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UNRAVELING CRYPTIC RETICULATE RELATIONSHIPS AND THE ORIGIN OF ORPHAN HYBRID DISJUNCT POPULATIONS IN *NARCISSUS*

Isabel Marques,[1,2,3] Gonzalo Nieto Feliner,[2] David Draper Munt,[4] Maria Amélia Martins-Loução,[1] and Javier Fuertes Aguilar[2]

[1]Universidade de Lisboa. Museu Nacional de História Natural, Jardim Botânico, Rua da Escola Politécnica 58. 1280-102 Lisboa, Portugal

[2]Real Jardín Botánico, CSIC. Plaza de Murillo, 2. 28014 Madrid, Spain

[3]E-mail: icmarques@fc.ul.pt; isabel.ic@gmail.com

[4]Dpto. Biologia Vegetal. E.T.S. Ingenieros Agrónomos. Universidad Politécnica de Madrid. Av. Complutense s/n 28040 Madrid, Spain

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Evolutionary consequences of natural hybridization between species may vary so drastically depending on spatial, genetic, and ecological factors that multiple approaches are required to uncover them. To unravel the evolutionary history of a controversial hybrid (*Narcissus* ×*perezlarae*), here we use four approaches: DNA sequences from five regions (four organellar, one nuclear), cytological studies (chromosome counts and genome size), crossing experiments, and niche modeling. We conclude that (1) it actually consists of two different hybrid taxa, *N.* ×*perezlarae* s.s. (*N. cavanillesii* × *N. miniatus*) and *N.* ×*alentejanus* (*N. cavanillesii* × *N. serotinus*); (2) both have been formed several times independently, that is, polytopically; (3) *N. cavanillesii* was the mother progenitor in most hybridization events. We also address the origin of orphan hybrid populations of *N.* ×*perezlarae* in eastern Spain, hundreds of kilometers away from *N. cavanillesii*. Although long-distance dispersal of already formed hybrids cannot be completely rejected, extirpation of *N. cavanillesii* via demographic competition is a more likely explanation. Low-reproductive barriers to fertilization by foreign pollen in *N. cavanillesii*, molecular footprints of the former presence of this species in the area, active asexual propagation by bulbs in *N.* ×*perezlarae*, and overlapping ecological niches are consistent with the extirpation scenario.

KEY WORDS: **cpDNA, ITS, mtDNA, niche competition, niche modeling, parental extirpation.**

The importance of hybridization as a diversity-generating mechanism is acknowledged not only in plants (Stebbins 1959; Grant 1971) but also in other groups of organisms (Grant and Grant 1992; Dowling and DeMarais 1993; Gogarten and Townsend 2005; Mallet 2005). Evidence is now profuse about evolutionary consequences derived from processes of hybridization (Arnold 1997; Rieseberg and Wendel 2004; Cozzolino et al. 2006). During the last two decades, two facts have been instrumental in shedding light on reticulate evolution. One is the generalized use of molecular evidence that has allowed documenting genetical case studies that had or had not been previously proposed based on other evidence (Rieseberg and Soltis 1991; Hegarty and Hiscock

2005). Recognizing and measuring the importance of ecological aspects, specifically adaptation, in hybridizing events is the other aspect that has contributed to our understanding of hybridization (Campbell et al. 1997; Choler et al. 2004; Gross and Rieseberg 2005).

Yet, one of the major difficulties when trying to understand the overall relevance of hybridization and making predictions about its evolutionary consequences is, in fact, the wide class of processes that it may actually involve. Hybrids may be as widely defined as offspring between individuals from populations "which are distinguishable on the basis of one or more heritable characters" (Harrison 1990; Rieseberg and Carney 1998). Thus, consequences derived from this wide-ranging topic are necessarily diverse (Barton 2001), ranging from the introgression of genes of one hybridizing species into the other (Rieseberg and Wendel 1993), or the reinforcement of internal reproductive barriers (Rundle and Schluter 1998) to what is considered the greatest possible contribution of hybridization in evolutionary terms, hybrid speciation (Rieseberg 1997). The different evolutionary fates of hybridization events and the models that better describe them depend on a number of factors. Some of the most determinant are the possible associated occurrence of polyploidy (allopolyploidy), the spatial structure of hybridizing processes (e.g., if a hybrid zone occurs), the degree of internal reproductive barriers, the viability of F_1 hybrids, the availability of new or intermediate niches to colonize, the genetic or phylogenetic proximity of hybridizing species, and the fitness of hybrid lineages (Arnold 1997; Rieseberg and Carney 1998).

Because of the potential complexity of natural hybridization, research mostly concentrates on specific aspects or models instead of trying to design studies that are representative of all the classes of phenomena and possible outcomes. In the present article, we first focus on the possibility that the same hybrid is formed independently in different places, that is, polytopically. Second, we explore the different evolutionary fates when this phenomenon occurs. A polytopic origin of hybrids has been reported particularly in polyploids (allopolyploids), for example, in North American hybrid species of *Tragopogon* originated repeatedly from crosses between Old world introduced species (Soltis and Soltis, 1991). However, the cohesiveness of the different polytopic hybrid populations formed has been questioned by the genomic changes detected already in less than one century (Soltis et al. 2004). Well-documented studies point to the opposite direction, that is, different evolutionary outcomes when the same species hybridize in different places and at different time (*Saxifraga*, Steen et al. 2000; *Helianthus*, Gross and Rieseberg 2005; *Senecio*, Kadereit et al. 2005).

Hybridization has been invoked as one of the causes for the high levels of inter- and intraspecific morphological diversity in *Narcissus* (Fernandes 1968; Fernandes 1975). This genus provides potential cases of polytopic hybrid origins, one of which is represented by *Narcissus* ×*perezlarae*. This hybrid, reported from different localities in the southern part of the Iberian Peninsula, was proposed to be a natural hybrid between *N. cavanillesii* (subgenus *Narcissus*) and *N. serotinus* (subgenus *Hermione*) (Font Quer 1927). Chromosome counts (Valdés and Müller-Doblies 1984) and the fact that the hybrid occurred in most populations in which both parents coexisted (Font Quer 1927; Valdés and Müller-Doblies 1984; Marques et al. 2005) supported the proposed hybrid origin of *N.* ×*perezlarae*.

Yet, two facts have raised doubts on the origin and parentage of this hybrid. First, a recent morphological and cytogenetic study of *N. serotinus* recognized two different species within this name. Consequently, populations in the western part of the Iberian Peninsula and northwestern Morocco were recognized as *N. serotinus* whereas the eastern Iberian populations were segregated in a new taxon, *N. miniatus* (Donnison-Morgan et al. 2005; = *N. obsoletus*; Díaz Lifante and Camacho 2007). Based on this segregation, the populations of *N.* ×*perezlarae* s.l. thought to have originated from *N. serotinus* were recently given a different name, *N.* ×*alentejanus* (Fernández-Casas 2008), although morphologically they are very similar to *N.* ×*perezlarae*. From here on, unless otherwise stated, we use *N.* ×*perezlarae* in a strict sense, that is, the putative hybrid between *N. cavanillesii* and *N. miniatus*.

The second fact that raises doubts on the previously proposed origin of *N.* ×*perezlarae* is the discovery of isolated (orphan) hybrid populations in eastern Spain, where the progenitor *N. cavanillesii* has never been reported (Soler 1998; Marques et al. 2007). Long-distance dispersal (LDD) is infrequent or absent in this genus, and the nearest populations of *N. cavanillesii* are too far (∼450 Km) to allow recurrent gene flow.

Thus, several questions are relevant concerning this case: (1) Are *N.* ×*alentejanus* and *N.* ×*perezlarae* two different hybrids or a single one with a polytopic origin? (2) Which are the species involved in their origin? (3) What are the causes for the existence of the orphan hybrid populations of *N.* ×*perezlarae*? To shed light on these questions, we carried out a multidisciplinary analysis purporting to unravel the evolutionary history of *N.* ×*perezlarae* s.l. This analysis consisted of four steps.

First, we surveyed natural populations of *N. cavanillesii*, *N. elegans*, *N. serotinus*, and *N. miniatus*, which either by flowering phenology or morphological traits could be considered putative progenitors of *N.* ×*perezlarae* and *N.* ×*alentejanus*. We used both maternally inherited (plastid and mitochondria) and biparentally inherited (ITS) sequences for distinguishing effects caused by hybridization from those created by lineage sorting.

Second, to assess the strength of reproductive barriers, we developed a crossing program between several populations of *N. cavanillesii*, *N. serotinus* and *N. miniatus*. We also included populations of *N.* ×*perezlarae* and *N.* ×*alentejanus* to test whether

hybrids are reproductively isolated or could represent a source of genetic assimilation for the parental species through repeated backcrossing.

Third, because meiotic behavior is a critical postpollination barrier to hybridization, we surveyed the chromosome number in all hybrid populations and their parents, including those in the isolated eastern Iberian populations, either by direct observation of chromosomes or by flow cytometry. Previous studies have reported that several populations of *N.* ×*perezlarae* have $2n = 29$ (Valdés and Müller-Doblies 1984), an intermediate chromosome number between those reported in *N. cavanillesii* ($2n = 28$) and *N. miniatus* ($2n = 30$) but chromosome number in *N.* ×*alentejanus* is unknown.

Finally, ecological predictive models were developed for each species to identify the niche requirements of the parental species and their respective hybrids. If niche differences between the progenitors and the hybrids occurred, the absence of the progenitors in eastern Iberian orphan populations could be associated to the lack of their ecological niche. If no ecological divergence were detected, the current absence of the progenitors should require other causes.

Materials and Methods

PLANT SAMPLING

The sampling was designed to cover the whole distribution range of the six species involved in the study (Table S1, Fig. S1). A significant sampling effort was performed, especially in the Iberian Peninsula, where several sympatric populations occur. With such a sampling effort we intended to minimize uncertainties in parentage determination across the whole distribution range of *N.* × *perezlarae* s.l., a hybrid that can be not only heterogeneous (involved more than two parents) but also polytopic (arising independently several times).

For the molecular study, 1450 individuals were included: 405 from *N. cavanillesii* (15 sympatric and 13 allopatric populations), 391 from *N. serotinus* (seven sympatric and 21 allopatric populations), 283 from *N. miniatus* (six sympatric and 15 allopatric populations), 215 from *N. elegans* (five sympatric and nine allopatric populations), 51 from *N.* ×*alentejanus* (four sympatric populations), 90 from *N.* ×*perezlarae* (three sympatric and three allopatric populations), as well as 15 individuals of *N. papyraceus* for rooting purposes. All the known hybrid populations were sampled. Generally, 15 individuals were sampled per population except otherwise indicated (Table S1). Because these species can propagate clonally, individuals were collected at a distance of more than 1 m from each other. Samples were preserved as bulbs, silica-gel dried leaves and/or stored at −80°C until ready for DNA isolation.

MOLECULAR ANALYSES

DNA isolation, PCR, cloning, and sequencing

Total DNA was isolated using the DNeasy™Plant Minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions and stored at −20°C. Four organellar regions were analyzed, two from the plastid genome (partial 5′-*ndhF* region, partial 5′-*matK*) and two from the mitochondrial genome (*cob, atpA*). Due to the great intraspecific variability previously detected in *Narcissus* in the ITS region (ITS1+5.8S+ITS2) from the nuclear ribosomal DNA (I. Marques, unpubl. data), this region was also sequenced. Primers used in the amplification were 1318 and 2110 for *ndhF* region (Olmstead and Sweere 1994), BF and CR (Ito et al. 1999) for *matK*, P1 and P6 (Städler and Delph 2002) for *cob*, F1 and B1 (Eyre-Walker and Gaut 1997; Davis et al. 1998) for *atpA*, and AB101 and AB102 for the ITS (Douzery et al. 1999). Polymerase chain reactions (PCRs) were performed in 25 μL reactions using PuReTaq Ready-To-Go PCR beads (Amersham Biosciences, Uppsala Sweden) after adding 1 μl of DMSO (4% v/v), 0.5 μl of each primer (10 μM), 1 μl of DNA (45–130 μg mL^{-1}). Amplifications were carried out on a GeneAmp 9700 PCR system (Perkin Elmer Biosystems, Foster City, CA) under conditions summarized in Table S2. All PCR products were purified using UltraClean PCR Clean-up Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's protocol. Purified PCR products of the ITS region were cloned because previous direct sequencing always produced multiple sequence signals. Cloning of the ITS region was performed using One Shot TOP10 protocol (Invitrogen, San Diego, CA). Competent TOP10F'cells were chemically transformed and the resulting colonies were screened for plasmids with inserts by PCR isolating 10 single recombinant colonies from each reaction. Amplifications were performed using the original amplification primers or the M13 plasmid primers. Purified PCR products were sequenced in both directions on a 3730 DNA ANALYZER (Applied Biosystems, Foster City, CA) at the Centro de Genómica y Proteómica, Parque Científico de Madrid (Spain). In total, 1450 sequences were obtained for each organellar region and 1590 cloned sequences were obtained for the ITS region. Sequence alignment was performed manually using BioEdit 7.0.0 Sequence Alignment Editor (Hall 1999), which was also used to check electropherograms. DnaSP version 3 (Rozas and Rozas 1999) was used to characterize DNA polymorphism. Within-species diversity was estimated with Nei's haplotypic diversity, Hd, and in terms of weighted sequence divergence with nucleotide diversity, π (Rozas and Rozas 1999).

Haplotype and ribotype analyses

Describing relationships among haplotypes and nuclear ribosomal alleles in a reticulate evolution scenario requires an approach in which the genetic variation, albeit differentially distributed among

the different populations, is contemplated as a whole. For this, we have used phylogeographic and phylogenetic analyses. In our study, five individual matrices (one per marker) were obtained and for each one, sequences were analyzed to detect variable sites and informative positions.

Organellar and ITS sequences were independently analyzed with Bayesian inferences (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). For efficient analysis, redundant sequences retrieved from the same accession were eliminated. As a result, the operational matrices obtained consisted of 123 organellar sequences and 208 cloned ITS sequences. For the organellar sequences, we also implemented partitioned Bayesian analysis that were chosen based on gene identity (i.e., *cob*, *atpA*, *ndhF*, *matK*). The best available model of molecular evolution was selected using hierarchical likelihood ratio tests (hLRT) and Akaike information criterion (AIC) as implemented in the software MrModeltest 1.1b (Nylander 2002), which considers only nucleotide substitution models implemented in PAUP and MrBayes 3.01 (Ronquist and Huelsenbeck 2003). GTR + I + G (Yang 1996), the symmetrical model, with some sites assumed to be invariable and variable sites assumed to follow a discrete gamma distribution was the best-fit for all the regions.

Four Markov chain Monte Carlo (MCMC) were run simultaneously ensuring that the likelihoods had reached a steady phase. For the orgDNA dataset, the steady phase was achieved after 2×10^6 generations whereas for the nrDNA dataset, 5×10^6 generations were run. At the end, 25% of the total number of trees saved was discarded as burnin whereas the remaining sample was used to create a 50% majority-rule consensus tree. The software Tracer 1.4 (Rambaut and Drummond 2007) in combination with MrBayes 3.01 was used to monitor the performance of the analyses, namely mixing and convergence among runs. The trace plots for all Bayesian MCMC analyses indicate stationary and convergence among runs. The potential scale reduction factor (PSRF) was always close to 1. Bayes factors were used to compare partitions and were estimated using the harmonic mean of the likelihood values sampled from the stationary phase of the MCMC run (Nylander et al. 2004). The negative log likelihood was −5206.48 for the partitioned organellar dataset and −5136.32 for the all dataset. Because a difference larger than five units between analyses indicates a "very strong support for the model with the highest likelihood" (Kass and Raftery 1995), adding partitions in the organellar dataset does not improve the likelihood value. Sequences have been deposited in GenBank (Table S1).

Relationships among haplotypes were also estimated by applying statistical parsimony criterion using TCS 1.21 (Clement et al. 2000) with gaps coded as missing data. This method reconstructs relationships as unrooted networks connecting only haplotypes with a high probability (<0.95) of being similar due to shared history and not homoplasy (Templeton et al. 1992). The complete dataset of the organellar matrix and the nuclear matrix were separately analyzed.

Recombinant copies and putative progenitors were identified using the recombinant detection methods implemented in RDP3 beta24 (Martín et al. 2005) with the default settings options. Six recombination tests were applied: RDP (Martin and Rybicki 2000), GENECONV (Padidam et al. 1999), BOOTSCAN (Salminen et al. 1995), MAXCHI (Maynard Smith 1992), CHIMAERA (Posada and Crandall, 2001), and SISCAN (Gibbs et al. 2000). Recombinant analyses were performed using correct *P*-values and considering only the results confirmed by at least two tests.

CROSSING EXPERIMENTS

In 2003, controlled pollinations were performed to evaluate the degree of reproductive isolation between *N.* ×*perezlarae* s.l. and its putative progenitors, *N. cavanillesii*, *N. serotinus*, and *N. miniatus*. Individuals were grown at the Jardim Botânico de Lisboa greenhouse under controlled conditions. Pollinations were performed using individuals of *N. cavanillesii* from PTA population, *N. serotinus* also from PTA population, *N. miniatus* from XAB population, *N.* ×*alentejanus* from PTA population, and *N.* ×*perezlarae* from MDS population and from the orphan hybrid population of OLI (Table S1). Each species was crossed with all other species either as maternal or paternal parent (30 combinations) and intraspecific pollinations were used as a control (six combinations). A total of 50 randomly selected flowers, corresponding to different individuals, were used in each of the 36 treatments resulting in 1800 cross-pollinations. All treated flowers were previously emasculated and bagged with 1 mm mesh nylon tulle to exclude pollinators. Flowers were monitored for fruit set after anthesis until complete dehiscence. Mean fruit set and mean seed production were obtained for each treatment and compared by nonparametric Mann–Whitney or Kruskal–Wallis tests. To evaluate seed viability, four replicates of 25 seeds from each pollination treatment were sown on moistened filter paper, in Petri dishes, following the conditions described in Marques et al. (2007). At the end of each assay, final germination percentage was assessed. Data were previously transformed and analyzed by one-way analysis of variance (ANOVA). All statistical analyses were carried out using SPSS 11.0 (SPSS, Inc., Chicago, IL).

CHROMOSOME NUMBER AND ESTIMATION OF NUCLEAR GENOME SIZE

Root tips of 10 different plants (except otherwise indicated: Table S1) were pretreated in 0.05% colchicine aqueous solution for 4 h at room temperature and then fixed in 3:1 ethanol:acetic acid. Chromosomes were stained using the Feulgen method (Feulgen and Rossenbeck 1924). For each individual, mitotic chromosome counts were determined upon observation of 10 different metaphases, except in the case of *N.* ×*alentejanus* where

only one metaphase plaque per population was possible to visualize, despite using several techniques and conditions. We applied flow cytometry to confirm the ploidy level and determine the nuclear genome size of the species in study (Zonneveld 2008).

Flow cytometry was performed in a total of 80 individuals: 15 from *N. cavanillesii*, 10 from *N. serotinus*, 15 from *N. miniatus*, 20 from *N. elegans*, 10 from *N.* ×*alentejanus*, and 10 from *N.* ×*perezlarae* (Table S1). For each sample, a fresh young leaf tissue was cochopped with an internal standard in 600 μl of Galbraith's buffer (Galbraith et al. 1983) supplemented with 100 μg mL^{-1} RNase II (Boehringer, Meylan, France) using a razor blade. *Pisum sativum* L. "Express Long" (2C = 8.37 pg) was selected as internal standard for flow cytometric measurements. A sample containing only the standard was first prepared and analyzed to determine peak position. Nuclei were filtered through a 33 μm nylon filter PA 1000 140/355–35W (SEFAR, Barcelona, Spain) before adding 50 μg mL^{-1} of propidium iodide (Sigma-Aldrich) and kept on ice for 10 min before measurement. Two samples from each individual were extracted and measured independently to ensure repeatability of results. All analyses were carried out using a Cell Lab Quanta Beckman Coulter (Brea, CA, U.S.A.) flow cytometer.

NICHE MODELING

A total of 641 point localities were considered, 139 from *N. cavanillesii*, 176 from *N. miniatus*, 262 from *N. serotinus*, 10 from *N.* ×*alentejanus*, and 18 from *N.* ×*perezlarae*. Occurrence data for all species were obtained directly in the field between 2001 and 2007, and complemented with herbarium material whose identification was previously confirmed (Fig. S1). In the few cases in which herbarium labels lacked coordinates, localities were geo-referenced using GEOLocate version 3.0 (http://www.museum.tulane.edu/geolocate). Ecological niche models were generated using the Maxent algorithm (Phillips et al. 2006) due to the scarce number of localities available for hybrid lineages. This algorithm is appropriate for presence-only data, and has proved to be a powerful tool in comparison with other methods (Elith et al. 2006) even in the presence of small datasets (Pearson et al. 2006). GIS layers used in the ecological niche model construction for the five *Narcissus* species included the 19 climate data variables from Worldclim (Hijmans et al. 2005) with a 30 arc-second resolution (approximately 1 km^2). Fitness of the models was assessed using the area under the curve (AUC) of a receiver-operating characteristics (ROC) plot (Greaves et al. 2006; Milne et al. 2006). To obtain a binary map, the threshold of the resulting models was determined as the minimum predicted value that included all records of each species so that a 0% omission error (proportion of observed presences incorrectly predicted) was attained (Sérgio et al. 2007). By assigning a 0% omission error, we enforce that all known populations are included in the predicted area. Similarity of niche models was assessed using interpredictivity measures by overlaying the point localities of one species into the predicted area of another (Peterson and Vieglais 2001; Yesson and Culham 2006). Similarity was quantified as the percentage of points that fall into the prediction model of the other species. Idrisi Kilimanjaro (Clark Labs, Clark University) was used to calculate and identify habitat segregation between hybrids and parental species.

Results

MOLECULAR ANALYSES

Haplotype and ribotype variation

The aligned matrix of the four-organellar regions (orgDNA) had 3285 bp, 2000 bp from the two mitochondrial regions (*cob*, *atpA*) and the remaining 1285 bp from the two plastid regions (*ndhF*, *matK*). Only four sites were found to be variable in the mitochondrial matrix (0.20%), all of them parsimony-informative while in the plastid matrix, 50 variable sites (3.89%) were detected, 44 (3.42%) of them, parsimony-informative. *Narcissus* ×*alentejanus* and *N.* ×*perezlarae* showed the highest levels of nucleotide variability with 38 and 34 variable sites, respectively (Table 1). The lowest level of variability was found in *N. cavanillesii* with seven variable sites, of which only one was parsimony informative.

The length of the ITS1–5.8S–ITS2 ranged between 840 and 870 bp. Alignment required the introduction of indels, especially in the case of *N. serotinus* and *N. miniatus* (Table 1). Consequently, the aligned matrix was 891 bp long. A high number of variable sites (304) were detected (34.12%), 297 of them (33.33%) were parsimony-informative. The highest number of variable sites was detected in *N. cavanillesii*, 251, and the lowest in *N. serotinus* with 42 sites (Table 1).

Statistical parsimony network

TCS analysis of the orgDNA sequences yielded one single network containing 19 haplotypes plus the unconnected outgroup *N. papyraceus* (Fig. 1). Three predominant haplotypes were distinguished (H1, H12, and H13), which were used to define three main groups (A, B, and C; Fig. 1). Group A gathered 71.2% of *N. cavanillesii* sequences, Group B encompassed 97.8% of *N. serotinus* sequences, and Group C consisted mainly of sequences from *N. miniatus* (56.2%) and *N. elegans* (35.1%). Among hybrids, 84.4% of *N.* ×*alentejanus* sequences and 92.2% of *N.* ×*perezlarae* sequences were grouped with *N. cavanillesii* in Group A (Fig. 1).

Of the five haplotypes detected in *N. cavanillesii*, two were shared with *N.* ×*alentejanus* (H1 and H2), one with *N.* ×*perezlarae* (H1), and three were exclusive and endemic to Moroccan populations (H3–H5). Four haplotypes were specific to *N. elegans* (H16–H19) and two were specific of *N. serotinus*

Table 1. Comparative information for orgDNA (cpDNA and mtDNA) and nrDNA surveyed.

	cpDNA					mtDNA					orgDNA					nrDNA				
	H_{cp}	N_{var}	N_{par}	H_d	π	H_{mt}	N_{var}	N_{par}	H_d	π	Horg	N_{var}	N_{par}	H_d	π	R	N_{var}	N_{par}	H_d	π
N. cavanillesii	5	7	1	0.509	0.00058	1	0	0	0	0	5	7	1	0.509	0.00058	11	251	250	0.515	0.05187
N. ×*alentejanus*	5	36	31	0.764	0.01137	2	2	2	0.545	0.00522	5	38	33	0.764	0.000547	4	112	108	0.582	0.03683
N. ×*perezlarae*	4	32	31	0.745	0.00956	2	2	2	0.545	0.00522	4	34	33	0.745	0.000537	8	115	110	0.923	0.05259
N. serotinus	2	16	11	0.259	0.00194	2	1	1	0.133	0.00662	2	17	12	0.259	0.00080	7	42	40	0.682	0.00832
N. miniatus	2	11	11	0.301	0.01255	2	1	1	0.290	0.01057	2	12	12	0.370	0.00095	23	215	215	0.968	0.07925
N. elegans	5	27	26	0.574	0.00722	2	1	1	0.382	0.00019	5	28	27	0.574	0.00294	14	246	246	0.933	0.09071

H, number of haplotypes (cp, cytotypes; mt, mitotypes; org, organellar types); R, number of ribotypes; N_{var}, number of variable sites; N_{par}, number of parsimony informative sites; H_d, Haplotype diversity (for each region); π, nucleotide diversity.

(H12 and H15), the latter being only found in eastern Mediterranean populations (Fig. 1). From the two haplotypes detected in *N. miniatus* (H13 and H14), the most frequent was also shared with *N. elegans* (H13). Finally, three haplotypes were exclusively found in *N.* ×*alentejanus* (H6–H8) whereas three other were only found in *N.* ×*perezlarae* (H9–H11). These six novel haplotypes resulted from combinations of two *matK* haplotypes predominant in *N. cavanillesii* and *N. serotinus* and three *ndhF* haplotypes present in *N. serotinus* and *N. miniatus* (Fig. S2).

Due to the high variability found in the ITS sequences, TCS identified 52 ribotypes distributed in 18 unconnected networks (not shown).

Phylogenetic analyses

BI analyses of the organellar sequences were highly congruent with the results yielded by TCS. The three main groups previously identified (A, B, and C; Fig. 1) were obtained in the 50%-majority rule consensus BI tree (Fig. S3). Haplotypes retrieved in Group A are mostly associated with *N. cavanillesii* (71.2%), followed by *N.* ×*perezlarae* (14.6%), *N.* ×*alentejanus* (7.6%) and *N. elegans* (6.6%). All the haplotypes of *N. cavanillesii* fall in this group. Within Group B, most sequences (97.8%) belonged to *N. serotinus* whereas the remaining 2.2% belong to *N.* ×*alentejanus*. All the sequences of *N. serotinus* fell in this group with the exception of three eastern-Mediterranean populations. Group C mainly consisted of sequences from *N. miniatus* (56.2%) and *N. elegans* (35.1%), which were identical. The remaining 7.3% grouped a small number of haplotypes of *N. serotinus* whereas 1.4% refers to a rare haplotype found in *N.* ×*perezlarae*.

BI analysis of the ITS dataset resulted in two predominant groups of ribotypes (R1 and R7), which included, respectively, 35.5% and 35.3% of the total number of sequences cloned (Fig. 2). Most of the sequences grouped in R1 were retrieved from *N. cavanillesii* (66.5%), *N.* ×*perezlarae* (14.9%), and *N.* ×*alentejanus* (14.2%). Moreover, R1 was also the predominant ribotype found in *N.* ×*alentejanus* and *N.* ×*perezlarae*, representing 93.8% and 86.7% of the sequences retrieved in each of the hybrids, respectively.

A small number of sequences, genetically similar to *N. cavanillesii* were found in three sympatric populations of *N. elegans* (2.1%), whereas the remaining 2.30% of R1 were retrieved from the Minorca population of *N. miniatus* (M. FOR) despite the fact that there has never been any record of the presence of *N. cavanillesii* in the island. Most of the sequences found in the R7 ribotype group were from *N. serotinus*. The rest of the sequences falling in the R7 group were from *N. miniatus* (32.3%), *N. cavanillesii* (1.6%), *N.* ×*perezlarae* (1.1%), *N.* ×*alentejanus* (0.9%), and *N. elegans* (0.5%).

From the 1590 cloned sequences, 39 (2.5%) representing clades R13 and R16 of the BI tree (Ψ; Fig. 2) are suspected to

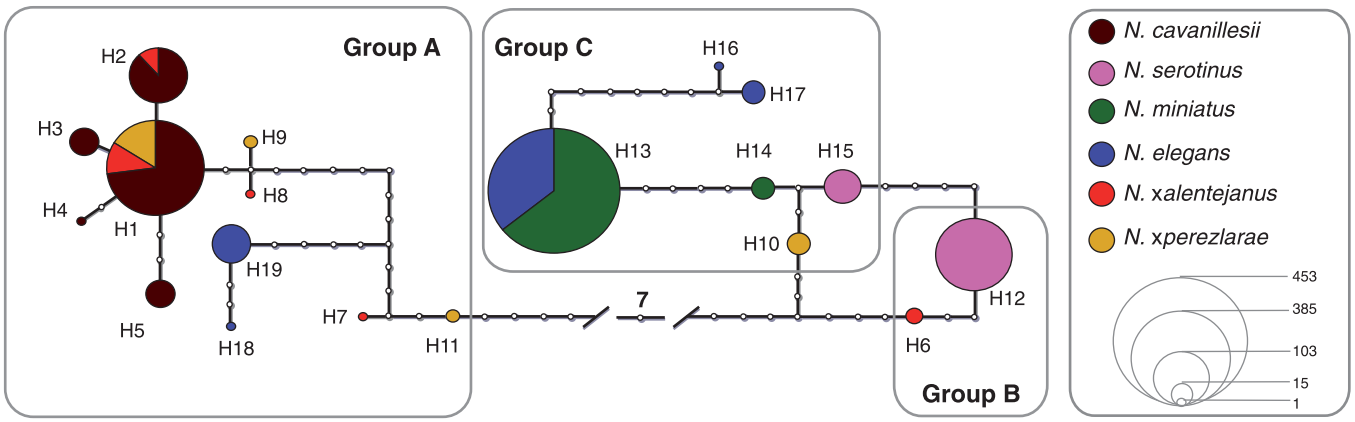

Figure 1. Statistical parsimony haplotype network based on combined sequences from two mitochondrial regions (*atpA* and *cob*) and two plastid regions (*ndhF* and *matK*). Small empty circles represent single mutational steps. Circle size is proportional to haplotype frequency. $N = 1435$ organellar sequences. Groups A, B, and C correspond to major lineages recognized by the phylogenetic tree obtained by Bayesian inference.

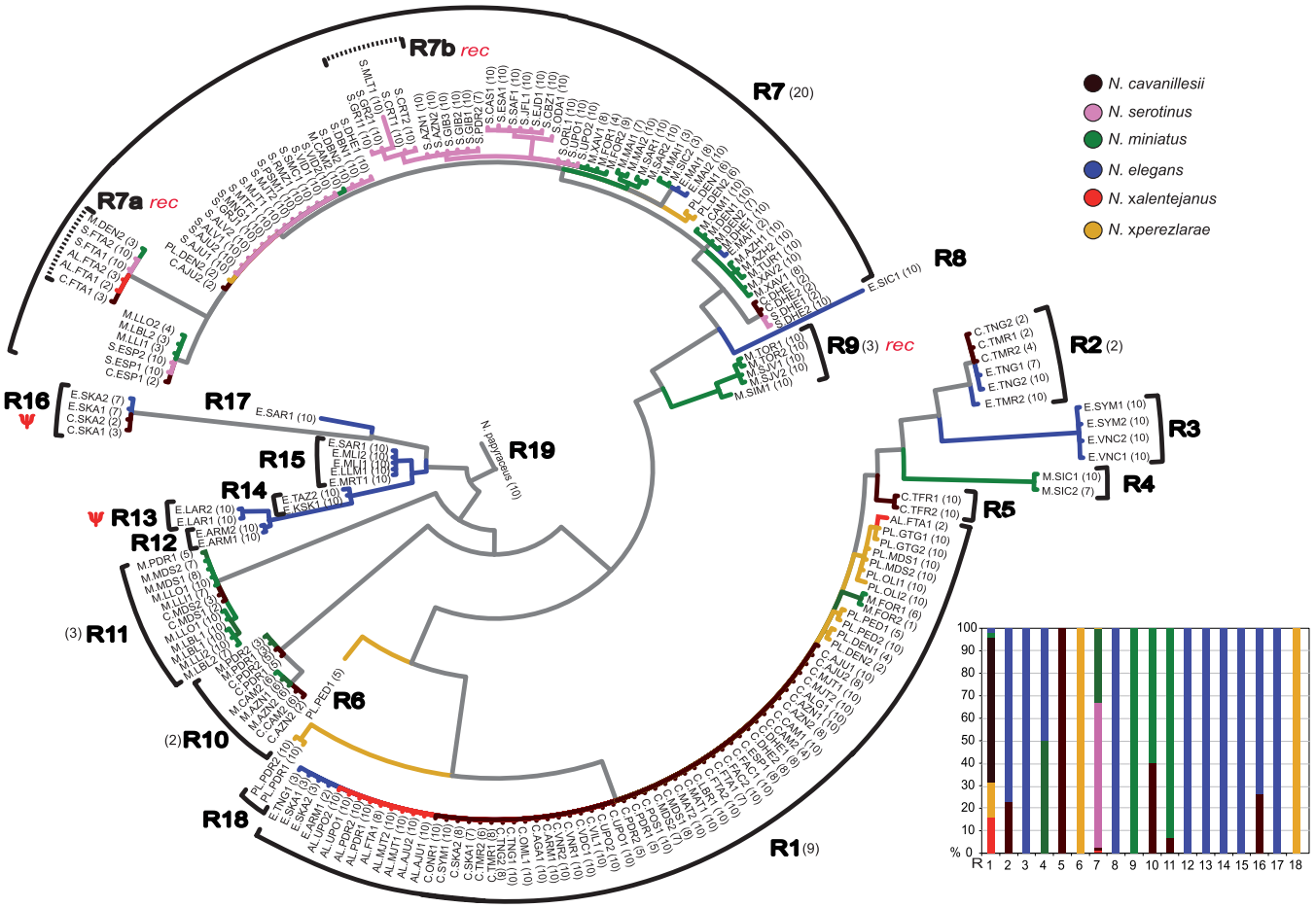

Figure 2. Fifty percent majority-rule rooted consensus tree of the ITS cloned sequences obtained by Bayesian inference. Due to the high ITS variability, 18 groups of ribotypes (plus the outgroup *N. papyraceus*) were defined along the tree (R1-R19). The number of different sequences for each group, when higher than one, is indicated between parentheses. Ψ indicates suspected pseudogenes and "rec" refers to sequences identified as recombinants. Distribution of the different groups of ribotypes across taxa, expressed as percentage, is depicted in the histogram. $N = 1590$ cloned sequences.

be pseudogenes because they have 1bp-indels in the conserved 5.8S region. This gap was found in position 383, in 20 copies of *N. elegans* (R13) and in position 504, in 19 copies of *N. cavanillesii* and *N. elegans* (R16). Of all cloned sequences, 131 (8%) were identified as recombinant sequences (rec; Fig. 2), by at least two recombinant tests (Table S3). Sequences from R7a and R7b groups were detected as recombinants between *N. elegans* (R12) and *N. miniatus* (R11) whereas sequences from R9 group were detected as recombinants between *N. elegans* (R3) and *N. serotinus* (R7).

CROSSING EXPERIMENTS

No strong reproductive isolation barriers were observed between *N. serotinus*, *N. miniatus*, and *N. cavanillesii*, although a significant asymmetry in fruit set was detected in some cases depending on the direction of crossings (Fig. 3). When *N. cavanillesii* acted as the mother progenitor, fruit set was significantly higher than when *N. serotinus* ($U = 950.000$, $P = 0.009$) or *N. miniatus* acted as such ($U = 850.000$, $P = 0.001$). Fruit set values in *N. cavanillesii* were similar whether coming from intra- or from interspecific pollinations ($H = 41.150$, $P = 0.781$) whereas in the other two species, fruit set of interspecific crosses was lower than fruit set of intraspecific crosses ($U = 800.550$, $P = 0.004$; Fig. 3). *Narcissus serotinus* was more compatible in interspecific crosses than *N. miniatus* ($H = 41.000$, $P = 0.007$; Fig. 3).

In contrast, the three hybrid populations were strongly isolated from their respective putative progenitors, especially the orphan hybrid population, where no fruit set was observed ($U = 800.000$, $P = 0.002$; Fig. 3). In the two sympatric hybrid populations, fruit set ranged from 5% in the progeny of the hybrids, with either *N. serotinus* or *N. miniatus* to 10% for offspring derived from crosses with *N. cavanillesii*. No fruit set occurred when either *N.* ×*alentejanus* or *N.* ×*perezlarae* acted as the mother progenitor (Fig. 3).

The same asymmetric pattern was found in the production of seeds. The number of seeds developed by *N. cavanillesii* in interspecific crosses was the same as in intraspecific crosses (4.5 ± 2.6 vs. 4.4 ± 2.1; $H = 39.425$ $P = 0.698$). In contrast, interspecific fertilizations resulted in a lower production of seeds than intraspecific fertilizations both in *N. serotinus* (1.6 ± 0.8 vs. 12.0 ± 6.5; $H = 40.050$ $P = 0.0001$) and in *N. miniatus* (1.6 ± 1.0 vs. 16.1 ± 9.4; $H = 40.500$ $P = 0.0001$). Independent of the hybrid population considered, the formation of seeds in backcross-pollinations was always very low, ranging from one seed per fruit in the progeny with either *N. serotinus* or *N. miniatus* to three seeds per fruit when considering backcrosses with *N. cavanillesii*.

In comparison with the putative progenitors, both hybrids produced fewer seeds ($U = 560.500$, $P = 0.002$) because 90% of them abort during the maturation phase. Nevertheless, the hybrid

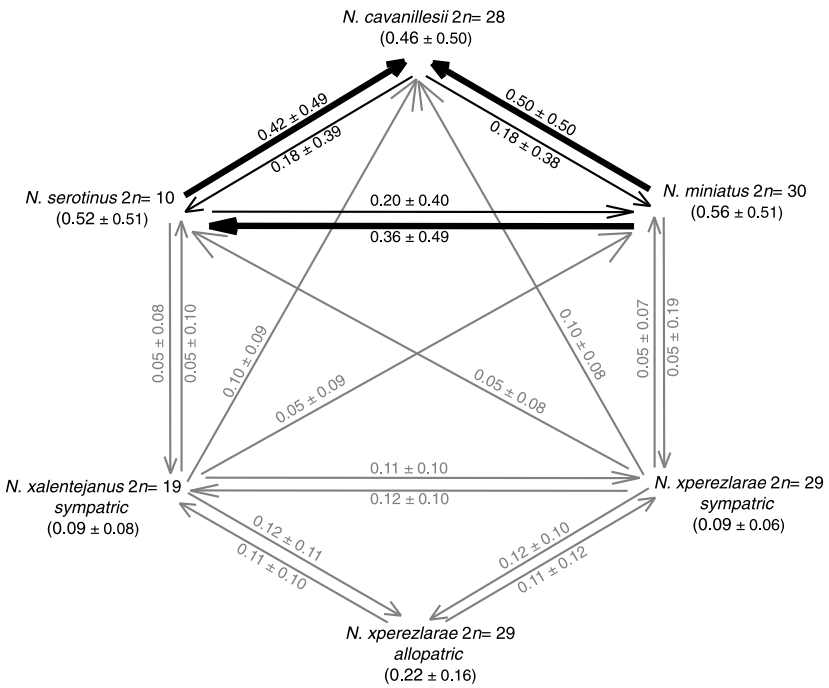

Figure 3. Reproductive isolation between the putative progenitors and the hybrids *Narcissus* ×*perezlarae* and *N.* ×*alentejanus* based on fruit set following experimental crosses. Arrows point to the maternal progenitors in the crossing experiments. Thickness is proportional to cross-compatibility. Numbers between parentheses indicate fruit set of intraspecific crosses. Mean ± SD. $N = 50$ per crossing treatment; $H = 34.869$, df = 35, $P = 0.0001$.

orphan population of *N.* ×*perezlarae* develops more viable seeds than the other two hybrid sympatric populations (3 vs. 1 seed per fruit; $U = 400.500$, $P = 0.0041$). Germination success was always higher than 88%, independently of the cross performed. No significant differences in germination percentage were found between seeds from interspecific pollinations and seeds from intraspecific pollinations ($P > 0.05$ in all crosses).

CHROMOSOME NUMBER AND ESTIMATION OF NUCLEAR GENOME SIZE

All studied populations consistently yielded 28 chromosomes for *N. cavanillesii*, 10 chromosomes for *N. serotinus*, 30 chromosomes for *N. miniatus*, and 20 chromosomes for *N. elegans*. The two hybrids had the expected intermediate chromosome numbers according to their parentage: *Narcissus* ×*alentejanus* $2n = 19$, *N.* ×*perezlarae* $2n = 29$, even in the orphan hybrid populations (Table S1).

Flow cytometry results were consistent with the two intermediate chromosome numbers found in the hybrids. Mean values for nuclear DNA amount were 31.94 ± 0.03 pg in *N. cavanillesii*, 20.71 ± 0.19 pg in *N. serotinus*, 51.18 ± 0.21 pg in *N. miniatus*, 26.62 ± 0.33 pg in *N.* ×*alentejanus*, and 41.83 ± 0.12 pg in *N.* ×*perezlarae*. No differences in the nuclear DNA content were found between sympatric individuals of *N.* ×*perezlarae* and those from orphan hybrid populations ($U = 124.150$; $P = 0.085$), suggesting the absence of polyploid complexes or hybrid swarms with changes in ploidy levels. *Narcissus elegans* had a mean DNA content of 30.67 ± 0.10 pg.

NICHE MODELING

High probability areas of occurrence generally matched the currently known distribution of the species with the exception of *N. cavanillesii* (Fig. 4, Fig. S1). In addition to its prediction in the southern part of the Iberian Peninsula and north-western Morocco where *N. cavanillesii* does occur, our results also point out the existence of suitable ecological conditions in the eastern part of the Iberian Peninsula, precisely where the orphan hybrid populations are located, as well as in the Balearic Islands, Kabilies, Tunisia, Sardinia and Sicily. Suitable conditions for the presence of *N. serotinus* were found in the southwestern part of the Iberian Peninsula, Morocco and eastern Mediterranean areas such as the Aegean and the Anatolian peninsula, where this species has been recorded (Fig. 4). Most of these areas match the ones found for *N. miniatus*, although suitable sites were also recorded for this species in the eastern part of the Iberian Peninsula, Balearic Islands, Kabilies, Tunisia, Sardinia, and Sicily (Fig. 4, Fig. S1).

When current known localities from one species were compared with the binary resulting model of another species, high degrees of niche similarity were recovered (Fig. 5). The two hybrids, *N.* ×*alentejanus* and *N.* ×*perezlarae* showed similar ecological requirements as the parental species (Fig. 5). Specifically, 100% of the localities of *N.* ×*alentejanus* overlapped with the niche model of *N. cavanillesii* and *N. serotinus*, and 96.34% of the localities of *N.* ×*perezlarae* covered the niche models of *N. cavanillesii* and *N. miniatus* (Fig. 5), which suggests the possibility of competition with the parental species, in both cases. The ROC plots for the training dataset exhibited high average AUCs for all the species (*N. cavanillesii*: 0.88, *N. serotinus*: 0.97, *N. miniatus*: 0.87, *N.* ×*alentejanus*: 0.90 and *N.* ×*perezlarae*: 0.89).

Discussion

HYBRID ORIGINS OF *NARCISSUS* ×*ALENTEJANUS*, AND *N.* ×*PEREZLARAE*

Evidence from the literature indicates that, in plants, hybridization between the same species may occur repeatedly at different times and geographical locations often leading to diverse evolutionary outcomes (Abbott and Lowe, 1996; Steen et al. 2000; Schwarzbach and Rieseberg, 2002; Soltis et al. 2004; Kadereit et al. 2005). In the present study, molecular, cytogenetic, and reproductive results indicate that three different species, one mother and two different fathers have given rise to two morphologically similar hybrids in different areas of the Iberian Peninsula (Fig. 6): *Narcissus* ×*alentejanus* (*N. cavanillesii* × *N. serotinus*) and *N.* ×*perezlarae* (*N. cavanillesii* × *N. miniatus*).

Further, our data indicate that *N. cavanillesii* has been in most cases the mother progenitor of the two hybrids, including the orphan populations of *N.* ×*perezlarae*. This is deduced from the haplotype network, where 92% of the organellar sequences of *N.* ×*perezlarae* and *N.* ×*alentejanus* were shared with *N. cavanillesii* (haplotypes H1 and H2; Fig. 1), but also from the ITS tree. The R1 group in the ITS tree contained 85.2% of the copies found in *N. cavanillesii*, 93.8% of the copies found in *N.* ×*alentejanus*, and 86.7% of the copies found in *N.* ×*perezlarae* (Fig. 2). Because differences in pollinators between hybrids and progenitors preclude the occurrence of backcrosses (I. Marques, unpubl. data), the similarity of ITS sequences between the two hybrids and *N. cavanillesii* can be explained by concerted evolution toward the mother species as detected in other hybrids (Franzke and Mummenhoff 1999; Lihová et al. 2004; Matyásek et al. 2007). The predominance of *N. cavanillesii* as the mother progenitor is also consistent with our crossing experiments because fruit set was significantly higher when *N. cavanillesii* acted as the ovule donor (Fig. 3).

Chromosome numbers found in *N.* ×*alentejanus* ($2n = 19$) and *N.* ×*perezlarae* ($2n = 29$) are intermediate between the parental species, which is also congruent with the amounts of nuclear DNA obtained. Although odd chromosome numbers result in irregular meiosis and thus the two hybrids are highly infertile, sterility is not complete. Our artificial pollinations reveal that the two hybrids are able to produce viable seeds (Fig. 3).

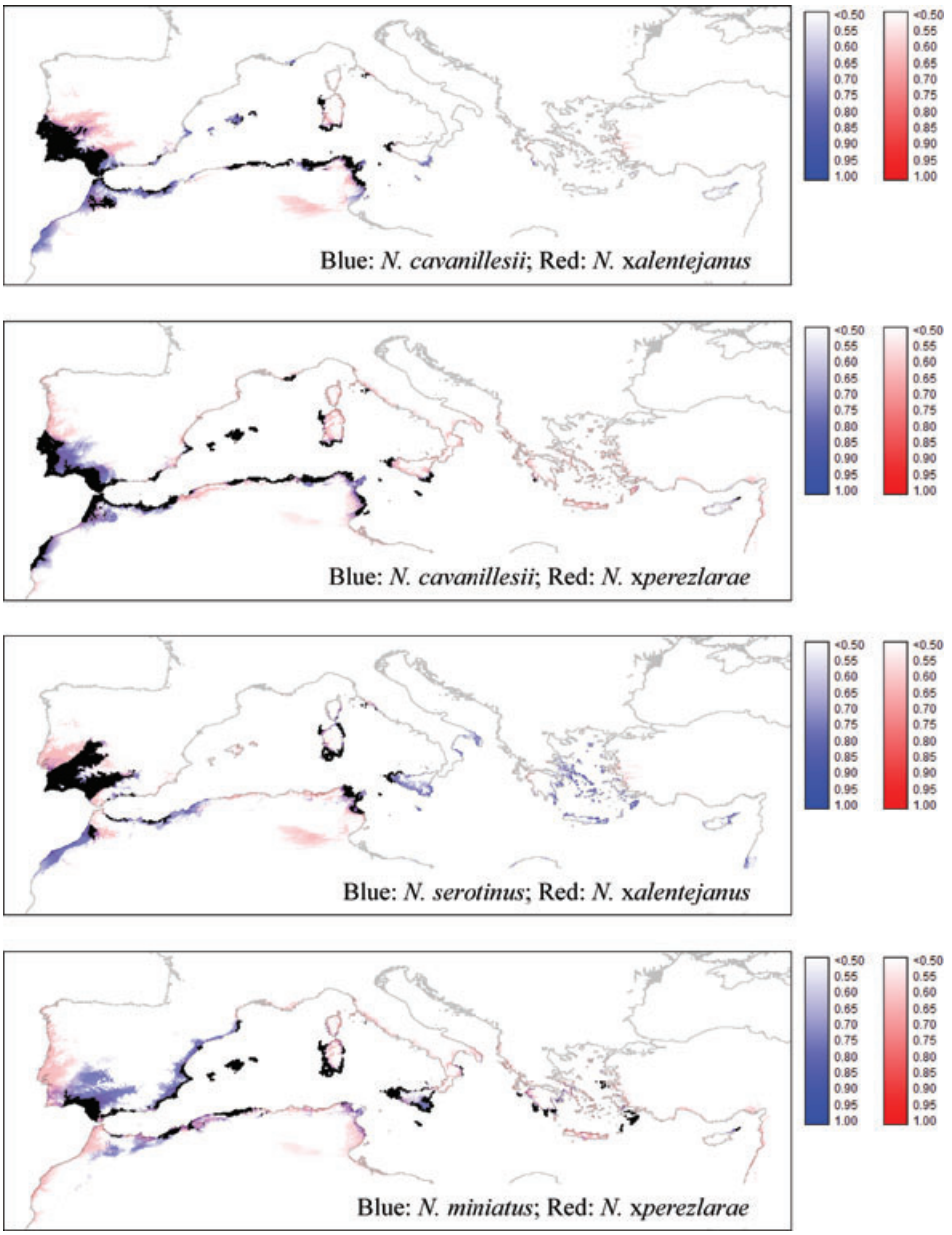

Figure 4. Predictive ecological models based on the Maxent algorithm of: ***N. cavanillesii*** **(AUC: 0.882),** ***N. serotinus*** **(AUC: 0.971),** ***N. miniatus*** **(AUC: 0.871),** ***N.* ×*alentejanus*** **(AUC: 0.898) and** ***N.* ×*perezlarae*** **(AUC: 0.892). The different maps assess similarity of niche models between one parental species and its hybrid using interpredictivity measures (see text). Predominance of one color indicates niche differentiation (blue scale vs. red scale) whereas dark color indicates overlapping of niche models.

localities \ niches	*N. cavanillesii*	*N. xalentejanus*	*N. xperezlarae*	*N. serotinus*	*N. miniatus*
N. cavanillesii	100	62.8	80.3	90.5	97.8
N. xalentejanus	100	100	90.9	100	100
N. xperezlarae	96.4	57.1	100	92.9	96.4
N. serotinus	91.7	82.7	82.3	100	95.7
N. miniatus	81.9	41.5	81.9	72.9	100

KEY (%)
0-45
45-60
60-85
85-100

Figure 5. Similarity of ecological niche between the five taxa represented as proportion of currently known populations (rows) versus the predicted niche model (columns).

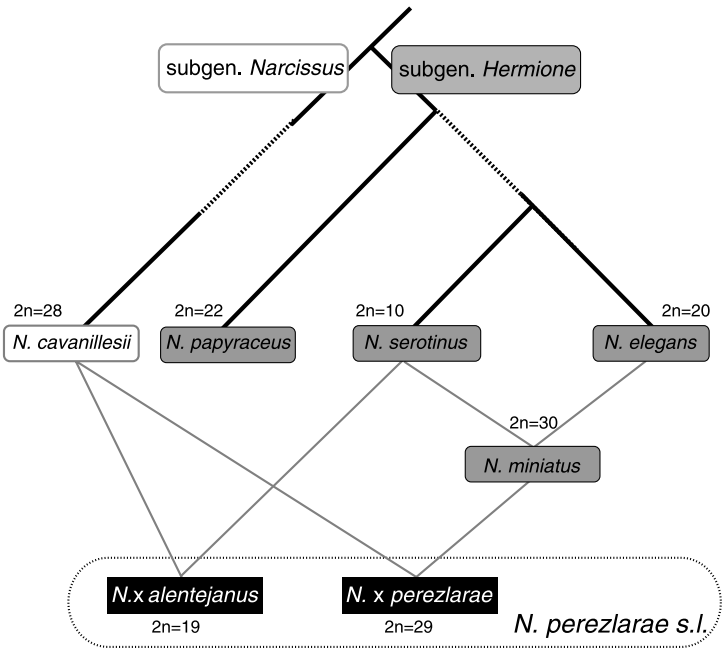

Figure 6. Scheme of the reticulate phylogenetic relationships among the studied species based on DNA sequences from two mitochondrial regions (*atpA* and *cob*), two plastid regions (*ndhF* and *matK*), and nrITS obtained in this study.

Our results also support that *N. miniatus*, the father progenitor of *N.* ×*perezlarae*, is an allopolyploid originated from *N. elegans* and *N. serotinus* as proposed by studies based on flow cytometry (Donnison-Morgan et al. 2005) and morphological characters (Díaz Lifante and Camacho 2007). *Narcissus miniatus* shared the same plastid haplotype with *N. elegans* (H13) but nrDNA alleles were grouped with either *N. serotinus* or *N. elegans* (Figs. 1 and 2). Our chromosome counts and flow cytometry results were consistent with the allopolyploid condition of *N. miniatus*, which also confirms recent genomic in situ hybridization (GISH) studies (Díaz Lifante et al. 2009).

GENETIC VARIATION IN HYBRIDS

One relevant feature of our results is the high haplotype diversity found in *N.* ×*alentejanus* and *N.* ×*perezlarae*. Six haplotypes were found to be exclusive to the two hybrid taxa. Certainly, the majority of the hybrids bear H1 or H2, which are predominant in the maternal progenitor *N. cavanillesii* as expected from the inheritance of cytoplasm markers. However, the haplotypes detected in 18% of the screened individuals of the two hybrids were not found in any other species in this study (H6–H11; Fig. 1). This percentage is worth of discussion, because lower nucleotide substitution rates, maternal inheritance and lack of, or at least, unlikeliness of recombination attributed to organellar markers make them less prone to change than the nuclear ones. Several factors may have contributed to this pattern. First, the haplotype distribution combined with the geographic locations of the two hybrids indicates that the origin of two hybrids is polytopic. These repeated hybridization events in different locations maximize the chances that genomes of different origins have merged. Second, asexual propagation plays an important role in the persistence of infrequent haplotypes. For instance, in some groups of animals in which sexual and asexual reproduction coexist in hybridizing populations, low-frequency haplotypes in sexual lineages increase their frequency in asexual lineages to the point that they become almost exclusive (Delmotte et al. 2003). In a similar way, asexual propagation in plants allows the persistence of rDNA alleles that otherwise would be eliminated due to concerted evolution following sexual reproduction (Suárez-Santiago et al. 2007). The same phenomenon may occur with the rare haplotypes detected in the two *Narcissus* hybrids, which despite their low fertility may persist in populations through bulb propagation. Third, all haplotypes that are exclusive to *N.* ×*perezlarae* and *N.* ×*alentejanus* are found in intermediate positions in the TCS network, which suggests that they might be the result of recombination within the plastid genome (Fig. 1, Fig. S2). Although infrequent in orgDNA, recombination has been reported in other plants (Ansell et al. 2007). Further, the fact that this putative recombinant haplotypes have been found in the two hybrids and not in the parental species suggests that recombination might be associated to the process of hybridization (Wolfe and Randle, 2002), a hypothesis that is consistent with similar findings in the mitochondrial genome (Jaramillo-Correa and Bousquet, 2005).

A high diversity of nrDNA sequences was also found in the two hybrids as compared to parental taxa. Several divergent ITS copies including recombinants, pseudogenes, and distinct

functional types have been observed in the same individual (Fig. 2). This may be explained by absence or retardation of concerted evolution, which is facilitated by the location of nrDNA loci and associated NORs on nonhomologous chromosomes (Sang et al. 1995; Campbell et al. 1997; Alvarez and Wendel 2003). This explanation seems to be particularly suitable for a genus such as *Narcissus* in which chromosomal rearrangements are active (Fernandes 1975) and may contribute to a marked instability in the number and position of nuclear ribosomal loci. In addition, as mentioned above, the ability of bulb propagation helps to perpetuate different nonhomogenized ribotypes. The active pseudogenization detected in the two hybrids is consistent with these two explanations.

CAUSES FOR ORPHAN HYBRID POPULATIONS IN EASTERN SPAIN

There are two plausible hypotheses to account for the origin of the orphan hybrid populations lying hundreds of kilometers apart from the nearest populations of *N. cavanillesii* and the remaining of *N. ×perezlarae.*

The first one is that LDD might have brought hybrids from western and southern Iberian Peninsula to eastern Spain. This implies that *N. cavanillesii* never occurred in eastern Spain, as it seems to be the case now, where it has never been reported despite being a floristically well-known region (Serra 2007, and references therein). Arguments against this hypothesis are that LDD is unknown in this genus, which lacks adaptive mechanisms for zoocory or anemocory. Second, although ecological niches found in eastern Spain are compatible with the requirements of both *N. cavanillesii* and *N. ×perezlarae*, it seems unlikely that stochastic phenomenon such as LDD should affect only *N. ×perezlarae* instead of *N. cavanillesii*, which is orders of magnitude more abundant than the hybrid. It might be argued that the hybrid could have been introduced by humans intentionally. This seems unlikely as well because *N. ×perezlarae* is horticulturally less attractive than its mother parent, although sequences are not conclusive to support or reject this hypothesis. The fact that the haplotype found in the orphan populations, is mostly shared with *N. cavanillesii* (H1) does not help and the ITS data are not decisive either. Ribotypes found in some individuals from the orphan populations (GT1, GT2, OLI1, OLI2) clustered with a western population (MDS1, MDS2) within R1 (Fig. 2), which might suggest the source of an LDD event. However, other eastern orphan populations did not cluster.

The second hypothesis to explain the origin of the orphan hybrid populations is that *N. cavanillesii* occurred in past times in eastern Spain, where it originated *N. ×perezlarae*. The disappearance of the mother progenitor in eastern Spain could be due to extirpation by hybridization through genetic swamping or demographic competition (Levin et al. 1996; Rhymer and Simberloff 1996; Carney et al. 2000; Wolf et al. 2001). With the available data, this is the most plausible hypothesis based (1) on the footprints of the former presence of *N. cavanillesii* in eastern Spain and (2) on the likeliness of the extirpation scenario.

The past presence of *N. cavanillesii* in eastern Spain is supported by the detection of ITS sequences that are almost exclusive to *N. cavanillesii* (2.25% of R1) in *N. miniatus* from the island of Minorca (Balearic islands; Fig. 2). It is important to note that the circumscription of R1 to *N. cavanillesii* is based not only in the present sampling but also on a thorough phylogenetic analysis performed at the genus level (I. Marques, unpubl. data). These *N. cavanillesii*-like ribotypes suggest the occurrence of nuclear introgression, which would require the past presence of *N. cavanillesii* in eastern Spain. In the absence of concerted evolution, and sustained by asexual mechanisms, these ribotypes may have originated thousands of years ago (Vargas et al. 1999; Silvertown 2004; Herben et al. 2005). The modeled niche of *N. cavanillesii* provides some further support for the past presence of this species in eastern Spain. Niche modeling has been successfully used to reconstruct evolutionary history of Mediterranean species (Jakob et al. 2007; Piñeiro et al. 2007). Paleotectonics and paleoclimatology in this complex area (Rosenbaum et al. 2002) indicate that there have been several occasions along an extended time-span in which, *N. miniatus* might have suffered introgression from populations of *N. cavanillesii* formerly occurring in eastern Spain. This explanation assumes that the niche has remained unchanged over time. Although a subject of discussion, several studies suggest that fundamental niches evolve little over evolutionary timescales and across spaces rather appearing late in the process of speciation (Bradshaw 1991; Holt 1996; Peterson et al. 1999; Ackerly 2004; Martínez-Meyer et al. 2004).

As for the likeliness of the extirpation scenario, in theory, this process might have occurred either through a disruption in demographic patterns or through genetic assimilation (Levin et al. 1996; Rhymer and Simberloff 1996; Carney et al. 2000; Wolf et al. 2001). There are several arguments against the occurrence of genetic assimilation through recurrent backcrosses, the main one being that no evidence of backcrossing has been found. Instead, hybridization between *N. miniatus* and *N. cavanillesii* seems to consist primarily of F_1 individuals. The chromosome number detected in the orphan populations of *N. ×perezlarae* was consistently $2n = 29$ and morphologies observed during field work over several years always followed clear intermediate patterns as compared to *N. miniatus* and *N. cavanillesii*, just as the hybrids in the remaining population did. The low formation of seeds in artificial pollinations of *N. ×perezlarae* and *N. ×alentejanus* with their progenitor species (Fig. 3) also suggests that backcrosses are very infrequent. Further, preliminary pollination studies suggest that potential backcrosses would be also hindered by a high degree of

pollinator constancy or preference in *N. ×perezlarae* (Marques and Draper 2004; I. Marques, unpubl. data).

In contrast, the extirpation of *N. cavanillesii* by demographic competition seems to be more feasible. This process occurs when hybridization interferes in the reproductive effectiveness of a plant thereby reducing the growth rate of its population and its competitive ability (Levin et al. 1996; Wolf et al. 2001).

Because no strong reproductive barrier constrains the fertilization by foreign pollen in *N. cavanillesii*, hybrid seeds can easily be produced by ovules that otherwise would generate a conspecific progeny (Levin et al. 1996; Huxel 1999; Wolf et al. 2001; Buerkle et al. 2003; Haygood et al. 2003). Because interspecific crosses use *N. cavanillesii* predominantly as the ovule donor, this species recurrently suffers a reduction in its growth rate and eventually can decline to levels below the ones required for persistence (Levin et al. 1996; Wolf et al. 2001). Once started, the process of extirpation can be reinforced because the numerical disadvantage of the narrowly distributed species is worsened by the proliferation of the hybrids, which compete for site establishment and resources. Even if hybrid plants are less fertile than parental species (Fig. 3), they can persist and compete through active bulb propagation (Ellstrand 1992; Ellstrand and Elam 1993; Haygood et al. 2003) which occurs three times faster than in parental species (Marques and Draper 2004). Theoretically, in species with weak reproductive barriers, hybridization could cause extinction in less than five generations (Wolf et al. 2001; Rieseberg 2006), so that this scenario is in fact a feasible explanation for the orphan hybrid eastern Spanish populations of *N. ×perezlarae*.

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Supporting Information

The following supporting information is available for this article:

Table S1. Species sampled, type of population (sympatric:S; allopatric:A), locality, voucher information, and GenBank accessions of DNA sequences analyzed in this study.
Table S2. PCR cycle conditions for each primer combination.
Table S3. Results of the recombinant analyses performed with RDP package.
Figure S1. Distribution maps of *Narcissus cavanillesii*, *N. serotinus*, *N. miniatus*, *N. elegans*, *N.* ×*alentejanus* and *N.* ×*perezlarae*, based on revised herbarium specimens, in addition to the localities sampled during this study.
Figure S2. Nucleotide variation in organellar regions.
Figure S3. A rooted 50% majority-rule consensus tree (BI) depicting the phylogenetic relationships among haplotypes of *Narcissus*.

Supporting Information may be found in the online version of this article.

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