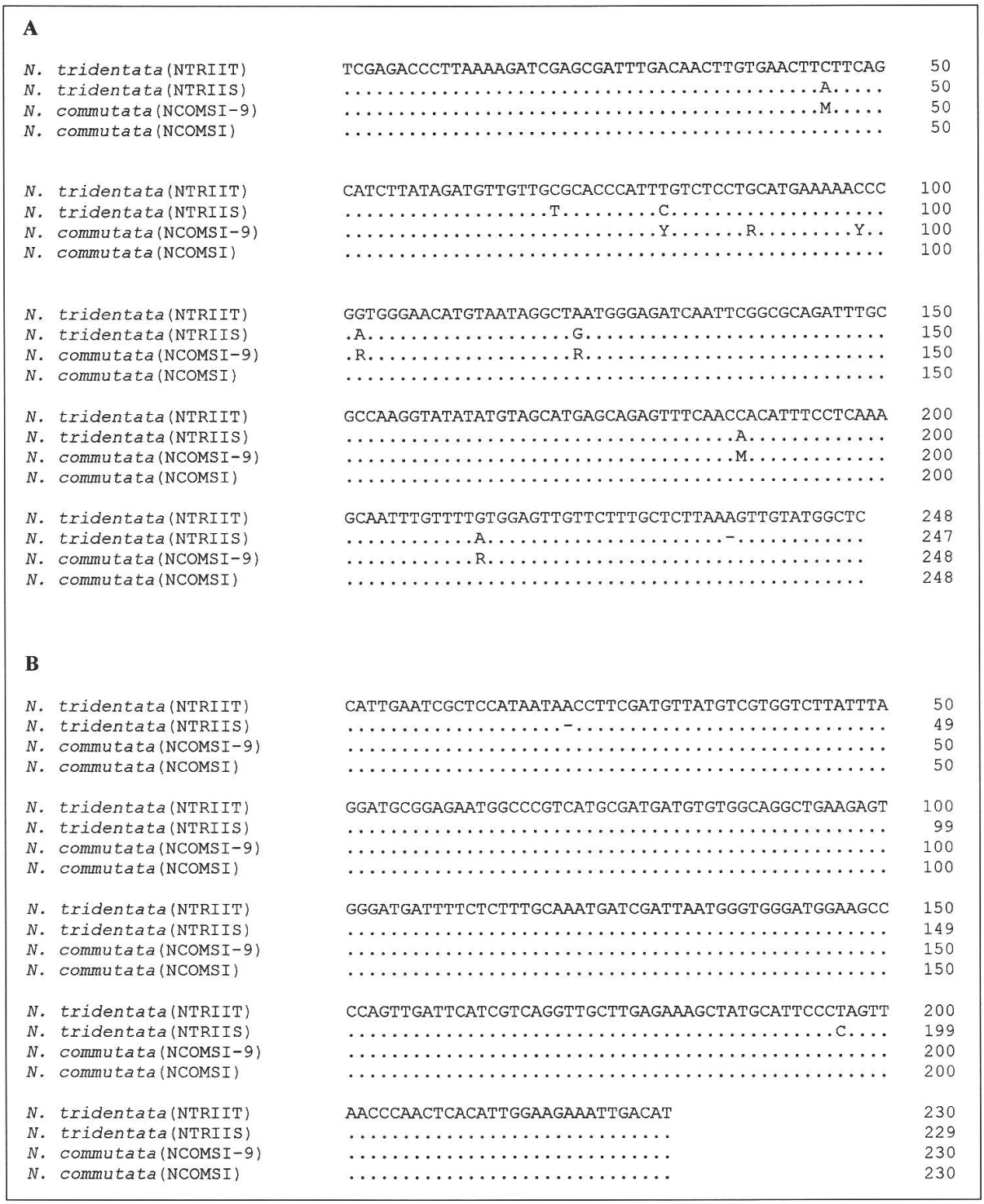

Fig. 2. Alignment of ITS1 (A) and ITS2 (B) sequences of *N. tridentata* from Italy (NTRIIT), *N. tridentata* from Israel (NTRIIS), *N. commutata* (NCOMSI) and a *N. commutata* accession (NCOMSI-9) from Arcia (ME). Dots indicate sequence matches to the first line.

The accessions of *O. brancifortii* from Sicily and Sardinia differ from *O. quadripunctata* for a single substitution (position 41 of ITS2). Similarly, *O. anatolica*, an eastern mediterranean closely related taxon (Delforge 1994) differs from *O. quadripunctata* for

one base substitution (position 160 of ITS1; Cozzolino et al. in press). The main differences between the two taxa are related to flower shape. The flower of *O. brancifortii* differs from the closely related *O. quadripunctata* flower for its shorter spur and the labellum always smaller than lateral tepals.

The sequence of *O. mascula* ssp. *ichnusae* is identical to those of *O. mascula* ssp. *mascula* from south France and northwestern Italy. It must be noted that *O. mascula* ssp. *ichnusae* sequences, while completely matching with those of *O. mascula* from northern Italy, differ for a single substitution from southern Italy accessions. Within the limits of the present analysis, this result may indicate a possible Sardinian dispersion starting from *O. mascula* populations inhabiting north-eastern Italy or southern France, rather than central or southern Italy. On a morphological standpoint, *O. mascula* ssp. *ichnusae* differs from the widespread *O. mascula* ssp. *mascula* (distributed from Portugal to Anatolia) for its flower size (smaller than in the typical supspecies), pink-lilac (never purple) tepal color, and spur length (always shorter than ovary; Corrias 1981).

The local pollinator community may exert strong selection on some morphological traits and, in conditions of geographical isolation, this may lead to the formation of geographic ecotypes (Grant 1963; Stebbins 1970), with slight floral differentiation, reflecting ongoing adaptation to local pollinator community (i.e. the changes in the "pollination climate" as defined in Grant 1963; Hodges and Arnold 1994). Within the limits of the present study, this may be a possible scenario for *O. brancifortii* and *O. mascula* ssp. *ichnusae*, in which the floral morphological differences, due to the few sequence differences found, do not find support in genetic distance with the widespread related taxa.

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