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Cytology and fertility of amphidiploid hybrids between *Nicotiana wuttkei* Clarkson et Symon and *N. tabacum* L.

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Abstract In this study, the morphology, cytology and crossability of amphidiploid hybrids ($2n = 8x = 80$; WWW′W′TTT′T′) between *Nicotiana wuttkei* × *Nicotiana tabacum* were studied. An R_1 progeny of the hybrids were produced through in vitro chromosome doubling of the *N. wuttkei* × *N. tabacum* cv. Wiślica amphihaploids. The resulting amphidiploids showed enough self-fertility to be perpetuated by self-pollination. The plants preserved their phenotypes and the number of chromosomes over subsequent R_2–R_4 selfed generations. During metaphase I the bivalent number increased progressively over the generations R_1 to R_4 and averaged 36.9, 37.4, 38.6 and 38.7, respectively. In addition, a progressive increase in pollen viability was observed: 50.8, 55.5, 62.3 and 65.7 % in generations from R_1 to R_4, respectively. Genomic in situ hybridization of R_4 plants revealed recombination between the parental species on two chromosomes. Reciprocal crosses of R_2 generation amphidiploids to *N. tabacum* cv. Wiślica yielded sesquidiploid hybrids ($2n = 6x = 64$; WW′TTT′T′). When *N. tabacum* cv. Wiślica is the pollinator, the amphidiploid gave rise to a total of 25 sesquidiploid plants with the cytoplasmic genome of *N. wuttkei*, while the reverse mating resulted in a mixed population consisting of a single sesquidiploid plant with the cytoplasm of *N. tabacum* and 203 plants of supposedly maternal origin. Our amphidiploid *N. wuttkei* × *N. tabacum* cv. Wiślica can be concluded as sufficiently stable and is regarded as a new artificial hybrid species.

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Introduction

The first plant hybridizations ever undertaken were between two *Nicotiana* species (*N. paniculata* × *N. rustica*) by Koelreuter in 1760 (Kostoff 1943). Hybridization barriers in *Nicotiana* developed relatively slower and were less hampering interspecific DNA transfer compared to many other genera (Goodspeed 1954). Up to now, more than 300 interspecific *Nicotiana* hybrids have been synthesized (Goodspeed 1954; Moav and Cameron 1960;

Gopinath et al. 1965; Smith 1968; Williams and Pandey 1974; Gerstel et al. 1979; Gangadevi et al. 1988; Laskowska and Berbeć 2003, 2012).The majority of the interspecific amphihaploid *Nicotiana* hybrids are sterile. In order to circumvent this obstacle, chromosome doubling of the sterile amphihaploid hybrids was performed to converted its to amphidiploids. If the conversion results in at least partial restoration of fertility, amphidiploids provide one of two, besides sesquidiploids, conventional points of issue for interspecific gene transfer in *Nicotiana* (Patel and Gerstel 1961; Reed and Collins 1978).

In the tobacco breeding, successful transfer of the disease resistance genes from wild *Nicotiana* species is of greatest importance. However, the application of wide hybridization and the creation of allopolyploids in *Nicotiana* did rarely result in cultivars with an added practical value and only a few *Nicotiana* species have been successfully used for the introgression of valuable genes into cultivated tobacco (Lewis 2011). Up to now resistances towards tobacco mosaic virus (TMV) from *N. glutinosa* (Holmes 1938; Henderson 1949; Heggestad 1966), blue mold and black root rot from *N. debneyi* (Clayton 1969; Miller 1987; Rufty 1989; Brandle et al. 1997; Lea 1999), black shank from *N. plumbaginifolia* (Apple 1962), wildfire from *N. longiflora* (Clayton 1947; Clayton et al. 1951), and tomato spotted wilt virus (TSWV) from *N. alata* (Gajos 1981, 1988) were introgressed in the cultivated *N. tabacum*.

Nicotiana wuttkei is one of recently reported new species in the section *Suaveolentes* (Knapp et al. 2004). Although *N. wuttkei* is an interesting source for resistance to *Peronospora hyoscyami* de Bary (*Peronospora tabacina* Adam.) (Laskowska and Berbeć 2003), causing blue mold in tobacco, it has never been explored for its breeding potential.

We successfully performed interspecific hybridization between *N. wuttkei* Clarkson and Symon (2n = 4x = 32) and *N. tabacum* L. (2n = 4x = 48). However, the initial amphihaploid seedlings were unviable. Therefore, in order to obtain viable *N. wuttkei* × *N. tabacum* hybrids, in vitro cotyledon culture was applied and yielded both amphihaploids (2n = 4x = 40), near-amphidiploids (2n = 78) and amphidiploids (2n = 8x = 80) (Laskowska and Berbeć 2012).

In the present study, the morphology, meiotic behavior, fertility and crossability of the amphidiploids *N. wuttkei* × *N. tabacum* are described.

Materials and methods

Nomenclature

Following the common nomenclature for *Nicotiana* allopolyploids (Webber 1930; Goodspeed 1954; Chaplin and Mann 1961; Burk 1975; Pittarelli and Sisson 1989; Zhou et al. 1997), our allopolyloid hybrids are referred to as amphidiploids and sesquidiploids, depending on their derivation and cytological status. The application of the terms "amphidiploid" (allotetraploid) and "sesquidiploid" (allotriploid) assumes that *Nicotiana* species can be regarded, at least cytologically, as functional diploids even if they are phylogenetically seen as allotetraploids, as is the case of *N. wuttkei* and *N. tabacum* (Chase et al. 2003). The validity of this approach was also confirmed by the recent studies on phylogeny and genome rearrangements in *Nicotiana* allotetraploids (Renny-Byfield et al. 2013).

Plant materials

Among the 38 viable plants regenerated from cotyledon culture of the amphihaploid seedlings *N. wuttkei* × *N. tabacum* cv. Wiślica WW′TT′ (2n = 4x = 40) (Laskowska and Berbeć 2012), seven were found to have 2n = 8x = 80 chromosomes. This amphidiploids WWW′W′TTT′T′ (R$_1$ generation) and their selfed progeny (R$_2$ to R$_4$ generations) provided the material for our investigation. The hybrid plants were grown in a greenhouse in pots filled with standard soil medium at 24 °C and with 16/8 h light–dark photoperiod.

Self-pollination and hybridization experiments

Self-pollination and interspecific hybridization was done under greenhouse conditions. Prior to hybridization stamens were removed from the juvenile flowers of the female parent. The pollen collected previously from the donor flowers was transferred to the stigmas of the emasculated female parent. For each self and

cross-pollination treatment, 7 plants of the amphidiploid R_1 generation and 10 plants of R_2, R_3 and R_4 were included, and approximately 20 flowers were pollinated on each plant. After pollination, all the other buds and flowers were removed from the plants and the pollinated inflorescences were covered with paper bags until capsule formation.

Seed germination rate was verified using the seed germination apparatus (Jacobsen) for 100 seeds per plant. The seed germination rate was calculated as the percentage of seeds that germinated over a period of 14 days per number of sown seeds. Seedling survival rate was expressed as the percentage of seedlings capable of growing into flowering plants per number of the germinated seeds.

Amphidiploid plants were briefly observed for their leaf and flower morphology.

Cytogenetic studies: chromosome counts and GISH

Mitotic chromosome counts were performed on juvenile corollas of greenhouse-grown plants using the method of Burns (1964). Corolla were pretreated for 5 h in a 0.44 % solution of 8-hydroxyquinoline with saturated maltose solution, added just before using (0.05 ml maltose per 3 ml 8-hydroxyquinoline). The pretreated material was fixed in the Carnoy solution (ethanol, chloroform and acetic acid—6:3:1) and stained with acetocarmine (1.5 %) after squashing the corolla fragments. Chromosome numbers were determined for each plant of the R_1 generation and 10 plants of R_2, R_3 and R_4, ten metaphase of each plant was analyzed.

Genomic in Situ Hybridization (GISH) were carried out on five plants of the amphidiploid R_4 generation. Young root tips were incubated for 5 h in a 0.44 % 8-hydroxyquinoline solution at room temperature. Then the roots were fixed for 2 h at room temperature in Farmer solution (3:1 ethanol: acetic acid). Enzyme incubation was done using an enzyme mixture containing 0.6 % Cellulase, 0.6 % Pectolyase, 0.6 % Cytohellicase for 1.5 h at 37 °C. Cell suspensions and chromosome slides were prepared according to the Steam Drop Method of Kirov et al. (2014). Total genomic DNA was extracted from young leaves using the CTAB protocol of Doyle and Doyle (1990) and purified by adding 1 μl RNAse (10 mg/ml). *N. wuttkei* genomic DNA was sonicated (ultrasonic Homogenizer Biologics) to obtain 1–3 kb

fragments and labeled with digoxygenin-11-dUTP (Dig-Nick Translation Mix, Roche) according to the manufacturer's indications. *N. tabacum* genomic DNA was sheared by autoclaving to obtain fragments of 200–400 bp and used as blocking DNA in 50 times excess than probe DNA. Denaturation, hybridization and detection was performed as described in Van Laere et al. (2010). A 82 % stringency wash was obtained by washing the slides twice in 0.1 % SSC at 37 °C for 15 min., twice in 0.1 % SSC at 52 °C for 7 min and in 2× SSC at room temperature (RT) for 5 min. Digoxigenin labeled signals were detected by anti-Dig-FITC (sheep) and anti-sheep-FITC (rabbit). The chromosomes were counterstained with 1 mg/ml DAPI and mounted in Vectashield. Chromosome analysis was done with an AxoImager M2 (Zeiss) fluorescence microscope equipped with an AxiocamMRm camera (Zeiss). Images were captured by ZEN software (Zeiss). Analysis of hybridization signals was carried out on at least 30 well-spread metaphases of each plant.

Analysis of meiotic configurations

Flower buds at the appropriate stage (determined by a preliminary microscopic examination of one of the five anthers in a bud) were fixed in the Carnoy's solution (ethanol, chloroform and acetic acid—6:3:1) for 24 h and stored in 70 % ethanol. Fixed anthers were squashed in a drop of 1.5 % acetocarmine. The meiotic analysis included observations of chromosome pairing at metaphase I, meiotic irregularities at further stages (chromatin bridges, laggards) and number of microspores and micronuclei produced at the tetrad phase. On the basis of chromosome pairing data, the means and ranges for the numbers of bivalents, univalent, trivalents and quadrivalents were estimated. Observations of meiotic configurations were performed in approximately 100 pollen mother cells (PMCs) prepared from anthers taken from four plants of each genotype.

Pollen viability tests

Pollen viability was determined as the percentage of mature pollen grains stainable in acetocarmine (1.5 %). Seven plants of amphidiploid R_1 generation and 30 plants of R_2–R_4 generations were analyzed, 1000 pollen grains of each plant.

Results

Self-fertility and hybridization experiments

The efficiency to produce a selfed offspring of the amphidiploid *N. wuttkei × N.tabacum* cv. Wiślica was low in the R_2 generation (Table 1). After pollination, 36.8 % of pollinated flowers yielded capsules with an average of 26.4 seeds per capsule. Seed germination capacity was 23.1 %, survival rate of seedlings—92.3 % and 416 plants were received (Table 1). The subsequent generations (R_3 and R_4) were substantially more self-fertile. Both the number of seeds per capsule and the germination rate increased by a factor more than 2 with practically the same survival rate of seedlings (91.9 and 92.2 % in R_3 and R_4 generations, respectively) (Table 1). As a consequence, the number of surviving progeny increased from 416 plants in generation R_2, to 2565 and 3666 in R_3 and R_4, respectively.

The ability of the R_2 amphidiploid *N. wuttkei × N. tabacum* cv. Wiślica to produce offspring by cross-pollination was considerably lower than by selfing, especially in terms of number of seeds per capsule and the survival rate of seedlings (Table 2). When used as female in crosses with 'Wiślica', the R_2 plants yielded on average 18.3 seeds per capsule and the seeds showed a germination capacity of 23.9 %. In total, 25 progeny plants were obtained, which is several times less when comparing with the obtained plants after selfing. This was caused by a massive dieback of the juvenile plants (9.3 % of surviving seedlings). All the plants that survived to maturity, were confirmed as true sesquidiploid hybrids (2n = 64; Fig. 1).

Data on the male fertility of the amphidiploid *N. wuttkei × N. tabacum* cv. Wiślica are ambiguous. Backcrossed to 'Wiślica', a population of 204 surviving plants was obtained (Table 2). However, only one progeny plants was confirmed as a sesquidiploid and the remaining were the maternal 'Wiślica'.

Pollen fertility of the amphidiploid hybrids

Flowers of the R_1 plants of the amphidiploids *N. wuttkei × N. tabacum* cv. Wiślica produced abundant pollen. The average pollen viability in R_1 generation was 50.8 % and varied among plants from 36.3 to 66.7 % (Table 3). The R_1 plants were able to produce seeds after self-pollination. In the subsequent selfed generations (R_2, R_3, R_4) the percentage of stainable pollen increased up to 65.7 % in R_4, varying from 49.1 to 75.1 % (Table 3).

Morphology of amphidiploid hybrids

Nicotiana wuttkei × N. tabacum cv. Wiślica amphidiploids showed good vigor and resembled amphihaploids (Laskowska and Berbeć 2012) for most morphological attributes. The leaves and flowers of the amphidiploids were a little smaller in size and thicker compared to those of the amphihaploids as well as much smaller than those of the cultivated *N. tabacum* parent (Fig. 2). The R_1–R_4 plants did not exhibit any phenotypic variations both within and between individual generations.

Chromosome counts and GISH

All the analysed plants from the R_1–R_4 amphidiploid generations had 80 chromosomes. For the R_4 generation the number of 80 somatic chromosomes was furthermore confirmed in the GISH analysis (Fig. 3).

By GISH, the parental genomes in the hybrid could be clearly discriminated (Fig. 3). The amphidiploid

Table 1 Fertility in terms of seed set and seed germination of the amphidiploid *N. wuttkei × N. tabacum* cv. Wiślica to produce selfed progenies

Selfed generation	No of self-pollinated flowers (pollination events)	Obtained capsules (%)	Average number of seeds per capsule	Germinated seeds (%)	Suvival rate of seedlings[a] (%)	Number of suviving progeny plants
R_2	201	36.8	26.4	23.1	92.3	416
R_3	200	42.9	58.3	55.8	91.9	2565
R_4	200	48.7	59.6	68.5	92.2	3666

[a] Seedlings capable to grow to maturity, calculated as (the number of viable seedlings/the germinated seeds) ×100

Table 2 Female and male fertility of the amphidiploid *N. wuttkei* × *N. tabacum* cv. Wiślica in the R_2 generation as measured by the reciprocal backcrosses with *N. tabacum* cv. Wiślica

Matting	No of self-pollinated flowers (pollination events)	Obtained capsules (%)	Average number of seeds per capsule	Germinated seeds (%)	Suvival rate of seedlings[a] (%)	Number of suviving progeny plants	Number of suviving true hybrid progeny plants
Amphidiploid R_2 × 'Wiślica' (female fertility)	212	29.8	18.3	23.9	9.3	25	25
'Wiślica' × amphidiploid R_2 (male fertility)	201	70.5	13.1	27.0	40.7	204	1

[a] Seedlings capable grow to maturity, calculated as (the number of viable seedlings/the germinated seeds) ×100
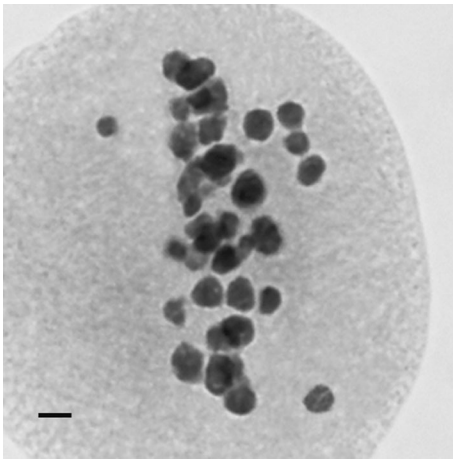
Fig. 1 Metaphase I in sesquidiploid-w hybrid *N. wuttkei* × *N.tabacum* 'Wiślica'(2n = 64); the bivalents in equatorial plate and univalents scattered in and around the plate. *Bar* 5 μm

Table 3 Viability of pollen produced by the amphidiploid *N. wuttkei* × *N. tabacum* cv. Wiślica

Generation	Percentage of viable pollen	
	Mean	Range
Amphidiploid R_1	50.8	36.3–66.7
Amphidiploid R_2	55.5	33.9–69.9
Amphidiploid R_3	62.3	24.2–72.2
Amphidiploid R_4	65.7	49.1–75.1

hybrid contained 31 chromosomes from the wild species *N. wuttkei*, 47 chromosomes from the cultivated *N. tabacum* and 2 recombinant chromosomes (Fig. 3a, b, c). The recombination occurred on the short arm of the chromosomes involved (Fig. 3d, e, f).

Meiotic analysis

In the majority of metaphase I configurations, chromosomes predominantly formed bivalents (Table 4) and normal metaphase plates with bivalents only (Fig. 4i, k) or with occasional unpaired chromosomes (Fig. 4a, b, g, h). Among the bivalents some weakly paired associations were observed (Fig. 4b, i).

Chromosome pairing observed at metaphase I varied among the four amphidiploid generations, increasing from 36.95 in R_1 to 38.71 in R_4 (Table 4; Fig. 4b—38 bivalents for R_1; Fig. 4g—39 bivalents in R_2; Fig. 4i—40 bivalents in R_3; Fig. 4k—40 bivalents in R_4). Highly regular meiosis in the amphidiploids was reflected by a relatively low number of unpaired chromosomes (univalents) per PMC, decreasing from R_1 to R_4 generation (5.75, 4.86, 2.69 and 2.52 in R_1, R_2, R_3 and R_4, respectively; Table 4). Also the number of trivalents decreased from 0.07 per PMC in R_1 generation to 0.01 in R_4 and quadrivalents from 0.05 to 0.004, respectively (Table 4).

Disjunctional abnormalities in later meiotic stages such as chromatin bridges and laggards at anaphase I and chromosomes not included in daughter nuclei at telophase II were rarely observed (Fig. 4d, e). Tetrads were formed in 97.4, 96.8, 98.0 and 98.8 % of the PMC's examined at late telophase II in R_1 to R_4 amphidiploid generations, respectively (Table 5). In the remaining PMC's abnormal microspores as tryads and pentads (Fig. 4f) were observed accounting for
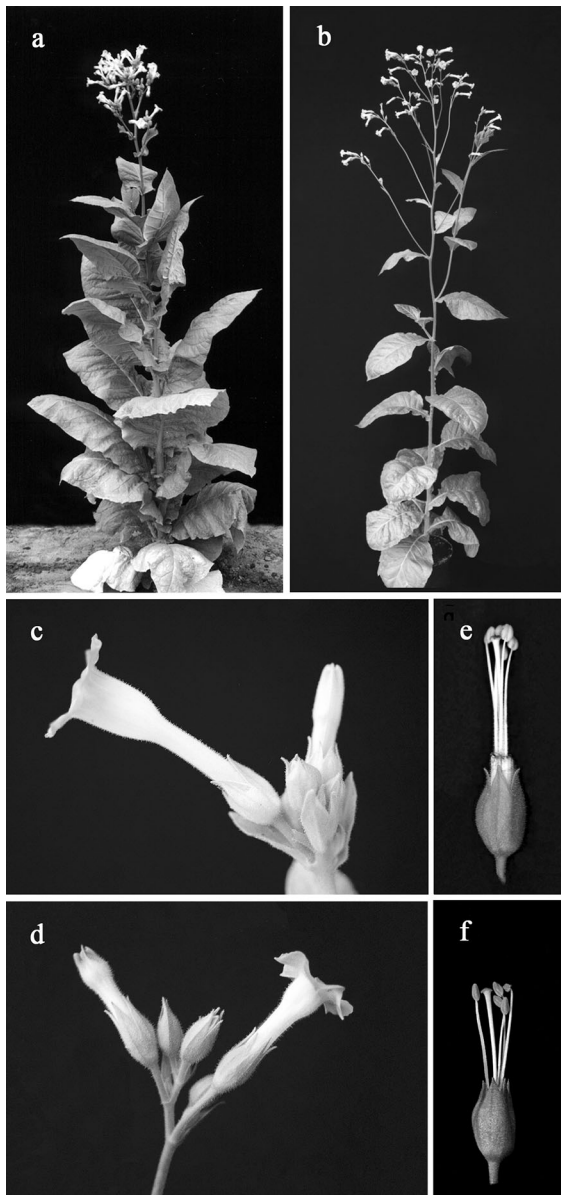
Fig. 2 Morphology of amphidiploid hybrid *N. wuttkei* × *N. tabacum* 'Wiślica' in comparison of *N. tabacum* 'Wiślica'. Plant growth: **a** 'Wiślica', **b** amphidiploid; flower morphology: **c** 'Wiślica', **d** amphidiploid; another position relative to the stigma: **e** 'Wiślica', **f** amphidiploid

0.4–2.0 % of the total number examined (Table 5). The micronuclei were found in 2.0 % of PMC's in R_1, 2.2 % in R_2 and 1.0 % in R_3 and R_4 (Table 5).

Some of the amphidiploid plants, especially from R_4 generation, were characterized by a completely regular meiosis (Fig. 4k, l).

Discussion

The closest relatives of *N. wuttkei* include *N. velutina* (2n = 32), *N. maritima* (2n = 32) and *N. amplexicaulis* (2n = 36) (Barbato 2009), all in the section *Suaveolentes*. Amphidiploids of these latter three species with *N. tabacum* have been reported (Wark 1970; Dorossiev et al. 1978; Berbeć and Doroszewska 1981; Palakarcheva and Dorossiev 1983; DeVerna et al. 1987; Nikova et al. 1991; Nikova and Vladova 2002). Our study here is the first to describe allopolyploids involving *N. tabacum* and *N. wuttkei*. In Laskowska and Berbeć (2012), the creation of the amphidiploids of *N. wuttkei* × *N. tabacum* (R_1 generation) was described. Now, self-pollination and hybridisation experiments were performed with the amphidiploids and the subsequent generations (R_1–R_4) were analyzed in detail.

Amphidiploids in *Nicotiana* are known to show many variation concerning their cytogenetic stability. At one end of the spectrum amphidiploids showing full cytological stability are found, the so-called "synthetic species" such as *N. edwardsonii*, the hybrid of *N. glutinosa* with *N. clevelandii* (Christie 1969; Christie and Hall 1979), *N. rustica* × *N. exigua* (Bolsunov 1971), *N. debneyi* × *N. tabacum* (Smith 1941; Clayton et al. 1967), *N.* × *digluta* from the hybrid *N. glutinosa* × *N. tabacum* (Clausen and Goodspeed 1925), *N. tabacum* × *N. glauca*—*N.* × *ditagla* (Ternovski 1934; Modilevski 1936). At the other end of the spectrum, there are the unstable amphidiploids which rapidly disintegrate due to chromosome loss: *N. tabacum* × *N. plumbaginifolia* (Davies 1974), *N. raimondii* × *N. tabacum* (Berbec 1988), *N. tabacum* × *N. africana* (Doroszewska and Berbec 2000) and *N. longiflora* × *N. tabacum* (Nikova et al. 2001). Some of the amphidiploids, notably *N. tabacum* × *N. glauca*, were reported as either very stable by Ternovski (1934) and Modilevski (1936) (*N. ditagla*) or very unstable (Shilagyi 1975). According to Sybenga (1992) species with little chromosome homology will tend to form more stable allopolyploids than closely related species.

We received amphidiploids of *N. wuttkei* × *N. tabacum* (2n = 80) showing enough self-fertility and cytological stability to be reproduced over successive generations. Increased self-fertility in the R_2 to R_4 generations resulted in a more regular chromosome pairing reaching the expected number of 40 bivalents
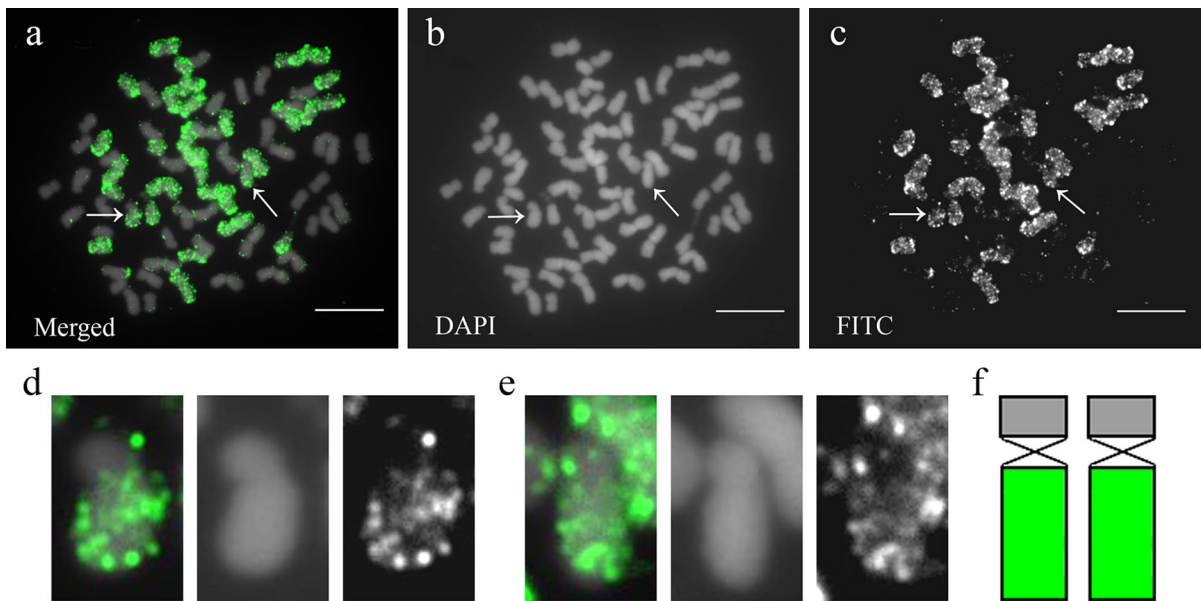
Fig. 3 GISH analysis of the *N. wuttkei* (*green fluorescence*) × *N. tabacum* amphidiploid hybrid of the R$_4$ generation. Pseudo colored merged image and grayscale version of the respective DAPI and FITC channels for complete metaphases (**a**, **b**, **c**) and for the pair of recombinant chromosomes on enlarged photos (**d**, **e**) are shown; **f** schematic drawing of the recombinant chromosomes. *Arrows* on **a**, **b**, **c** indicate the recombinant chromosomes; *bar* 10 μm

Table 4 Chromosome pairing in metaphase I of the amphidiploid *N. wuttkei* × *N. tabacum* cv. Wiślica

Generation	Number of PMCs observed	Chromosome association							
		Univalents		Bivalents		Trivalents		Quadrivalents	
		Mean per cell	Range	Mean per cell	Range	Mean per cell	Range	Mean per cell	Range
Amphidiploid R$_1$	428	5.75	0–20	36.95	30–40	0.07	0–2	0.05	0–1
Amphidiploid R$_2$	500	4.86	0–18	37.40	31–40	0.05	0–1	0.04	0–1
Amphidiploid R$_3$	382	2.69	0–12	38.59	34–40	0.03	0–1	0.01	0–1
Amphidiploid R$_4$	493	2.52	0–10	38.71	35–40	0.01	0–1	0.004	0–1

and elimination of higher chromosome associations (trivalents and tetravalents).This result is in contrast with the results obtained for the allopolyploids involving *N. velutina* and *N. maritima,* the closest relatives of *N. wuttkei* (Nikova and Zagorska 1987). In this study, the near-amphidiploids (82–84 chromosomes) regenerated from the amphihaploid hybrid *N. velutina* × *N. tabacum*, showed high cytological instability, segregating into male fertile and male sterile types. It was explained by massive disintegration of the allopolyploid genome through selective

chromosome elimination in the first selfed generation, resulting in the appearance of *N. tabacum*-like male sterile phenotypes.

The strong tendency for chromosomes to associate as bivalents in the amphidiploid *N. wuttkei* × *N. tabacum*, can be explained by the existing structural differences between genomes of the *N. wuttkei* and *N. tabacum* and the consequently preferential pairing between duplicated chromosomes of each species. The GISH technique provide a valuable tool to verify the relationship and homology between species. In the

present study, parental chromosomes in the amphidiploid R$_4$ generation could be clearly distinguished by standard GISH. According to Schwarzacher et al. (1989) genomes sharing 80 % or less sequence homology can be discriminated by standard GISH conditions.

One pair of recombinant chromosomes in the amphidiploid *N. wuttkei* × *N. tabacum* detected by GISH was most probably the result of a single crossover event. It demonstrates that homoeologous pairing between these two species is possible, albeit very low and that it may occur within the first few generations in an amphidiploid lineage. Intergenomic translocations were also reported in the synthetic allopolyploid *N. sylvestris* × *N. tomentosiformis* of S$_4$ generation (Skalicka et al. 2005) as well as in the hybrid *N. tabacum* × *N. rustica* (Kitamura et al. 1997).

The decreasing self- and cross fertility of the amphidiploids *N. wuttkei* × *N. tabacum* can be, at least partly, related to cytoplasmic male sterility (CMS). All 16 combinations of *Suaveolentes* cytoplasms with the nuclear genome of *N. tabacum* are known to have resulted in CMS (Gerstel 1980; Kubo 1985; Kaul 1988; Berbeć 2001; Nikova and Vladova 2002), *N. wuttkei* being no exception (Laskowska and Berbeć 2007). CMS may manifestate in the first generation of the hybrid, as in *N. excelsior* × *N. tabacum* amphihaploids (Nikova et al. 1997). CMS was also observed in highly unstable populations of the allopolyploid *N. maritima* × *N. tabacum* (Nikova and Zagorska 1987) and in the near-amphidiploid regenerants of the hybrid *N. velutina* × *N. tabacum* where anthers contained no sporogenic tissue (Nikova and Vladova 2002). Since the efficiency to produce true hybrid offspring is low, the amphidiploid *N. wuttkei* × *N. tabacum* may be considered as having its male fertility drastically reduced (single sesquidiploid plant out of more than 200 pollination events). On the other hand, the cross between *N. tabacum* as female and the amphidiploid *N. wuttkei* × *N. tabacum* as pollen donor, resulted in a large population of fully fertile non-hybrid plants, completely resembling the maternal parent. This phenomenon is described earlier in interspecific hybridization in *Nicotiana*. At first, Goodspeed (1915) dismissed diploid maternals (apomictic or otherwise) as products of experimental errors in disagreement to East (1930) but later on he revoked his former view (Goodspeed 1954). Alleged

Fig. 4 Meiosis of the amphidiploid hybrids *N. wuttkei* × *N. tabacum* 'Wiślica' (2n = 80); R$_1$ generation: **a** the bivalents in equatorial plate and univalents scattered in and around the plate, **b** 4 I, 38 II and **c** 6 I, 37 II in metaphase I, **d** lagging chromosomes in anaphase I, **e** chromosomes not included in daughter nuclei in telophase II, **f** tetrad stage—pentad; R$_2$ generation: **g** 2 I, 39 II and **h** 2 I, 37 II, 1 IV in metaphase I; R$_3$ generation: **i** 40 II and **j** 7 I, 35 II, 1 III in metaphase I; R$_4$ generation, PMC's with regular meiosis: **k** 40 II in metaphase I, **l** anaphase I, **m** telophase II, **n** tetrad. *Bar* 5 µm; univalents, trivalents and tetravalents marked by *arrows*

apomictics have continued to be reported either as induced by external factors (Pandey and Phung 1982) or as spontaneous by-products or even sole products of interspecific mattings (Berbeć and Doroszewska 1981; Sarychev 1987; Murthy and Subbarao 2004). According to Naumenko (2012) diploid maternals resulting from the cross *N. tabacum* × *N. alata*, described as "pseudogamic", can be generated on a regular basis, even with *N. tabacum* male sterile plants used as female parents. Similar phenomenon were maternal haploids obtained in several *Nicotiana* cross combinations (Clausen and Mann 1924; Kumashiro and Oinuma 1985). One of these crosses, *N. tabacum* × *N. africana*, produces maternal haploids very regularly and this approach became one of the methods to generate haploid plants in *N. tabacum* (Burk et al. 1979; Nielsen and Collins 1989). Investigation of this phenomenon should get more attention than it has hitherto received.

The amphidiploids *N. wuttkei* × *N. tabacum* of this report could be successfully backcrossed to *N. tabacum* but, with a single exception, only used as females. This resulted in the substitution of an alien cytoplasm in the backcross products (Laskowska and Berbeć 2007) which is a serious limitation in terms of introgression since further backcrosses in alloplasmic lineages usually irrevocably lead to cytoplasmic male sterility. However, based on the results from this study, in which also a single sesquidiploid plant with *N. tabacum* cytoplasm was obtained, there is a theoretical possibility to circumvent the barrier. Therefore, similar work should be done on a larger scale.

In conclusion, the stable amphidiploid of *N. wuttkei* × *N. tabacum* can be regarded as a new artificial hybrid species which can extend the genetic variation within *Nicotiana*. This hybrid can serve as a starting point for transfer of useful genes, such as *Peronospora tabacina* resistance, from *N. wuttkei* to *N. tabacum*.
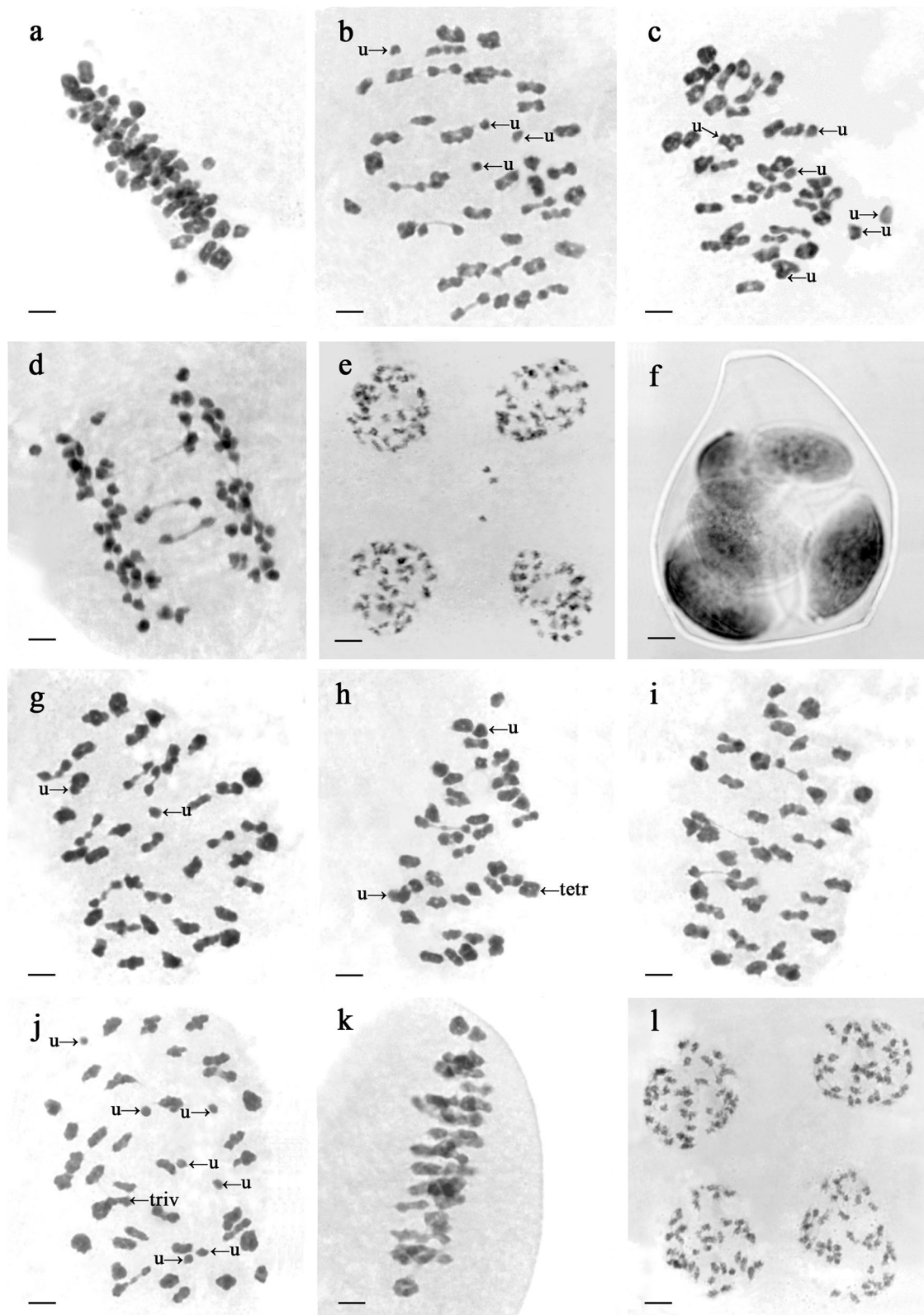
Table 5 Abnormal sporads produced by the amphidiploid *N. wuttkei* × *N. tabacum* cv. Wiślica

Generation	Number of PMC's analyzed	Sporad type			
		Tryads	Tetrads	Pentads	Micronuclei
Amphidiploid R_1	502	6 (1.2)	489 (97.4)	7 (1.4)	10 (2.0)
Amphidiploid R_2	500	6 (1.2)	484 (96.8)	10 (2.0)	11 (2.2)
Amphidiploid R_3	500	4 (0.8)	490 (98.0)	6 (1.2)	5 (1.0)
Amphidiploid R_4	499	2 (0.4)	493 (98.8)	4 (0.8)	5 (1.0)

Absolute values given without parentheses; percentages of the total number in parentheses

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