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REGULAR PAPER

Nicotiana suaveolens accessions with different ploidy levels exhibit different reproductive isolation mechanisms in interspecific crosses with *Nicotiana tabacum*

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Abstract

Reproductive isolation, including prezygotic and postzygotic barriers, is a mechanism that separates species. Many species in the *Nicotiana* section *Suaveolentes* exhibit reproductive isolation in crosses with *Nicotiana tabacum*. In this study, we investigated whether the chromosome numbers and ploidy levels of eight *Nicotiana suaveolens* accessions are related to the reproductive isolation after crosses with *N. tabacum* by flow cytometry and chromosome analyses. Additionally, the internal transcribed spacer (ITS) regions of the eight *N. suaveolens* accessions were sequenced and compared with the previously reported sequences of 22 *Suaveolentes* species to elucidate the phylogenetic relationships in the section *Suaveolentes*. We revealed that four *N. suaveolens* accessions comprised 64 chromosomes, while the other four accessions carried 32 chromosomes. Depending on the ploidy levels of *N. suaveolens*, several types of reproductive isolation were observed after crosses with *N. tabacum*, including decreases in the number of capsules and the germination rates of hybrid seeds, as well as hybrid lethality and abscission of enlarged ovaries at 12–17 days after pollination. A phylogenetic analysis involving ITS sequences divided the eight *N. suaveolens* accessions into three distinct clades. Based on the results, we confirmed that *N. suaveolens* accessions vary regarding ploidy levels and reproductive isolation mechanisms in crosses with *N. tabacum*. These accessions will be very useful for revealing and characterizing the reproductive isolation mechanisms in interspecific crosses and their relationships with ploidy levels.

Keywords Internal transcribed spacer region · Interspecific hybridization · Phylogenetics · Polyploidy · Reproductive isolation · Tobacco

Introduction

Reproductive isolation, including prezygotic and postzygotic barriers, is a mechanism that separates species and plays a crucial role in the evolution of animals and plants (Rieseberg and Blackman 2010; Stebbins 1966). In plants, prezygotic barriers include the inhibition of pollen adhesion to the stigma, pollen germination, pollen tube growth in the style, and pollen tube penetration of the ovule micropyle (Dickinson et al. 2012). Postzygotic barriers, which occur after a successful fertilization, include seed abortion as well as the weakness, inviability, and sterility of the F_1 hybrid plants or their offspring (Bushell et al. 2003; Guo et al. 2016; Ichitani et al. 2007; Kuboyama et al. 2009; Li et al. 1997; Tezuka et al. 2010). These barriers hinder plant breeding programs such as wide hybridizations which incorporates backcrossing of F_1 hybrids between cultivated and related wild species

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to the cultivated species. To facilitate wide hybridization breeding, the mechanisms underlying reproductive isolation should be characterized and methods for overcoming the reproductive isolation will need to be developed.

Many species in the *Nicotiana* section *Suaveolentes* have been investigated for reproductive isolation by crosses with the cultivated species *N. tabacum* (2n = 48), which belongs to the section *Nicotiana* (Tezuka 2012; Tezuka et al. 2010). The section *Suaveolentes* includes 25 wild species, which are endemic to Australasia except for the African wild species *N. africana*. All species in section *Suaveolentes* are allotetraploids (Leitch et al. 2008) and the haploid number of chromosomes within this section can be 16–24, although species carrying 17 pairs of chromosomes have not been identified (Goodspeed 1954; Ladiges et al. 2011). Although *N. suaveolens* is one of four species with 16 pairs of chromosomes, Wheeler (1935) reported that two of the many *N. suaveolens* races carry 32 pairs of chromosomes. The section *Suaveolentes* is a monophyletic group based on internal transcribed spacer (ITS) regions (Chase et al. 2003), plastid genes (Clarkson et al. 2004), and nuclear-encoded chloroplast-expressed glutamine synthetase (Clarkson et al. 2010).

Three types of reproductive isolation have been observed in reciprocal crosses between *N. suaveolens* JT (this accession name is described in the "Materials and methods") and *N. tabacum*. Although hybrid seeds that germinate normally can be obtained in the *N. suaveolens* JT (♀) × *N. tabacum* (♂) cross after a conventional hand pollination (Manabe et al. 1989; Yamada et al. 2000), unilateral incongruity (UI) occurs and seeds cannot be obtained in the reciprocal cross after a conventional hand pollination. This incongruity can be overcome with a test-tube pollination and ovule culture to obtain hybrid seedlings (Tezuka and Marubashi 2004). However, hybrid lethality (a phenomenon responsible for the death of hybrid plants) characterized by browning of the hypocotyl and roots (Type II lethality) is observed in reciprocal hybrid seedlings (Marubashi and Onosato 2002; Tezuka and Marubashi 2004, 2006; Yamada et al. 1999). Although the Type II lethality can be overcome or bypassed by several methods, including high temperature cultivation, and tissue culturing, as well as by the lack of the chromosome causing hybrid lethality, the resulting hybrid plants are sterile (Inoue et al. 1994; Manabe et al. 1989; Tezuka and Marubashi 2006). In our previous studies, *N. suaveolens* JT, which has 16 pairs of chromosomes (Japan Tobacco Inc. 1994), was solely used for crosses with *N. tabacum* (Tezuka and Marubashi 2004, 2006).

In the present study, we used *N. suaveolens* accessions with an unknown number of chromosomes as well as the JT accession, and investigated types of their reproductive isolation and their phylogenetic relationships. We discuss whether the chromosome numbers and ploidy levels of the *N. suaveolens* accessions are related to the reproductive isolation, and estimate the evolutionary order and timing of the acquisition of the reproductive isolation in the *N. suaveolens* accessions.

Materials and methods

Plant materials

The following eight accessions of wild *N. suaveolens* were analyzed in this study: PI 230960, PI 555561, PI 555563, PI 555565, PI 555566, PI 555567, PI 555568, and JT. The one accession obtained from the Leaf Tobacco Research Center (Japan Tobacco Inc., Oyama, Japan) was named JT (2n = 4x = 32) to distinguish it from the other accessions because the accession number and line name were not provided. The accessions with Plant Introduction (PI) numbers were obtained from the United States *Nicotiana* Germplasm Collection (Lewis and Nicholson 2007). Information regarding the seven PI accessions is available in the Genetic Resources Information Network database of the United States National Plant Germplasm System (https://www.ars-grin.gov/npgs/index.html) (USDA ARS National Genetic Resources Program 2010). The eight accessions were reciprocally crossed with the *N. tabacum* 'Red Russian' cultivar (2n = 4x = 48). The 'Red Russian' seeds were provided by the Leaf Tobacco Research Center. All plants except plants used for investigation of flower and leaf morphology were cultivated in a greenhouse (natural day length; Osaka Prefecture University, Sakai, Osaka, Japan). Plants used for investigation of flower and leaf morphology were grown under fluorescent lamps (FLR40S·BRN; Toshiba, Tokyo, Japan) in a cultivation room (16-h light/8-h dark; approximately 70 μmol m^{-2} s^{-1}; 25 °C).

Investigation of flower and leaf morphology

Three plants of each *N. suaveolens* accession were grown in separate plastic pots (13 cm diameter, 12 cm deep; soil volume approximately 1.2 L) filled with a 1:3 mixture of vermiculite (Type GS; Nittai, Osaka, Japan) and culture soil (Sakata Super Mix A; Sakata Seed, Kanagawa, Japan). Flower morphology was analyzed by recording the flower color as well as the corolla-limb diameter and corolla tube length. Ten mature flowers from three plants (3 flowers/2 plants, 4 flowers/1 plant) were examined for all eight *N. suaveolens* accessions. The maximum length and width of cotyledons and 1st to 15th rosette leaves (total two cotyledons and 15 rosette leaves per plant) were measured, with three plants analyzed for each of the eight *N. suaveolens* accessions. The oldest leaf was defined as the 1st rosette leaf. The aspect ratio was calculated by dividing the leaf width by the leaf length.

Self-crosses and interspecific crosses

Five plants of each *N. suaveolens* accession and *N. tabacum* were used in a cross experiment. The same five *N. tabacum* plants were used for all reciprocal interspecific crosses and self-crosses, but the number of flowers per plant differed among crosses (Table 2). The plants were pollinated in 2009, 2012, and 2017–2018. In summer, the minimum temperatures in the greenhouse were 20–26 °C and the maximum temperatures were 35–45 °C. In winter, the minimum temperatures were maintained above 15 °C with an air heating system and the maximum temperatures were 30–35 °C. The flowers of plants used as the female parents were emasculated 1 day before anthesis and then pollinated with pollen grains from the male parents. To assess the reproductive isolation after reciprocal crosses between the eight *N. suaveolens* accessions and *N. tabacum*, we observed whether pollinated flowers dropped, ovaries enlarged and dropped, capsules were obtained, and seed morphology was normal. Self-crosses of *N. suaveolens* and *N. tabacum* were conducted as a control. Seeds obtained from self and interspecific crosses were sterilized with 5% sodium hypochlorite for 15 min. The sterilized seeds were sown in Petri dishes (60 mm diameter, 17 mm deep) containing 8 mL half-strength Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 1% sucrose and 0.2% Gelrite (pH 5.8), and then incubated at 28 °C under continuous illumination (approximately 150 μmol m^{-2} s^{-1}). About 100 seeds per cross were initially sown, but when seeds germinated poorly, additional seeds were sown. We determined whether seeds could germinate, counted the seedlings obtained at 30 days after sowing, and assessed whether the seedlings exhibited lethality at 30 days after germination (Table 2).

Statistical analysis

Data were analyzed with the SPSS program (version 22) (IBM Corp., Armonk, NY, USA). Chi square tests were used to compare the number of capsules obtained and seeds that germinated. The tests were performed separately for self-crosses, *N. suaveolens* × *N. tabacum*, and *N. tabacum* × *N. suaveolens*. The corolla-limb diameter, corolla tube length, and maximum length, width and aspect ratio of cotyledons and rosette leaves were compared among the eight *N. suaveolens* accessions using Tukey's multiple comparison test.

Flow cytometry

The ploidy levels of the *N. suaveolens* accessions were analyzed by flow cytometry to clarify why diverse cross results were obtained with the various accessions. Young leaves were placed on a Petri dish and then finely chopped with a sharp razor blade in 400 μL ice-cold Otto I buffer (Doležel and Bartos 2005; Otto 1990). The resulting extract was filtered through a 30-μm nylon mesh, after which 1.6 mL Otto II buffer (Doležel and Bartos 2005; Otto 1990) containing 2 μg mL^{-1} 4′,6-diamino-2-phenylindole was added. For each sample, the DNA content of at least 10,000 nuclei was analyzed with a CyFlow Space flow cytometer (Partec GmbH, Münster, Germany). Previous studies indicated that *N. suaveolens* JT consists of 32 chromosomes and is an allotetraploid species (Clarkson et al. 2010; Japan Tobacco Inc. 1994). The G1 peak (4C) of *N. suaveolens* JT was positioned on channel 10^1 on the histogram by adjusting the instrument gain settings in every analysis (Fig. S2h). The same instrument gain settings were used to determine the ploidy levels of the other seven accessions. Two individuals were observed for *N. suaveolens* JT, but three individuals were observed for the other accessions.

Chromosome analysis

The *N. suaveolens* root tips were pretreated with distilled water for 24 h at 4 °C and with 2 mM 8-hydroxyquinoline for 4 h at 18 °C, after which they were fixed in ethanol/acetic acid (3:1) overnight to determine chromosome numbers. The root tips were hydrolyzed in 1 N HCl for 8 min at 60 °C, stained with Schiff's reagent, and squashed in 45% acetic acid. For each plant, the number of chromosomes in at least five root tip cells was counted with a BX50 light microscope (Olympus, Tokyo, Japan). Two individuals were observed for *N. suaveolens* JT, but three individuals were observed for the other accessions.

ITS amplification and cladistic analysis

The rDNA ITS region was sequenced for all eight *N. suaveolens* accessions. The DNA was extracted from leaves following a CTAB method (Murray and Thompson 1980). The ITS DNA region was amplified using the primers described by Baldwin (1993) and Sun et al. (1994). The PCR mixtures consisted of 1 × standard buffer (BioAcademia, Osaka, Japan), 0.2 mM each dNTP, 0.2 μM each primer, 20 ng template DNA, and 1.0 U *Taq* DNA polymerase (BioAcademia) in a total volume of 20 μL. For all reaction mixtures, 3% DMSO was added because GC-rich regions in the ITS sequences cause premature termination of most strands within 100–150 bp of the initiation point (Chase et al. 2003). The PCR program was as follows: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min; 72 °C for 3 min. The PCR products were separated by 1.5% agarose gel electrophoresis with TBE buffer, and were visualized by staining with ethidium bromide. The target DNA fragments were extracted and purified from the agarose gels with the High Pure PCR Product Purification

Kit (Roche, Mannheim, Germany). The purified DNA fragments were directly sequenced with the BigDye Terminator (version 3.1) Cycle Sequencing kit (Applied Biosystems, USA) and an ABI PRISM 3130xl genetic analyzer (Applied Biosystems). The obtained sequences, which were deposited in the DNA Data Bank of Japan as LC202850–LC202857 (Table 1), were aligned using MUSCLE in the MEGA6 program (Tamura et al. 2013) with other ITS sequences downloaded from GenBank (Table 1, Chase et al. 2003), including sequences from 22 *Suaveolentes* species, one artificial hybrid *N.* × *didepta* (*N. debneyi* × *N. tabacum*), *N. tabacum*, and *Petunia axillaris*, which served as the ultimate outgroup.

Table 1 GenBank/DNA Data Bank of Japan accession numbers for rDNA ITS sequences of *Suaveolentes* and related species

Species	Accession no. of ITS[a]
Nicotiana africana Merxm.	AJ492393
Nicotiana amplexicaulis N. T. Burb.	AJ492394
Nicotiana benthamiana Domin	AJ492409
Nicotiana cavicola N. T. Burb.	AJ492395
Nicotiana debneyi Domin	AJ492439
Nicotiana eastii Kostoff	AJ492396
Nicotiana excelsior J. M. Black	AJ492399
Nicotiana exigua H.-M. Wheeler	AJ492391
Nicotiana fragrans Hook.	AJ492397
Nicotiana goodspeedii H.-M. Wheeler	AJ492401
Nicotiana gossei Domin	AJ492390
Nicotiana hesperis N. T. Burb.	AJ492402
Nicotiana ingulba J. M. Black	AJ492403
Nicotiana maritima H.-M. Wheeler	AJ492404
Nicotiana megalosiphon Van Huerck and Müll. Arg.	AJ492392
Nicotiana occidentalis H.-M. Wheeler	AJ492417
Nicotiana rosulata (S. Moore) Domin	AJ492405
Nicotiana rotundifolia Lindl.	AJ492406
Nicotiana simulans N. T. Burb.	AJ492407
Nicotiana suaveolens Lehm.	AJ492438
Nicotiana suaveolens Lehm. PI 230960	LC202850
Nicotiana suaveolens Lehm. PI 555561	LC202851
Nicotiana suaveolens Lehm. PI 555563	LC202852
Nicotiana suaveolens Lehm. PI 555565	LC202853
Nicotiana suaveolens Lehm. PI 555566	LC202854
Nicotiana suaveolens Lehm. PI 555567	LC202855
Nicotiana suaveolens Lehm. PI 555568	LC202856
Nicotiana suaveolens Lehm. JT	LC202857
Nicotiana umbratica N. T. Burb.	AJ492400
Nicotiana velutina H.-M. Wheeler	AJ492408
Nicotiana × *didepta*	AJ492398
Nicotiana tabacum L.	AJ492447
Petunia axillaris (Lam.) Britton	AJ492460

[a]AJ accessions are from Chase et al. (2003)

The evolutionary history was inferred using MEGA6 with the maximum-likelihood method based on the General Time Reversible model (Nei and Kumar 2000). Branch support was assessed by the bootstrap resampling with 1,000 replications.

Results

Flower and leaf morphology of *N. suaveolens* accessions

The flowers of all accessions were white. The corolla-limb diameter and corolla-tube length was not uniform among the eight *N. suaveolens* accessions (Fig. 1). The corolla-limb diameter ranged from 2.49 ± 0.07 cm (mean and standard error; PI 555563) to 3.09 ± 0.06 cm (PI 555566), and the corolla-tube length ranged from 3.84 ± 0.09 cm (PI 230960) to 4.5 ± 0.05 cm (PI 555566). Accession PI 555566 had the largest corolla-limb diameter and the longest corolla tube.

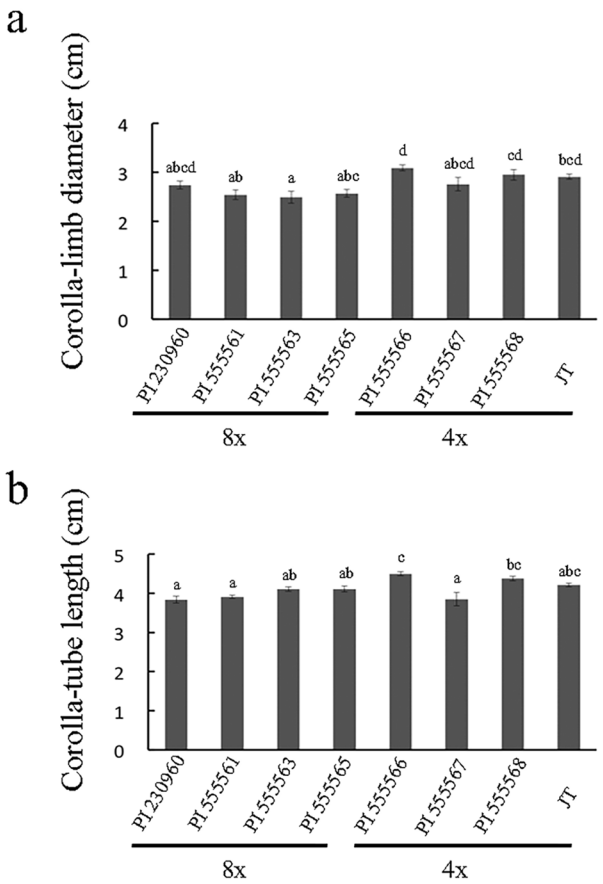

Fig. 1 Flower morphology compared among eight *N. suaveolens* accessions. 4x indicates tetraploid and 8x indicates octoploid. Data are presented as the mean ± standard error for ten flowers. Different lower case letters indicate significant differences at the 0.05 level (Tukey's test)

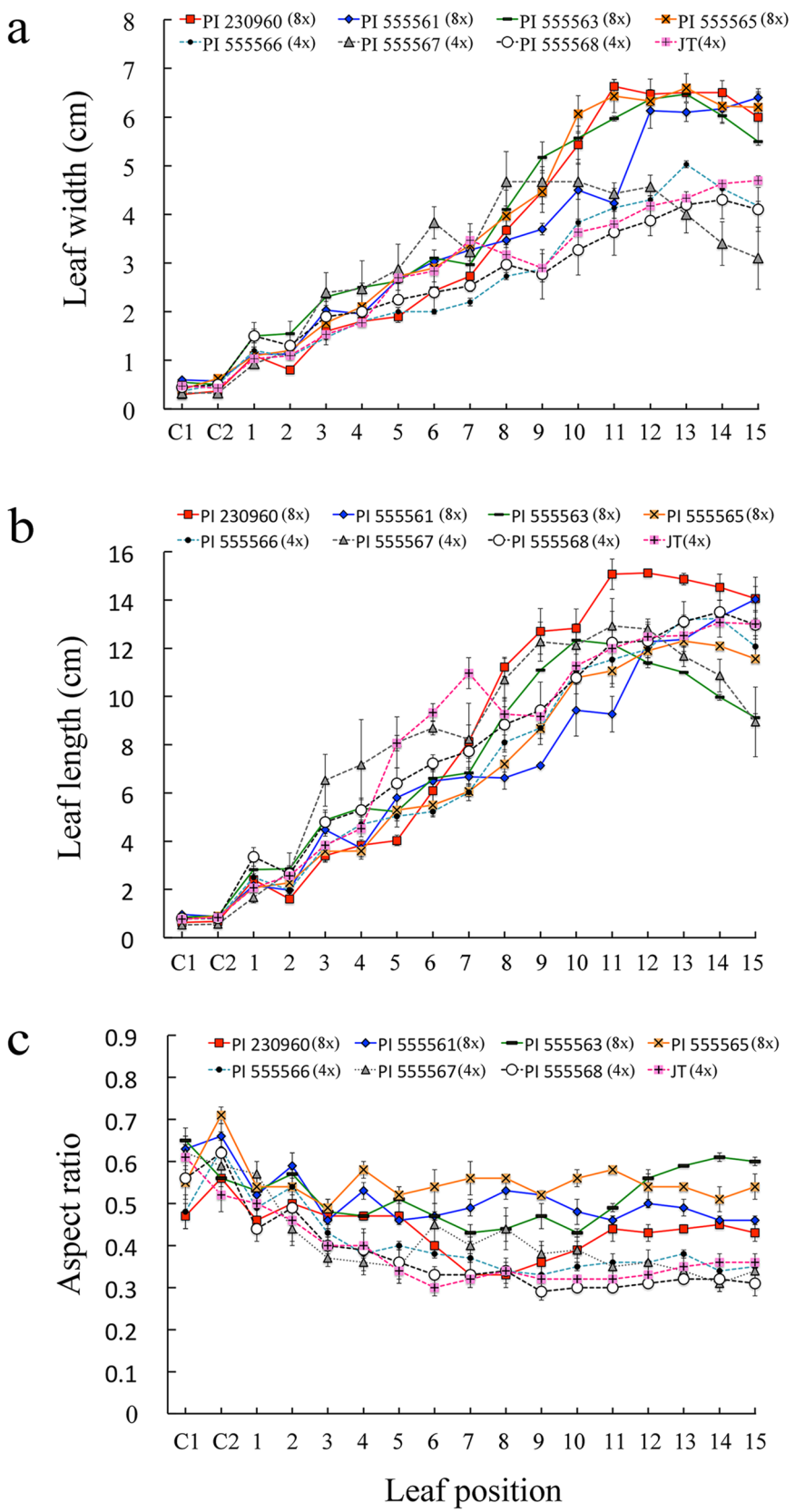

Fig. 2 Sizes of cotyledons and rosette leaves in eight *N. suaveolens* accessions. **a** Maximum width of cotyledons and rosette leaves. **b** Maximum length of cotyledons and rosette leaves. **c** Aspect ratio of the maximum width and the maximum length. C1 and C2 are cotyledons and the leaves at positions 1–15 are 1st to 15th rosette leaves. Error bars indicate the standard error for three individuals

Moreover, the size of the leaves varied among the eight *N. suaveolens* accessions. The maximum leaf widths tended to be greater at the upper leaf positions (10th–15th leaves) in PI 230960, PI 555561, PI 555563, and PI 555565 (Fig. 2a, Table S1). Regarding the maximum leaf lengths, no clear correlation was observed among the eight accessions, although some differences were detected. Accession PI 230960 had the longest leaves (8th–15th leaves) (Fig. 2b, Table S2). The leaf aspect ratios tended to be larger at the upper leaf positions (7th–15th leaves) in PI 230960, PI 555561, PI 555563, and PI 555565 (Fig. 2c, Table S3). Subsequent experiments using flow cytometry and chromosome analyses revealed that the ploidy levels of these four accessions were higher than those of the other four accessions.

Interspecific crosses between *N. suaveolens* accessions and *N. tabacum*

The results of the reciprocal crosses between the eight accessions of *N. suaveolens* and *N. tabacum* as well as the self-crosses of eight *N. suaveolens* accessions and *N. tabacum* are presented in Table 2. Selfed seeds were obtained from all accessions, with 91.2–100% of the flowers producing capsules with seeds. Hybrid seeds were obtained from all interspecific crosses between *N. suaveolens* accessions as females and *N. tabacum* as the male, except for the cross involving PI 555565. Conversely, hybrid seeds were not obtained when *N. tabacum* was used as the female parent in crosses with eight *N. suaveolens* accessions, and all flowers dropped at about 7 DAP without the ovaries enlarging, suggesting that fertilization did not occur. Thus, UI was observed in these interspecific crosses. In three interspecific crosses (*N. suaveolens* PI 230960 × *N. tabacum*, PI 555561 × *N. tabacum*, and PI 555563 × *N. tabacum*), only 42.1–54.5% of the flowers produced capsules with seeds, which were significantly lower compared with each self-cross. In contrast, in the other four interspecific crosses (*N. suaveolens* PI 555566 × *N. tabacum*, PI555567 × *N. tabacum*, PI 555568 × *N. tabacum*, and JT × *N. tabacum*), more than 78.5% of the flowers produced capsules with seeds, which were not significantly different from each self-cross (Fig. 3a). Additionally, in the cross between PI 555565 and *N. tabacum*, all of the ovaries were enlarged after pollination, suggesting that fertilization had occurred. However, the ovaries dropped at 12–17 days after pollination (DAP) and seeds were never obtained. In all self-crosses and interspecific crosses between *N. suaveolens* and *N. tabacum* excluding that involving PI 555565, several flowers did not develop into capsules and dropped at about 7 DAP without the ovaries enlarging, suggesting that fertilization did not occur.

The selfed seeds of eight *N. suaveolens* accessions are presented in Fig. S1, and the germination rates (percentages of hybrids obtained after sowing) of all accessions were greater than 80% (Fig. 3b). We identified two kinds of hybrid seeds after the interspecific crosses. Most of the hybrid seeds from three crosses (*N. suaveolens* PI 230960 × *N. tabacum*, PI 555561 × *N. tabacum*, and PI 555563 × *N. tabacum*) were fragile and empty, and were obviously different from the seeds resulting from self-crosses (Fig. S1b, d, f). Meanwhile, the hybrid seeds from the other four crosses (*N. suaveolens* PI 555566 × *N. tabacum*, PI555567 × *N. tabacum*, PI 555568 × *N. tabacum*, and JT × *N. tabacum*) appeared normal and were similar to the seeds resulting from self-crosses (Fig. S1i, k, m, o). In the crosses between three *N. suaveolens* accessions (PI 230960, PI 555561 and PI 555563) and *N. tabacum*, the germination rates of the hybrid seeds were low (0% for PI 230960 × *N. tabacum*, 4.2% for PI 555561 × *N. tabacum*, and 1.7% for PI 555563 × *N. tabacum*), and were significantly lower compared with each self-cross. Conversely, the germination rates were high (≥ 87.6%) for the other four interspecific crosses, and were not significantly different from each self-cross (Fig. 3b). Almost all of the hybrid seedlings obtained from all crosses exhibited lethality at 30 days after germination at 28 °C. The characteristic symptom of hybrid lethality in all crosses was the browning of the hypocotyl and roots, which was consistent with Type II hybrid lethality. However, one hybrid seedling from the PI 555566 × *N. tabacum* cross and two hybrid seedlings from the JT × *N. tabacum* cross exhibited no lethal symptoms after 30 days after germination (Table 2).

Ploidy levels and chromosome numbers in *N. suaveolens* accessions

The DNA contents of the eight *N. suaveolens* accessions clearly indicated differences in the ploidy levels (Fig. S2). In *N. suaveolens* JT, a large G1 (4C) peak and a smaller G2/M (8C) peak were observed (Fig. S2h). Additionally, 4C and 8C peaks were detected in three *N. suaveolens* accessions (PI 555566, PI 555567 and PI 555568) (Fig. S2e–g). In contrast, 8C (+ 16C) peaks were detected in four *N. suaveolens* accessions (PI 230960, PI 555561, PI 555563 and PI 555565) (Fig. S2a–d). Thus, four of the *N. suaveolens* accessions (PI 230960, PI 555561, PI 555563 and PI 555565) were plausibly octoploid, whereas the other three accessions (PI 555566, PI 555567 and PI 555568) were tetraploid, similar to JT. Four *N. suaveolens* accessions (PI 230960, PI 555561, PI 555563 and PI 555565) comprised 64 chromosomes (Fig. S3a–d), while the other four accessions (PI 555566, PI 555567, PI 555568 and JT) carried 32 chromosomes (Fig. S3e–h). These results were consistent with the flow cytometry analysis.

Table 2 Interspecific crosses between eight *N. suaveolens* accessions and *N. tabacum*

Cross (♀×♂)	Pollination period[a]	No. of flowers pollinated	No. of capsules obtained	No. of seeds sown	No. of hybrids obtained[b]	
					Viable	Lethal
PI 230960 (8x) self	4th round	10	10	100	97	0
PI 555561 (8x) self	4th round	14	14	95	83	0
PI 555563 (8x) self	4th round	10	10	111	102	0
PI 555565 (8x) self	4th round	15	14	104	84	0
PI 555566 (4x) self	4th round	12	11	115	101	0
PI 555567 (4x) self	4th round	10	10	110	96	0
PI 555568 (4x) self	4th round	10	10	100	86	0
JT (4x) self	4th round	12	12	100	88	0
N. tabacum self	4th round	11	11	100	98	0
PI 230960 × *N. tabacum*	1st and 2nd rounds	19	8	214	0	0
PI 555561 × *N. tabacum*	1st and 2nd rounds	10	5	286	0	12
PI 555563 × *N. tabacum*	1st and 2nd rounds	11	6	525	0	9
PI 555565 × *N. tabacum*	1st and 2nd rounds	25	0[c]	–	–	–
PI 555566 × *N. tabacum*	1st and 2nd rounds	14	11	197	1	186
PI 555567 × *N. tabacum*	1st and 2nd rounds	10	8	185	0	162
PI 555568 × *N. tabacum*	1st and 2nd rounds	16	16	148	0	143
JT × *N. tabacum*	3rd round	11	11	164	2	149
N. tabacum × PI 230960	4th round	9	0	–	–	–
N. tabacum × PI 555561	4th round	7	0	–	–	–
N. tabacum × PI 555563	4th round	7	0	–	–	–
N. tabacum × PI 555565	4th round	9	0	–	–	–
N. tabacum × PI 555566	4th round	9	0	–	–	–
N. tabacum × PI 555567	4th round	8	0	–	–	–
N. tabacum × PI 555568	4th round	7	0	–	–	–
N. tabacum × JT	4th round	8	0	–	–	–

[a]1st round, Jun.–Sep. 2009, 2nd round, Jun.–Sep. 2012, 3rd round, Jun.–Sep. 2015, 4th round, Oct. 2017–Apr. 2018

[b]Number of hybrids was assessed at 30 days after germination

[c]Ovaries enlarged, but dropped at 12–17 days after pollination

Phylogenetic relationships between *N. suaveolens* accessions and other *Suaveolentes* species inferred from the ITS

The phylogenetic analysis revealed that the eight *N. suaveolens* accessions could be divided into three clades mainly corresponding to their ploidy levels (Fig. 4). The first clade comprising PI 555567, *N. debneyi*, and *N.* × *didepta* (bootstrap < 50), was related with other *N. suaveolens* accessions across two clades. Three tetraploid accessions, PI 555566, PI 555568 and JT, formed a clade that included *N. amplexicaulis* (bootstrap = 71). This clade was a sister to a clade that included the four octoploid accessions, PI 230960, PI 555561, PI 555563 and PI 555565, as well as the *N. suaveolens* accession (ITS Accession no. AJ492438) used by Chase et al. (2003) (bootstrap = 70).

Discussion

In *N. suaveolens*, in addition to lines with 16 pairs of chromosomes, lines with 32 pairs of chromosomes were described by Wheeler (1935); however, since then, lines with 32 pairs of chromosomes have not been reported, and it was unclear which lines possess 32 pairs of chromosomes. In the present study, we confirmed that four accessions, PI 230960, PI 555561, PI 555563 and PI 555565, have 32 pairs of chromosomes. Although flower sizes were not correlated with ploidy levels, the maximum widths and the aspect ratios of leaves tended to be larger in these accessions. Our molecular phylogenetic analyses implied that PI 555567 is not grouped with the other three tetraploid *N. suaveolens* accessions, and instead belongs to the clade with *N. debneyi* and *N.* × *didepta*. Although this implies that PI 555567 may not be *N. suaveolens*, the present study is not sufficient to revolve the taxonomy of this accession.

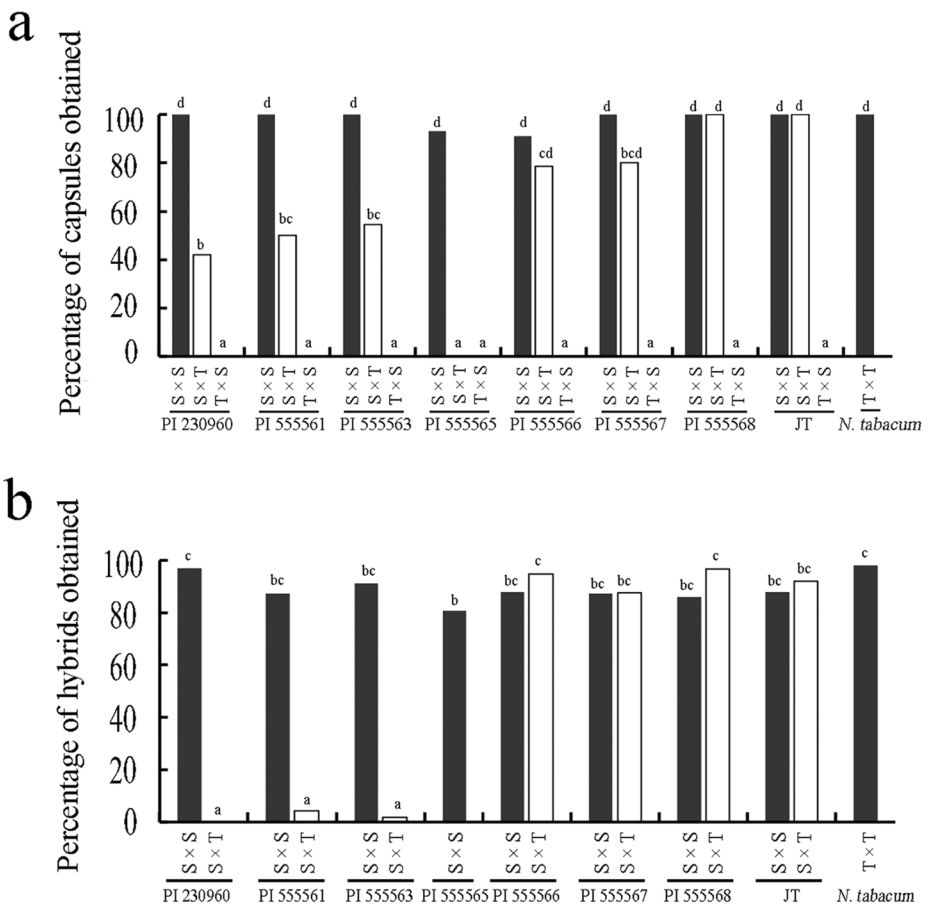

Fig. 3 Percentages of capsules obtained after pollination (**a**) and hybrids obtained after sowing **b** in self and interspecific crosses. S indicates *N. suaveolens* and T indicates *N. tabacum*. Different lower case letters indicate significant differences at the 0.05 level (Chi square test)

On the basis of our phylogenetic tree constructed from ITS sequences as well as an evaluation of ploidy levels in *N. suaveolens* accessions, we estimated the evolutionary order and timing of the acquisition of the reproductive isolation mechanisms in the *N. suaveolens* lineage observed after crosses with *N. tabacum*. First, because Type II hybrid lethality was observed in all *N. suaveolens* accessions that produced hybrid seedlings, Type II hybrid lethality was likely acquired in the progenitors of *N. suaveolens*. Most *Suaveolentes* species exhibit Type II hybrid lethality in crosses with *N. tabacum*, and it is thought that the ancestral diploid species of the section *Suaveolentes*, the allotetraploid ancestor derived from crosses between the ancestral diploid species, or the allotetraploid descendent acquired Type II hybrid lethality during the evolutionary process (Tezuka 2013; Tezuka and Marubashi 2012).

A few hybrid seedlings that did not express lethality were obtained in two crosses in which hybrid seedlings usually express lethality (PI 555566 × *N. tabacum* and JT × *N. tabacum*). We previously determined that a few viable hybrids are occasionally obtained in generally lethal crosses because either the whole Q chromosome has been eliminated or the region that includes a hybrid lethality gene or genes on the Q chromosome has been deleted (Tezuka et al. 2010, 2012). However, other mechanisms are also possible because several viable hybrids appeared to have intact Q chromosomes in the previous study (Tezuka et al. 2010).

Second, UI was observed when *N. tabacum* was used as the female parent in crosses with eight *N. suaveolens* accessions. Moreover, UI was also observed when other *Suaveolentes* species were used for crosses with *N. tabacum*, although hybrid seeds were obtained relatively easily when *N. africana* and *N. fragrans* were used (Tezuka and Marubashi 2012; Tezuka et al. 2010). Therefore, we think that UI may have been acquired by the ancestral species of the section *Suaveolentes* after the divergence of *N. africana* and *N. fragrans*. Additionally, possible prezygotic barriers were observed in crosses between three octoploid *N. suaveolens* accessions (PI 230960, PI 555561, and PI 555563) and *N. tabacum*, but not in crosses between tetraploid *N. suaveolens* accessions and *N. tabacum*. This implied that the possible prezygotic barriers were acquired during or after the chromosome doubling that occurred in the clade with the octoploid *N. suaveolens* group. However, there is some controversy over the *N. suaveolens* octoploid accession PI 555565 because prezygotic barriers appeared to be absent in crosses with *N. tabacum*. Nevertheless, we cannot exclude the possibility that some prezygotic barriers led to

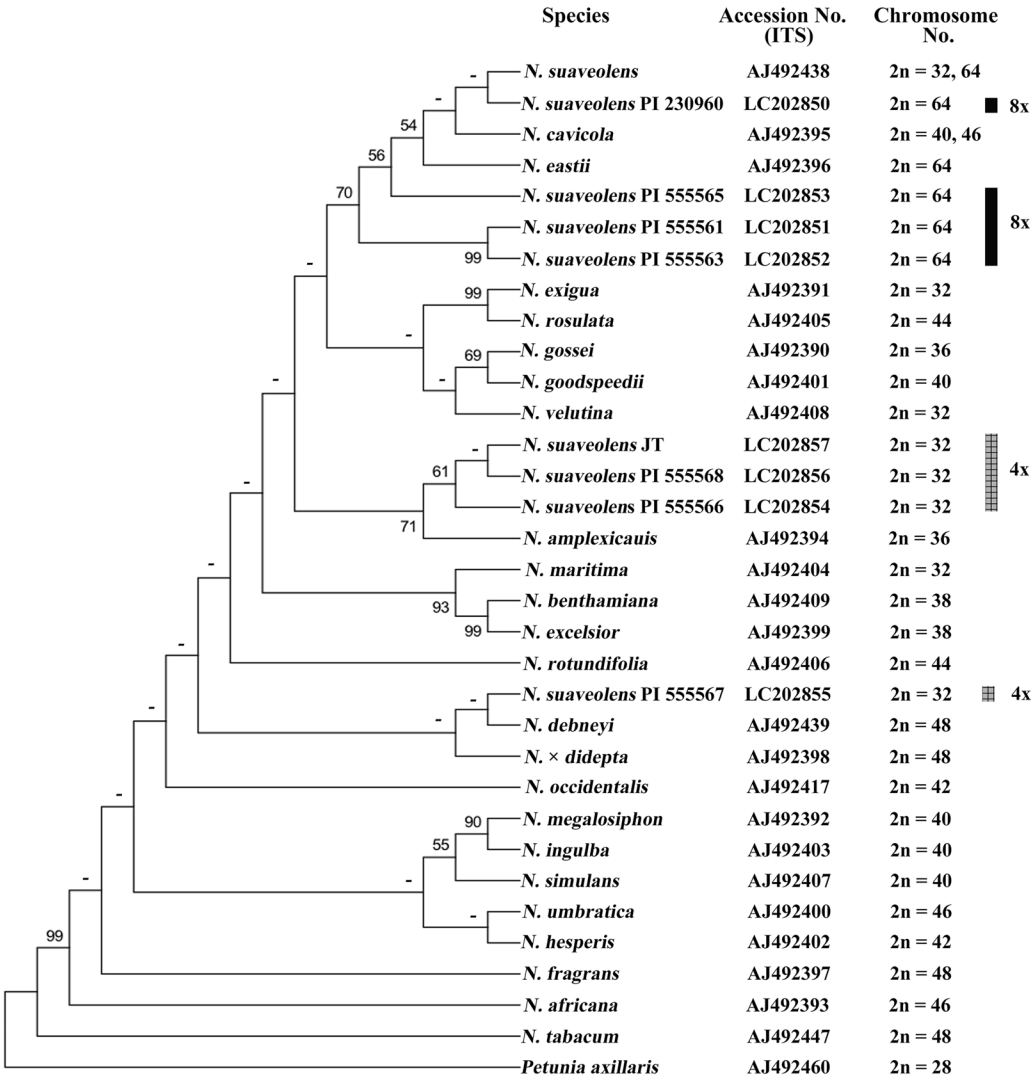

Fig. 4 Molecular phylogenetic tree based on a maximum-likelihood analysis of rDNA ITS sequences in the section *Suaveolentes*. *Petunia axillaris* was used as an outgroup. The eight *N. suaveolens* accessions were sequenced. Additionally, the sequences of 22 *Suaveolentes* species, the artificial hybrid *N. didepta*, *N. tabacum*, and *P. axillaris* were obtained from the GenBank database. Chromosome numbers were based on published articles by Goodspeed (1954), Purdie et al. (1982), and Japan Tobacco Inc. (1994). Bootstrap = 1,000 replicates (any clade with a hyphen has a BP < 50)

the abscission of the enlarged ovaries in the cross between PI 555565 and *N. tabacum*. Future studies will need to verify or disprove this possibility.

Third, seed abortion was observed after crosses between *N. tabacum* and three octoploid *N. suaveolens* accessions (PI 230960, PI 555561 and PI 555563), but not with the tetraploid *N. suaveolens* accessions. This suggested that seed abortion mechanisms were acquired during or after the chromosome doubling events. In interploidy crosses, endosperm breakdown is widely believed to be responsible for seed failure (Bushell et al. 2003; Kradolfer et al. 2013). Scott et al. (1998) described the seed failure in *Arabidopsis thaliana* interploidy crosses, in which the seeds contained different doses of maternally or paternally expressed imprinted loci that affected endosperm development. Similar phenomena have also been observed in the seed failure of interspecific crosses between species with the same ploidy level (Ishikawa et al. 2011; Sekine et al. 2013). Additionally, Rebernig et al. (2015) reported that interspecific crosses between *Capsella rubella* and *C. grandiflora*, which have the same ploidy level, resulted in seed abortion due to failed endosperm cellularization, and crosses in the opposite direction resulted in the formation of small seeds (with precociously cellularized endosperm) capable of germinating. Therefore, there are two possible causes of the seed abortion in crosses between octoploid *N. suaveolens* accessions and *N. tabacum*. First, the chromosome doubling event(s) in *N. suaveolens* may be the sole cause of seed abortion. Second, genetic changes

accumulated in the lineage leading to the octoploid lines before or after the chromosome doubling event(s), and the subsequent lines developed the mechanisms responsible for seed abortion.

Fourth, in this study, the abscission of enlarged ovaries (immature fruits) was only observed in crosses between the *N. suaveolens* octoploid accession PI 555565 and *N. tabacum*. Although we did not investigate pollen tube growth, fertilization appeared to have occurred because the ovules in the dropped ovaries were enlarged. Therefore, we speculated that some postzygotic barriers were present during the developmental processes of seeds and/or fruits in this cross. Similar to seed abortion, there are two possible causes for this abscission; i.e., the chromosome doubling event(s) in *N. suaveolens* or the acquisition of genetic factors responsible for the abscission of enlarged ovaries in PI 555565.

In summary, in the present study, we confirmed that *N. suaveolens* accessions vary regarding ploidy levels and reproductive isolation mechanisms in crosses with *N. tabacum*. These accessions will be very useful for revealing and characterizing the reproductive isolation mechanisms in interspecific crosses and their relationships with ploidy levels.

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