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# Cytological and Molecular Characterization of Intertribal Hybrids between the Geophytes *Anemone coronaria* L. and *Ranunculus asiaticus* L. (Ranunculaceae)

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## ABSTRACT

*Anemone coronaria* L. and *Ranunculus asiaticus* L. both belong to the Ranunculaceae, a large plant family with many ornamentals of horticultural importance. There are considerable differences between these species leaves, flower morphology and flower colour therefore intergeneric crosses between the species might result in new interesting hybrids. Crosses between *Anemone coronaria* L. and *Ranunculus asiaticus* L. were performed and the F1 progeny was examined. In this study, the F1 hybrid generation was investigated at morphological, molecular and cytogenetic levels. More than 85% of the F1 plants had very similar flowers to the maternal plants and although seeming to have a limited paternal contribution, AFLP analyses confirmed the partial hybrid character of the F1 plants. GISH experiments revealed that the F1 plants were mixoploids (plants composed of cells with different chromosome numbers) or showed many chromosome rearrangements.

**Keywords:** AFLP, distant crosses, GISH, intergenera, wide hybridization
**Abbreviations:** **AFLP**, Amplified Fragment Length Polymorphism; **DAPI**, 4',6-diamidino-2-phenylindole; **GISH**, Genomic *In Situ* Hybridization

## INTRODUCTION

In horticulture there is a ceaseless search for more variation. One option to introduce variation to a species comprises interspecific or intergeneric crosses. This results in hybrids with combined traits normally not observed in a single species (Sharma 1995; Van Tuyl and De Jeu 1997; Van Tuyl and Lim 2003). However, intergeneric hybridization has been shown to trigger genome stress, which induces wide-spread genomic reorganisation (Madlung and Comai 2004). For example, distant hybridization can result in the formation of allopolyploids but unanticipated, genetic and epigenetic changes such as sequence elimination, chromosome rearrangements, gene silencing, DNA methylation and activation of transposons have been observed in distant crosses of several plant systems (Soltis and Soltis 1995; Comai 2000; Comai *et al.* 2000; Madlung and Comai 2004; Ma and Gustafson 2005). In some cases, attempted intergeneric hybridization can lead to haploid progeny (Doré *et al.* 1996; Sidhu *et al.* 2006).

The Ranunculaceae are a moderately large family with 59 genera and *circa* 2500 species (Tamura 1995a). Most are perennials and mainly herbaceous geophytes (Tamura 1995b). The Ranunculaceae harbours several ornamentals with beautiful flowers. For example, *Anemone coronaria* L. and *Ranunculus asiaticus* L., are two species with distinct and complementary characteristics. Combining several interesting characteristics of both genera into one hybrid could result in crossing products with new traits. Since *Anemone coronaria* L. and *Ranunculus asiaticus* L. are situated in separate tribes (Hoot 1995), fertilization barriers are present (Dhooghe *et al.*, unpublished data). Over the years methods have been developed to overrule these barriers artificially (Sharma 1995) and intergeneric crossing products of *Anemone coronaria* L. x *Ranunculus asiaticus* L. have been obtained (Dhooghe *et al.*, unpublished data).

Previous reports showed that morphological analysis can distinguish hybrids obtained after interspecific and intergeneric crosses from the parent plants (Nomura *et al.* 2002; Reed *et al.* 2008). However, keeping in mind the possible genome reorganisation events, it is important to combine this morphological data with a molecular or cytogenetic screen to confirm the interspecific or intergeneric origin of the crossing products.

In this study, we have scored plants obtained from intergeneric crossing on their hybrid characteristics using morphological comparison, Amplified Fragment Length Polymorphism (AFLP) analysis and Genomic *in Situ* Hybridization (GISH).

## MATERIALS AND METHODS

### Plant material and growth conditions

*In vitro* seedlings of intergeneric crosses between *Anemone coronaria* L. ('Mistral® Wine', 'Mistral® Fucsia' and 'Wicabri® Blue') and *Ranunculus asiaticus* L. ('Success® Alfa' and 'Krisma') were acclimatized in the greenhouse from December to March to obtain rhizomes. These rhizomes were harvested and dried properly. During the next growing season (in September), these rhizomes were replanted together with their respective parent plants in a peat-mixture ($pH_{H2O}$ 6.5-7.5) with 5% perlite, 5% clay and fertilizers (NPK 14-16-18, 1 $kg.m^{-3}$). Standard nursery practices were used for watering, fertilization and pest control. Plants were grown in a greenhouse (natural photoperiod regime, Gent, Belgium) with climate condition settings of 18°C day temperature and 5°C night temperature. During the day (8 a.m. – 4 p.m.) extra assimilation light was given (assimilation light HPI-T Plus Philips, PAR: 25 – 30 $\mu mol.m^{-2}.s^{-1}$).

Received: 4 January, 2011. Accepted: 20 December, 2012.

Original Research Paper

## Morphological analysis of F1 progeny and their parent plants

Quantitative data of plant characteristics were collected from February to April for both F1 plants and their corresponding parent plants. Vegetative plant characteristics (plant height, number of rosette leaves) were measured after full vegetative development (February). Plant height was determined in stretched conditions of the leaves. Flower characteristics measured were number of flowers, number of sepals or bracts, number of tepals or petals, flower stem length, flower diameter, flower colour. Flower colours were determined using the Royal Horticultural Society Colour Chart (London).

## Molecular analysis of F1 progeny and their parent plants

DNA-extraction of young leaf tissue was done using the DNeasy Plant Kit (Qiagen) according to the manufacturer's conditions. AFLP procedures were performed as described by Vos *et al.* (1995) but modified according to Nissim *et al.* (2004) and Fang and Paz (2005) because the standard *Eco*RI/*Mse*I combination for DNA digestion did not result in a useful fingerprinting pattern (pers. obs., Nissim *et al.* 2004; Fang and Paz 2005). Therefore, DNA was digested with the hexacutter *Eco*RI as single restriction enzyme. Pre-amplification was done with *Eco*RI adaptor primers (E) extended with one selective nucleotide (E-A). Selective amplification was tested using five different primer combinations: E-ACA/E-ACG, E-ACG/E-AGG, E-ACT/E-ACT, E-AGG/E-ACG, E-ACG/E-ACT. The first primer of each combination was end-labelled with [$\gamma$-$^{33}$P]ATP. Following amplification, an equal volume of formamide loading dye was added to the PCR products. After denaturation, the products were separated electrophoretically on 5% denaturing polyacrylamide gels using a run time of about 5.5 h to 6 h. Fingerprint patterns were visualised on Fuji autoradiography films, following an exposure of seven days. The DNA fingerprints were visually scored.

## Cytogenetic analysis of F1 progeny and their parent plants

Chromosome spreads were made of young root tips pre-treated with a saturated solution of $\alpha$-bromonaphthalene in water. After overnight incubation at 4°C, the roots were fixed in an ethanol: acetic acid solution (3:1) for 1 h and stored at -20°C until use. Roots were rinsed in water before incubation in an enzymatic solution (0.1% cellulase 'Onozuka' RS (Duchefa), 0.1% pectolyase Y23 (Duchefa), 0.1% cytohelicase (Sigma) in a 10 mM citrate buffer pH 4.5) for 35 min. After enzymatic digestion, the roots were transferred to water. The root tips were squashed on a slide, where a drop of acetic acid (50%) was added. Slides were then incubated on a heating plate (42°C) for 2 min. The chromosomes were fixed using ethanol:acetic acid (3:1) and were rinsed with ethanol (100%). The chromosomes were counted using Differential Interference Contrast microscopy (OLYMPUS BX51) and photographed using a Nikon digital camera (DS-5M-L1).

For Genomic *in Situ* Hybridization (GISH), total genomic DNA was extracted from 2 g fresh leaf tissue according to Rogers and Bendich (1988). *In situ* hybridization of F1 plants was done using labelled genomic DNA from the pollen parent as probe and unlabelled genomic maternal DNA as blocking DNA. Probe labelling was done using biotin-16-dUTP using a Biotin-Nick Translation Mix (Roche) according to manufacturer's instructions. Biotin labelled DNA was detected with Cy™3-conjugated streptavidin (Jackson Immuno Research Laboratories) and amplified with biotinylated anti-streptavidin (Vector Laboratories). The slides were counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy (Olympus IX81).

# RESULTS

## Morphological analysis of F1 progeny and their parent plants

233 F1 plants were retrieved after acclimatization of which 204 plants produced flowers that could be compared with the parent plants. A unilateral preference existed. Although a similar number of crosses were done in both cross-directions, the crosses in which *Anemone coronaria* L. was used as maternal plant resulted in 203 acclimatized plants, while only one plant was obtained when *Ranunculus asiaticus* L. was used as maternal parent.

The crossing product of *Ranunculus asiaticus* 'Krisma' x 'Mistral Wine' had very similar plant parameters to the maternal plant except a slightly reduced number of petals

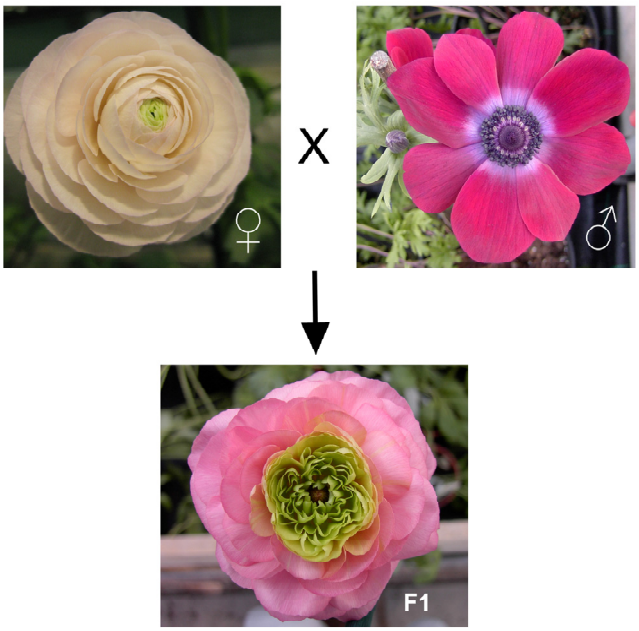


**Fig. 1 Crossing product (center) with its respective parent plants *Ranunculus asiaticus* 'Krisma' (left) as maternal plant and *Anemone coronaria* 'Mistral Wine' (right) as paternal plant.**

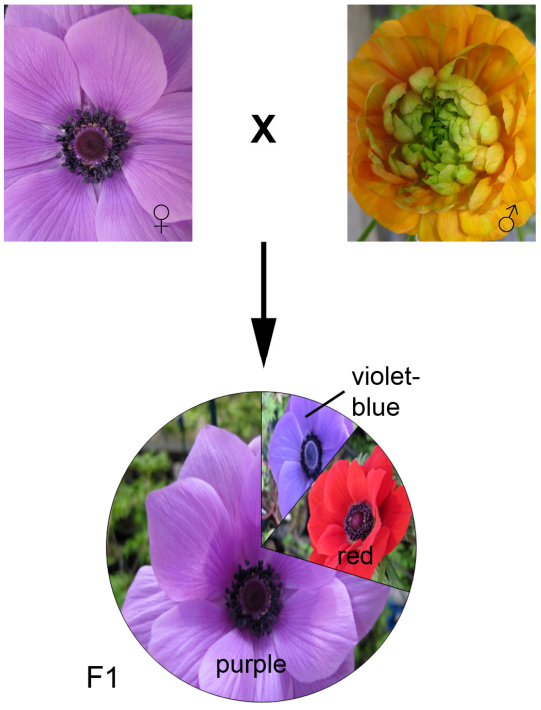


**Fig. 2 Distribution of the flower colours of the crossing products of *Anemone coronaria* 'Mistral Fucsia' x *Ranunculus asiaticus* 'Alfa'.**

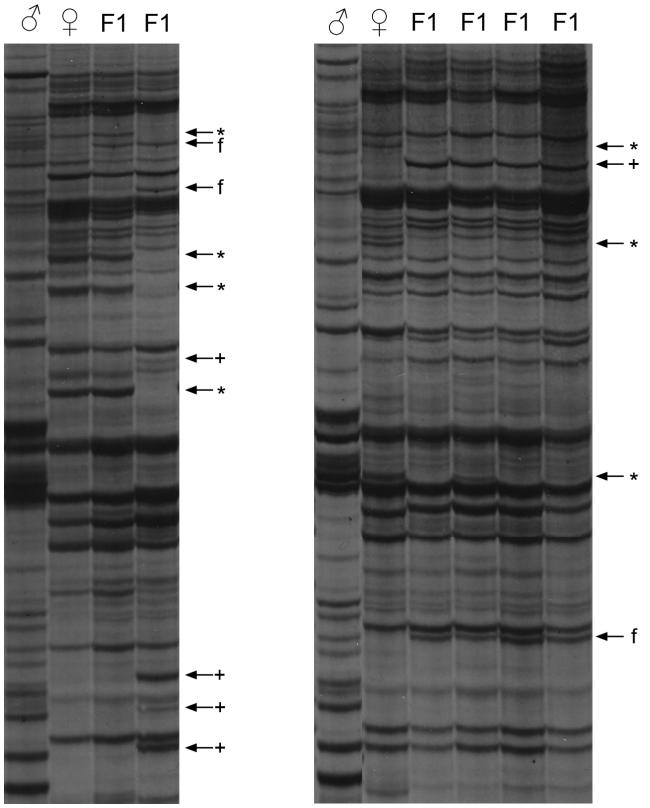


**Fig. 3 AFLP analyses of F1 progeny and their respective parent plants. Left: *Anemone coronaria* 'Mistral Fucsia' x *Ranunculus asiaticus* 'Alfa'. Right: *Anemone coronaria* 'Mistral Wine' x *Ranunculus asiaticus* 'Alfa'.** (f = band specific for male plant (father), + = novel band not present in both parent plants, * = band deleted from female plant)

(data not shown) and a more intense pink flower colour (**Fig. 1**).

The morphological observations of the rhizomatous F1 plants of the other cross direction *Anemone coronaria* L. x *Ranunculus asiaticus* L. revealed some trends. First, the vegetative and generative plant characteristics were highly variable and hence not optimal to distinguish parent plants from the hybrids. Second, there was a high resemblance of the F1 progeny to their maternal parents. Third, the most striking observation was the dominance of a blue flower colour (violet-blue group - RHS Colour Charts) among the F1 population, that was not related to the flower colour of the maternal parent used; there were also some unexpected flower colours in the F1 progeny. For example, a cross between *Anemone coronaria* 'Mistral Fucsia' with *Ranunculus asiaticus* 'Alfa' resulted in crossing products with three main colour classifications: 70.3% had a similar pink colour as the maternal plant, 18.9 % had a red border and a white centre and 10.8% had a violet-blue colour (**Fig. 2**). Similarly, a cross between *Anemone coronaria* 'Mistral Wine' with *Ranunculus asiaticus* 'Alfa' showed an interesting intense pink flower colour in the progeny.

## Molecular analysis of F1 progeny and their parent plants

80 F1 plants were analysed by AFLP to confirm the hybrid status on a molecular level. The plants were found to have inherited a very limited percentage (0.0 to 15.4%) of the unique markers of the male parent and their AFLP pattern was very similar to the female parent (**Fig. 3**). Although the genetic input of the male parent appeared rather low, specific paternal bands could be observed. Besides this, 13.3% (mean value) of the bands present in the maternal profile were not detected in the F1 plants, and 10.0% (mean value) novel bands, which were not present in both parents, could be observed in the F1 progeny.

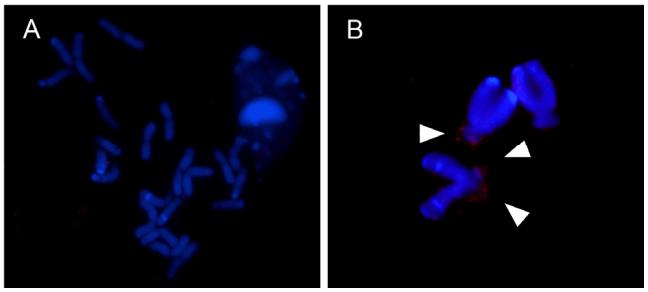


**Fig. 4 GISH analysis of crossing products of *Anemone coronaria* 'Mistral Fucsia' x *Ranunculus asiaticus* 'Alfa'.** (**A**) Twenty two chromosomes are observed in a cell with no hybridization apparent in this cell. (**B**) Hybridization of paternal probe DNA (arrowheads) to some chromosomes in one of the progeny.

## Cytogenetic analysis of F1 progeny and their parent plants

To analyse the putative hybrids on a cytogenetic level, chromosome counts were performed on 41 F1 plants and parent plants. Both *Anemone coronaria* L. and *Ranunculus asiaticus* L. have a diploid chromosome number of 16. Hence hybrids should posses eight *Anemone* and eight *Ranunculus* chromosomes. Surprisingly, in root tip cells of individual F1 plants a wide range of chromosome numbers could be detected indicating that the F1 plants are mixoploids (**Table 1**). Some cells contained less than eight chromosomes (less than the haploid level of the parent plants), while other cells displayed more than 32 chromosomes (more than double the chromosome number). These analyses suggest that the F1 plants suffer from chromosome instability. In an attempt to further investigate whether specific chromosomes might be preferably eliminated during genomic reorganisation, GISH experiments were performed. GISH analysis of 31 F1 plants from 11 different intergeneric crosses between *Anemone coronaria* L. and *Ranunculus asiaticus* L. confirmed elimination of chromosomes or an unexpectedly high number of chromosomes even within the same plant (**Fig. 4**). The results also argue for a non-specific elimination of parental chromosomes. The GISH experiments confirmed the low input of the paternal parent observed by AFLP. All cells observed lacked even a single chromosome from the paternal parent, though sometimes paternal probe DNA was hybridized on some chromosomes (**Fig. 4**).

## DISCUSSION

In this study, the F1 progeny obtained from intergeneric crosses between *Anemone coronaria* L. and *Ranunculus asiaticus* L. was investigated on morphological, molecular and cytological characteristics to determine the hybrid status of each plant.

Upon interspecific and intergeneric crosses, unilateral incongruity is often observed. This means that a cross is successful in one direction, whereas the reciprocal cross fails (Van Tuyl and De Jeu 1997). In the intergeneric crosses between *Anemone coronaria* L. and *Ranunculus asiaticus* L., no crosses were observed which totally failed but unilateral preference did exist. The cross *Anemone coronaria* x *Ranunculus asiaticus* resulted in a significantly higher number of acclimatized seedlings than the reverse cross.

Morphological analysis revealed a striking similarity between maternal parent plants and their F1 progeny, with only some small differences were noticed. The ornamental production sector is constantly seeking new flower colours and/or flower morphologies. The majority of the F1 plants produced blue flowers. The blue colour is probably a dominant characteristic which prevails in the progeny of *Anemone coronaria* × *Ranunculus asiaticus*. However, totally unexpected colours sometimes appeared, for example the

red colour in a cross of *Anemone coronaria* 'Mistral Fucsia' × *Ranunculus asiaticus* 'Alfa'. In the other cross-direction *Ranunculus asiaticus* 'Krisma' × *Anemone coronaria* 'Mistral Wine', flowers with a colour intermediate to the parental flower colours appeared.

AFLP confirmed that most of the crossing products were hybrids, but also demonstrated the low paternal contribution. Furthermore, AFLP analysis of the F1 plants revealed high rates of new or deleted bands. The observation of novel and deleted AFLP markers might be a consequence of intergenomic translocations promoted by the stressful conditions in distant crosses. This abnormal status of the genome was revealed by chromosome counts and GISH, which showed that F1 plants were composed of cells with different chromosome numbers and that the chromosomes were predominantly derived from the maternal plant. In accordance with our results, AFLP analysis of intergeneric hybrids between *Brassica* and *Orychophragmus violaceus* showed that the frequency of marker deletion from the female parent was much higher than the presence of novel bands or markers from the male parent (Liu and Li 2007). On top of this, intergeneric hybrids between *Brassica* and *O. violaceus* mainly exhibited maternal parent-like phenotypes and showed a variety of chromosome origin in their somatic cells (cells with only maternal chromosomes, cells with a doubled number of maternal chromosomes, cells with maternal chromosomes with or without one or two intact paternal chromosomes or cells with maternal chromosomes with one or more paternal chromosomal segments) (Li *et al.* 1998; Hua *et al.* 2006; Liu and Li 2007). In F1 plants generated from crosses between *Brassica carinata* and *Orychophragmus*, not a single *Orychophragmus* chromosome could be detected, although some AFLP bands were specific for *Orychophragmus* (Hua *et al.* 2006). Other studies of intergeneric crosses between *Brassica* and *Capsella* (Chen *et al.* 2007) or between *Brassica* and *Lesquerella* (Du *et al.* 2008) reported a similar maternal-like F1 progeny, a comparable chromosome number variation and analogous AFLP patterns.

The mechanism of alien chromosome elimination after wide crosses is not known. Some hypotheses include incorrect spindle interactions, asynchrony in essential mitotic processes, spatial separation of genomes during different phases in mitosis or formation of micronuclei (Gernand *et al.* 2005). In wheat × maize crosses, elimination of one or more maize chromosomes occurred at the first division of about 70% of hybrid embryos and all maize chromosomes appeared to be lost by the time the embryo had eight cells (Laurie and Bennett 1989). These results suggest instability of the F1 genome which might lead to an elimination of parental chromosomes.

## CONCLUSION

The initial aim of producing intergeneric hybrids between *Anemone coronaria* L. and *Ranunculus asiaticus* L. was to expand the variation available for creating new cut flower varieties; our intergeneric crossing experiments generated some interesting products. Importantly these hybrids also increase our insight into the genetics of distant crosses. Based on the combination of techniques used, we assume that upon hybridization of *Anemone coronaria* L. and *Ranunculus asiaticus* L. genomes, a process took place which is intermediate between allopolyploidization and chromosome elimination; processes regularly observed during distant sexual hybridization and often referred to as a genomic shock.

## ACKNOWLEDGEMENTS

The authors thank T. Versluys and C. Petit for technical support. Istituto Regionale per la Floricoltura and Biancheri Creations are acknowledged for supplying the rhizomes of *Anemone coronaria* L. and *Ranunculus asiaticus* L.

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