Interspecific Hybridization in Lily (*Lilium*): Interploidy Crosses Involving Interspecific F₁ Hybrids and Their Progenies

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ABSTRACT

Despite a long history of cultivation, a large number of lily (*Lilium*) cultivars were still diploid (2n=2x=24) till recently. But polyploid cultivars are rapidly increasing. In addition to having robust stems, large flowers, thicker and larger leaves, polyploids can also serve to combine desirable characters from species of different taxonomic sections; not only from the cultivated groups, viz., Sinomartagon, Archelirion and Leucolirion, but also from sections that include non-cultivated species. Because the F₁ hybrids between the species of different taxonomic sections are highly sterile, it is imperative that breeding has to be carried out at the polyploidy level. This chapter is based on our results on using intersectional interspecific hybrids of Longiflorum x Asiatic (LA) and Oriental x Asiatic (OA) groups of lilies and their polyploid backcross progenies BC1, BC2 and BC3). In order to cross genotypes of different ploidy levels, i.e., interploidy crosses, a knowledge of embryo and endosperm ploidy levels is valuable because, unlike in most other plant species, lily has very large chromosomes and the embryo sac formation is of tetrasporic 8-nucleate type. In order to highlight the differences, a comparison is made with the most commonly occurring, monosporic 8-nucleate type of diploid potato, with 24 chromosomes (same as lily). Some of the limitations and constraints associated with interploidy crossing are considered.

1. INTRODUCTION

Generally, polyploid plants differ from their diploid counterparts in various ways. As a result of increase of chromosome numbers as well as DNA content, polyploids may possess robust stems, larger flowers, thicker leaves and can be more attractive in appearance in some cases. Besides these, some physiological features such as flower initiation, duration of flowering, altered water relations and photosynthesis may also be affected (for review, see Ramsey and Schemske 2002). Therefore, not surprisingly, horticultural plant breeders have selected polyploid forms in numerous ornamental plants in order to create new varieties (Darlington 1967; Van Tuyl *et al.* 2002). Although lilies (*Lilium*) have been cultivated for several centuries (Evans 1921, 1930; Woodstock and Stearn 1950), a large number of cultivars were probably diploids (2n=2x=24) till recently. At present, however, a large majority (80% or more) of cultivars are polyploids. Not all polyploid cultivars of lilies have been listed so far. However, in Asiatic group of lily hybrids, more than 120 polyploid cultivars were listed and these include triploids (2n=3x=36), tetraploids (2n=4x=48) as well as a few aneuploids (Schmitzer 1991). In addition to these autopolyploids, allopolyploid lily cultivars are becoming increasingly popular. These include interspecific polyploids involving Longiflorum x Asiatic hybrids (LA), Longiflorum x Oriental hybrids (LO), Oriental x Asiatic hybrids (OA) and Oriental x Trumpt lily hybrids (OT). Recent statistics indicate that the afore-mentioned groups of allopolyploids occupy nearly a quarter of the area used for lily bulb production in the Netherlands (Chapter by Lim *et al*. in this series). Although no critical data on the chromosome numbers of all allopolyploid lily cultivars are yet available, it appears that both triploid and tetraploid forms are being cultivated. A recent survey of chromosome constitutions in a number of Dutch lily cultivars has shown that allotriploids of interspecific origin, involving Longiflorum and Asiatic lilies, are predominant among them (Zhou 2007).

Despite the recognition of the presence of both auto- and allopolyploid cultivars of lilies, little is known about the exact origins of most of the polyploid cultivars, with the exception of a few cultivars for which detailed information on the parentage and the chromosome constitution are available (Van Tuyl *et al*. 2002; Zhou 2007). In these cases, both somatic doubling of chromosomes by colchicine or oryzalin as well as sexual polyploidization, (i.e., induced by unreduced (*2n*) gametes) have been used (Van Tuyl 1989).

When hybrids between the species belonging to different taxonomic sections, viz., Leucolirion, Sinomartagon and Archelirion, are made, the diploid interspecific F$_1$ hybrids are invariably sterile and as such they cannot be used in further breeding. Sterility in these hybrids can be overcome either by doubling the chromosome numbers or through the use of *2n* gametes (previous chapter). Further more, polyploidy can also be helpful for combining important horticultural traits from species of different sections and at the same time select cultivars that are more vigorous and attractive.

Regardless of whether the polyploid lilies are produced through somatic doubling or through *2n* gametes, there is one unavoidable consequence: i.e., it becomes necessary to hybridize genotypes of different ploidy levels for the purpose of breeding. This type of interploidy crossings among *Lilium* species hybrids and their progenies is in some ways unique as compared to a majority of other plant species for two reasons. 1. *Lilium* species possess huge amounts of DNA in their genomes as compared to other plant species. This may be a limiting factor for producing genotypes of higher ploidy levels. 2. The formation of the female gametophyte in lilies is of the so-called tetrasporic 8-nucleate type (Maheshwari 1971). In this case, the secondary nucleus of the female gametophyte (i.e., embryo sac) invariably possesses chromosome number that is twice that of the sporophyte. This means, the presence of excessive amount of DNA in the secondary nucleus, and eventually in the endosperm, can impose limitations for the development of the embryos in certain combinations of crossings. Thus, the endosperm-embryo ploidy levels in lily it is expected to be quite different from most of the other plant species that have the monosporic 8-nucleate embryo sacs. In view of this, our results on interploidy crosses involving the species, F$_1$ interspecific hybrids and their backcross progenies (BC$_1$, BC$_2$ and BC$_3$) among Longiflorum, Asiatic and Oriental lilies are considered in this chapter. Some of the implications of interploidy crosses in lily for introgression of characters and to achieve optimal ploidy levels that may be required for cultivars are highlighted.

2. INTERPLOIDY CROSSINGS

If the primary objective is to breed polyploid cultivars, one may double the chromosome number of a diploid species, or a cultivar, and then use the resulting autotetraploid as a parent to produce either triploid or tetraploid progenies through appropriate crossings (e.g., 2x–4x or reciprocal and 4x–4x respectively). Although there are instances of autotriploid and autotetraploid lily cultivars known, not many data are available regarding the types of crosses that were made and their consequences. Nevertheless, there are some data on the origins of allotriploids, allotetraploids, allopentaploids together with the results on intercrossing of such genotypes (Lim *et al*. 2003; Barba-Gonzalez *et al*. 2006; Zhou 2007). Two unique features of these polyploids are: a) the ploidy levels of a large number of progenies have been determined by DNA measurement through flow cytometry and b) the chromosome constitutions of the parents and the progenies have been critically determined through genomic in situ hybridization (GISH) in a large number of cases. With this background knowledge, an assessment is made in the following pages of the various types of crosses that were made involving genotypes of different ploidy levels and the expected results based on embryo/endosperm relationship that occur in each case (**Table 1**). In this Table, in addition to the crosses of lily, which has tetrasporic 8-nucleate type of embryo sacs, a list of analogous crosses in a monosporic 8-nucleate type of embryo sac is also included for the sake of comparison. The chromosome numbers mentioned for monosporic type in the **Table 1** represent diploid potato (2n=2x=24), that has the same chromosome number as lily. For interploidy crossings in lily, both somatically doubled as well as sexual polyploids have been used. Cross combinations shown for lily in **Table 1** are those in which plants have been successfully obtained (with only one or two exceptions) through ovule or embryo rescue methods. However, it should be pointed out that the fruits were allowed to develop *in vivo* for about 6-7 weeks before culturing ovules or embryos and to this extent they are comparable to normal development of fruits and seeds.

2.1. 2x–2x crosses

The main reason for including crosses between two diploid parents in this table is that, at times, they can produce polyploid progenies due to *2n* gametes and in this sense they are more or less similar to those of interploidy crosses. Further more, distant hybrids such as LA, OA, produce only *2n* gametes which give rise invariably to polyploid progenies. However, some genotypes of LA hybrids produce a low frequency of haploid (*n*) as well as *2n* gametes. In such cases it was possible to obtain both diploid (near diploid in some cases) as well as triploid progenies. The remarkable difference between tetrasporic 8-nucleate and monosporic 8-nucleate type is that in the former the secondary nucleus remains invariably 4x regardless of the chromosome number of the egg nucleus, whereas in the latter the ploidy level of the secondary nucleus is related to the ploidy level of the egg (always possesses twice the number). This also means that in the case of monosporic 8-nucleate type the variation of the ratio of embryo: endosperm ploidy level is smaller when compared to tetrasporic 8-nucleatype (1:1.3–1:1.7 vs 1:1.6–1:3.0

Table 1 Types of interploidy crosses made by using interspecific lily hybrids and their progenies together with the ploidy levels of egg, secondary nucleus (sec. nu), embryo (emb) and endosperm (endo) in each cross. To highlight the differences between monosporic 8-nucleate and tetrasporic 8-nucleate types of embryo sacs, analogous crosses of diploid potato are also included. Diploid parents that contribute *2n* gametes are marked as '*ur*' (unreduced).

Type of cross	Monosporic 8-nucleate embryo sac			Tetrasporic 8-nucleate embryo sac		
	Ploidy level of			Ploidy level of		
	Egg	Sec. nu	Emb: Endo ratio (Chromosome nr.)	Egg	Sec. nu	Emb: Endo ratio (Chromosome nr.)
2x - 2x	x	2x	1:1.5 (24:36)	x	4x	1:2.5 (24:60)
2x *ur* - 2x	2x	4x	1:1.7 (36:60)	2x	4x	1:1.7 (36:60)
2x – 2x *ur*	x	2x	1:1.3 (36:48)	x	4x	1:2.0 (36:72)
2x *ur* – 2x *ur*	2x	4x	1:1.5 (48:72)	2x	4x	1:1.5 (48:72)
2x-3x (x)*	x	4x	1:1.5 (24:36)	x	4x	1:2.5 (24:60)
2x – 3x (2x)*	x	2x	1:1.3 (36:48)	x	4x	1:2.0 (36:72)
2x – 3x *ur*	x	2x	1:1.3 (48:60)	x	4x	1:1.75 (48:84)
3x (x)* – 2x	x	2x	1:1.5 (24:36)	x	6x	1:3.0 (24:72)
3x (2x)*- 2x	2x	4x	1:1.7 (36:60)	2x	6x	1:2.3 (36:84)
3x (2x)* – 4x	2x	4x	1:1.5 (48:72)	2x	6x	1:2.0 (48:96)
3x *ur*- 4x	3x	6x	1:1.6 (60:96)	3x	6x	1:1.6 (60:96)
4x – 2x	2x	4x	1:1.7 (36:60)	2x	8x	1:3.0 (36:108)
2x – 4x	x	2x	1:1.3 (36:48)	x	4x	1:2.0 (36:72)
4x – 4x	2x	4x	1:1.5 (48:72)	2x	8x	1:2.5 (48:120)
2x -5x (2x)*	X	2x	1:1.3 (36:48)	x	4x	1:2.0 (36:72)
5x (2x)* - 2x	2x	4x	1:1.7 (36:60)	2x	10x	1:3.6 (36:132)

respectively). Almost as a general rule, the ratios of embryo: endosperm in different types of crosses of the monosporic type is smaller than those of tetrasporic type. In 2x–2x crosses, progenies of three ploidy levels can be obtained depending upon the types of gametes that are produced: when the hybrid produces haploid gametes (as in the case of LA hybrids), diploid BC_1 progenies are produced; when *2n* gametes are produced either by male or the female parent, triploid BC_1 progenies are produced and when both parents produce *2n* gametes (bilateral sexual polyploidization) tetraploid progenies are produced.

2.2. 2x–3x crosses and reciprocals

Triploids are generally highly sterile because of the unequal segregation of chromosomes during meiosis. However, in some exceptional cases triploid do produce considerably high frequencies of fertile spores either during micro- or megasporogenesis. Such spores generally possess aneuploid (ranging from 12+1 to 36-1) as well as euploid chromosome numbers (x=12, 2x=24, 3x= 36), the former being predominant. It has been possible to produce large numbers of progenies by using triploids both as female and male parents in the case of LA and OA hybrids (Lim *et al.* 2003; Barba-Gonzalez *et al.* 2006; Zhou 2007). A notable feature of 2x–3x, or reciprocal, cross is that there is a tendency for the production of progenies with nearly diploid chromosome numbers. However, when the triploid is used as the female parent mostly aneuploids are produced but when the same parent is used as a male parent the progenies are more likely to be diploid. This is probably due to the higher rate of transmission aneuploid gametes by the female parent as has been observed in several other plant taxa (Khush 1973; Brandham 1982). An interesting feature of triploid female parent is that it can produce haploid gametes, and on fertilization with haploid male gametes, it gives rise to diploid progeny. In such a case, a remarkably high ratio of embryo: endosperm of 1:3.0 (**Table 1**) can result. This implies that the presence of a massive amount of DNA in the endosperm nucleus is not an impediment for the development of a diploid embryo in lily hybrid. Diploid or near diploid progenies derived from 2x–3x, or reciprocals, involving Asiatic x allotriploid, ALA and Oriental x allotriploid, AOA give rise to progenies with predominantly Asiatic chromosomes (see later).

2.3. 3x–4x and other crosses

Allotriploids ALA as well as AOA have been successfully used as female parents to cross with tetraploid parents. But the reciprocal 4x–3x crosses have not been successful in both cases. The explanation might be that in the case of 3x–4x cross, the secondary nucleus has only 6x ploidy level and this results in an embryo: endosperm ratio of 48:96 (**Table 1**) which is obviously viable. On the contrary, in 4x–3x cross, the secondary nucleus is expected to possess 8x ploidy level, and assuming that a *2x* game is functional from the triploid male parent, gives to an embryo: endosperm ratio of 48:120 (not shown in **Table 1**) which may not be viable. It should also be pointed out that when a *2n* gamete from the triploid parent is functional in a 3x–4x cross, progenies can be obtained because the embryo: endosperm ploidy levels are 60:96, a viable combination. One notable result is that in the case of 3x–4x crosses, the progenies possess either 4x or circa 4x chromosome numbers. Although the results from crosses involving 4x female parents with both ALA and AOA allotriploid have not been successful, it cannot be concluded that the former may not be used as female parents. There are indeed some cases where 4x parents have been successfully used as female parents to produce progenies (Schmitzer 1991). Compared to 4x–3x crosses, it is relatively easy to produce progenies from 2x–4x crosses rather than their reciprocals. Also in the case of 2x–5x crosses, where the diploid parent is used as female parent, progenies have been obtained but not in the reciprocal crosses. In the latter case the endosperm possesses 11x ploidy level (**Table 1**) and therefore not viable.

3. RELEVANCE OF INTERPLOIDY CROSSING FOR BREEDING

In order to circumvent F_1 sterility in distant hybrids, one or the other of the following approaches can be use: 1. Double the chromosome numbers of the prospective diploid parent species and use the auto polyploids to make hybrids. This can results in an allotetraploid that is fertile. 2. Produce an F_1 hybrid between two diploid species and double the chromosome number through colchicine or oryzalin to produce an allotetraploid. 3. Select genotypes of F_1 hybrids that produce *2n* gametes and use directly for crossing. 4. In rare cases, such as in LA hybrids, the F_1 hybrids may produce functional haploid gametes that can be used for producing backcross progenies. All these approaches have been successful in lilies but each of these has advantages as well as disadvantages. The allotetraploids produced through the first and second approaches can probably be utilized through 2x–4x (back) crossings but the resulting allotriploids do not posses any recombinant chromosomes and, more over, the plants will be sterile once again. The great drawback of allotetraploids is that they cannot give rise to genetic variation in their progenies because of the lack of intergenomic recombination. The third approach of using *2n* gametes is attractive when genotypes of F_1 hybrids produce such gametes are available. However, genotypes that produce high frequencies of either *2n* pollen or *2n* eggs appear to be relatively rare in lilies. But, unlike the *2x* gametes produced by typical allotetraploids, *2n* gametes from F_1 hybrids can possess high frequencies of recombinant chromosomes and generate considerable amount of genetic variation in the progenies they produce. In fact, several cultivars of ALA constitution have been directly selected by the lily breeders in BC_1 progenies of LA hybrids backcrossed to Asiatic parent (Zhou 2007). However, if further crossing is required between such triploid progenies derived from any of the first three approaches, interploidy hybridizations such as 2x–3x, or reciprocal, and 3x–4x crossings are necessary. Fortunately, it has been possible to find allotriploid genotypes of ALA as well as AOA which can be used for further crossing. One important virtue of allotriploids is that they can produce haploid, diploid as well as aneuploid gametes that are viable. Because of this it is possible to generate diploid (or near 2x) and tetraploid (or near 4x) progenies by making either 2x–3x or 3x–4x crosses respectively. Similar to allotriploids, it is also possible to produce progenies on a large scale by crossing allopentaploid with a diploid, i.e., 2x–5x cross, because the embryo: endosperm ploidy levels can be viable in certain combination (**Table 1**). This shows that odd-polyploidy may not be a bottle neck for using them as parents. However, one general phenomenon observed in the backcross progenies of such odd-polyploid crosses is that the chromosomes of the Asiatic parent will be predominant. So much, that almost all chromosomes of the other alien genomes of Longiflorum as well as Oriental are selected out from the progenies (**Fig. 1**). In some cases the progenies of odd-polyploids do produce plants with a balanced euploid chromosome number but the parental genomes are not represented in the expected proportion. Such euploids are known as 'pseudoeuploids' (**Fig. 2**). The significance of such pseudoeuploids is that a part of the chromosome complement will be a hybrid between the two parents but another part of the same complement will not show hybridity. This means there is a potential for genetic
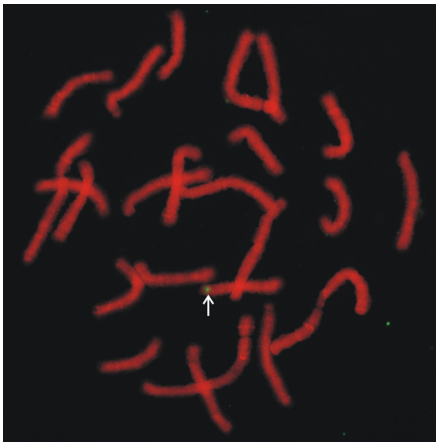
Fig. 1 Chromosome complement of a diploid (2n=2x =24) progeny plant derived from crossing a diploid Asiatic parent with an allotripolid with 24 A+ 12 L chromosomes (AA x LAA). The arrow indicates the segment of Longiflorum chromosome, the only alien segment of Longiflorum still surviving in the progeny.
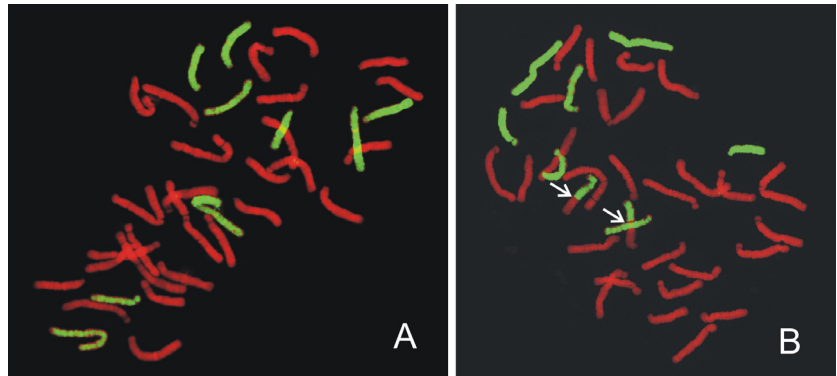

Fig. 2A, 2B. Chromosome complements of two triploid progenies (2n=3x=36) derived from 2x-5x crosses of AA (12A) x ALALA (24L+36A). The triploids, despite possessing a balanced euploid chromosome number of 36, the L genome (green fluorescence) is represented in both cases with only nine chromosomes. These are pseudoeuploids in which hybridity is present for only a part of the chromosome complement. 2A has no recombinant chromosomes whereas in 2B there are two recombinant chromosomes (arrows).

variation in the progenies from odd-polyploids. Thus, apart from intergenomic recombination and chromosome substitutions that might result from the use of *2n* gametes (Chapter by Barba-Gonzalez, in this series), there are other modes of origin of genetic variation in the interploidy crosses of lily.

The difficulties of crossing genotypes with different ploidy levels have been well recognized in several plant species. But, as was mentioned earlier, the situation in lily is unique in the sense that the genomes possess enormous amounts of DNA as compared to other plant species, such as potato for example. And secondly, the embryo sac is of tetrasporic 8-nucleate type which gives rise to much higher ploidy level in its endosperm. This also leads to extremely high disparity in the amount of DNA between the embryo and the endosperm as compared to the monosporic type such as potato. In spite of these differences, there is a tendency to explain the failure of interploidy crosses in lily based on the phenomena observed in other plant species. For example, the so-called 'triploid block', endosperm balance number', among others, are invoked in the case of lily. It should be emphasized that what is applicable in the case of potato and other plants may not be valid in the case of lily.

As regards the ploidy levels of ornamental plants, there is an interesting view that the plant breeders have selected (unconsciously) genotypes with 4C, DNA values corresponding to about 100-120 pg per cell (Brandham 1995). Some examples are: *Narcissus* (4x), *Hyacinthus* (2x, 3x) and *Tulipa* (2x). In the case of lily it is yet known whether triploid or tetraploid cultivars are superior although both of these ploidy levels are under cultivation at present. Considering the 1C value 35-36 pg of DNA in *Lilium* species (Bennett and Smith 1976, 1991), the optimum levels in lily cultivars may be attained at the triploid level. Indeed, triploids appear to be predominant among the cultivars derived from LA hybrids in the Netherlands (Zhou 2007; Zhou *et al.* 2008). However, a more detailed survey on the polyploidy cultivars of lily is needed in order to determine the optimum ploidy level that is required.

4. CONCLUSION

In conclusion, it may be pointed out that the following technical developments have facilitated more efficient breeding of interspecific polyploids: embryo and ovule culture methods to make interspecific hybrids and their progenies on a large scale; rapid determination of ploidy levels of large numbers of progeny through flow cytometric method and, finally*, in situ* DNA hybridization techniques for an accurate analysis of the hybrids and their progenies. Although the present methods of breeding at the polyploidy level may not be too different from the traditional approaches, which produced only modest results in the past, the techniques available at present have opened new perspectives for breeding. Lily serves as a model plant to produce new cultivars through the application of the new techniques.

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REFERENCES

Barba-Gonzalez R, Van Silfhout AA, Visser RGF, Ramanna MS, Van Tuyl JM (2006) Progenies of allotriploids of Oriental × Asiatic lilies (*Lilium*) examined by GISH analysis. *Euphytica* **151**, 243-250

Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **274**, 227-274

Bennett MD, Smith JB (1991) Nuclear DNA amounts in angiosperms. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **334**, 309-345

Brandham PE (1982) Inter-embryo competition in the progeny of autotriploid *Aloineae* (Liliaceae). *Genetica* **59**, 29-42

Brandham PE, Fraser E, West JP (1995) DNA amount, polyploidy and selection of cultivars of decorative monocotyledons. In: Brandham PE, Bennett MD (Eds) *Proceedings of the Kew Chromosome Conference IV*, Kew Publishing, Kew, pp 37-46

Darlington CD (1967) *Chromosome Botany and the Origin of Cultivated Plants*, George Allen and Unwin Ltd., London, 231 pp

Evans A (1921) *The Palace of Minos at Knossos* (Vol I), MacMillan and Co., London, 603 pp

Evans A (1930) *The Palace of Minos at Knossos* (Vol III), MacMillan and Co, London, p 131 fig. 86

Khush GS (1973) *Cytogenetics of Aneuploids*, Acadmic Press, New York, 301 pp

Lim KB, Ramanna MS, De Jong JH, Jacobsen E, Van Tuyl JM (2003) Evaluation of BC2 progenies derived from 3x-2x and 3x-4x crosses of *Lilium* hybrids: a GISH analysis. *Theoretical and Applied Genetics* **106**, 568-574

Maheshwari P (1971) *An Introduction to the Embryology of Angiosperms*, McGraw-Hill Book Company, Inc, New York, 453 pp

Ramsey J, Schemske DW (2002) Neopolyploidy in flowering plants. *Annual Review of Ecology and Systematics* **33**, 589-639

Schmitzer E (1991) A survey of named polyploid lilies of the Asiatic section. *Quarterly Bulletin of the North American Lily Society* **45**, 6-12

Van Tuyl JM (1989) Research on mitotic and meiotic polyploidisation in lily breeding. *Herbertia* **45**, 97-103

Van Tuyl JM, Lim K-B, Ramanna MS (2002) Interspecific hybridization and introgression. In: Vainstein A (Ed) *Breeding for Ornamentals: Classical and Molecular Approaches*, Kluwer Academic Publishers, Dordrecht, pp 85-103

Woodcock HBD, Stearn WT (1950) *Lilies of the World. Their Cultivation and Classification*, Country Life Ltd., London, pp 15-22

Zhou S (2007) Intergenomic recombination and introgression breeding in Longiflorum × Asiatic lilies (*Lilium*). PhD Thesis, Wageningen University, the Netherlands, 111 pp

Zhou S, Ramanna MS, Visser RGF, Van Tuyl JM (2008) Genome composition of triploid lily cultivars derived from sexual polyploidization of Longiflorum × Asiatic hybrids (*Lilium*). *Euphytica* **160**, 207-215