POLYPLOIDY AND HYBRIDISATION IN THE *RORIPPA* X *ANCEPS* HYBRID COMPLEX

POLYPLOIDY AND HYBRIDISATION IN THE *RORIPPA* X *ANCEPS* HYBRID COMPLEX

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Marc Stift

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Promotiecommissie*:*

Promotor:	Prof. dr. P. H. van Tienderen
Co-promotor:	Dr. J. G. B. Oostermeijer
Overige leden:	Prof. dr. S. B. J. Menken
	Prof. dr. M. K. H. Veith
	Prof. dr. J. H. D. Wolf
	Dr. B. K. Mable
	Dr. E. J. W. Visser

Faculteit der Natuurwetenschappen, Wiskunde en Informatica

Instituut voor Biodiversiteit en Ecosysteem Dynamica

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CHAPTER 1

General introduction

Hybridisation – evolutionary dead-end or driving force?

For decades, there has been controversy over the significance of the phenomenon of hybridisation in evolution. Many see hybrids as an accidental by product of sexual reproduction that will be wiped out by selection due to hybrid inferiority or hybrid breakdown:

> '*There is overwhelming evidence that hybrids between closely related species generally have lower fitness than their parents. With the possible exception of allopolyploidy, this should render speciation through hybridization an improbable event.*'
> Schemske 2000, pp1072

The opposing view is mainly advocated in the plant literature, inspired mostly by well-studied examples of hybrid speciation in Louisiana irises (Arnold 1997) and sunflower (Rieseberg 1997). Schemske emphasised that human disturbance triggered hybridisation in these cases and subsequently claimed:

> '*There is now little evidence that hybrid speciation is a significant mechanism of speciation in undisturbed natural populations.*'
> Schemske 2000, pp1073

While Schemske meant to underscore the exceptional status of hybridisation, this point actually provides a very good argument for the reverse: disturbance is actually far from exceptional. Whether or not related to human action, there are innumerable processes and chance events that cause various levels of disturbance, e.g. grazing of herbivores, tidal cycles,

wave and ice action along shores, meandering rivers, urban development, heavy precipitation, drought spells, landslides, hurricanes, seasonal and diurnal fluctuations, road construction, et-cetera. If hybridisation is indeed associated with disturbance, it need not be an infrequent process. In the following paragraphs I will provide some background on the process of hybridisation, and elucidate under which conditions it could be an evolutionary driving force.

Hybridisation

In the broadest sense, the offspring of any two cross-fertilized individuals could be considered hybrids. Generally, the terms 'hybrid' and 'hybridisation' are applied to crosses between individuals belonging to different species (Stebbins 1950). This may seem straightforward, but in fact it is a rather problematic definition, because it depends on the definition of species, which has been the subject of endless debate among scientists for decades. The definition of hybridisation that will be used in this thesis follows Grant's formulation:

> '*Interbreeding between populations which have undergone a previous history of divergence to the level of disjunct races, semispecies, or species, and which are separated by partial ecological or reproductive isolation or both*'
> Grant 1981, pp195

The consequences of hybridisation depend on three factors: (i) the likelihood of the formation of hybrids between two parental lineages, (ii) their fitness with respect to the parents and (iii) the degree of reproductive isolation between parents and hybrids.

(i) Formation of hybrids

If there is little or no opportunity for hybrid formation (separation in time or space, different habitats, different pollinators, etc), and the viability and/or fertility of hybrids is low, it seems reasonable to assume that hybrids are literally a dead-end. If hybrids are strongly selected against and produced at sufficient frequency to be formed at the expense of non-hybrid offspring, hybridisation may result in what has been called 'reinforcement' (Dobzhansky 1937). Reinforcement is defined as the action of natural selection to increase reproductive isolation between diverged lineages by the evolution of traits that prevent hybridisation (e.g.,

Servedio & Noor 2003). If selection against hybrids is strong and gene flow is limited, reinforcement could eventually lead to speciation of the parental lineages involved. While theoretically sound, there is little evidence for reinforcement speciation under natural conditions (Servedio & Noor 2003), but see Hoskin *et al.* (2005) for an example of reinforcement driving rapid allopatric speciation. The rarity of reinforcement speciation has been attributed to the weakening of reinforcing selection in systems with multiple mating (Marshall *et al.* 2002) and the countering effects of gene flow (Servedio & Kirkpatrick 1997).

(ii) Fitness

If hybrids can establish frequently, hybrids could have a lower average fitness than their parents, e.g. due to the breaking up of co-adapted gene complexes (Dobzhansky & Pavlovsky 1958). However, even if the mean hybrid fitness is low, some hybrid genotypes may be as fit or even fitter than the parents (Arnold 1992; Arnold & Hodges 1995; Arnold 1997). Moreover, heterosis may lead to hybrid vigour (Rieseberg & Carney 1998). Although heterosis normally breaks down in later generations, further hybrid generations could also give rise to transgressive phenotypes (phenotypes with trait values beyond the range of phenotypes present in the parents), some of which may have an increased fitness under particular environmental conditions (Rieseberg *et al.* 1999).

(iii) Reproductive isolation between hybrids and parental lineages

If hybrids are not reproductively isolated from the parental species, hybrids may form a bridge between the parental species through backcrossing. This could lead to gene exchange in successive generations: *introgression* (Anderson 1949; Rieseberg & Wendel 1993). Even if hybrids establish rarely, introgression can be substantial (Arnold *et al.* 1999). The creation of open spaces by natural or human disturbance (Rieseberg & Carney 1998), or the availability of suitable hybrid habitat (Anderson 1948) can further promote establishment of hybrids and introgression. This may eventually lead to the formation of a hybrid swarm, in which introgression has led to a range of intermediate types bridging the two hybridising species. Introgression of traits could be unilateral due to genetic incompatibilities (Keim *et al.* 1989) or if pollen transfer to one of the parental species is more likely to occur, for example if the hybrid habitat overlaps with only one of the parent habitats (Anderson & Hubricht 1938).

If hybrids are reproductively isolated from their parents, this may lead to hybrid speciation. There are two main ways by which reproductive isolation and ultimately hybrid speciation can arise. First, hybrids can become instantly isolated from their parents by allopolyploidisation, the situation in which hybrids become polyploid (treated in more detail below). Secondly, in a homoploid situation (i.e., both parents and their hybrids are diploids), ecological and karyotypic divergence may lead to reproductive isolation of newly formed hybrids and eventually to ecological and recombinational speciation (Arnold 1992; Rieseberg 1997; Buerkle *et al.* 2000). In plants, ecological divergence could be related to changes in hybrid flower morphology or phenology (e.g., reviewed in Rieseberg 1997). Hybrids may also possess a suite of traits that allows them to grow in a different habitat (Welch & Rieseberg 2002). Karyotypic divergence caused by chromosomal rearrangements in early generation hybrids (reviewed in Rieseberg 1997) may impose a chromosomal sterility barrier between later generation hybrids and their parents.

The role of hybridisation in evolution

It will have become apparent from the previous theoretical considerations, that hybridisation can play a role in evolution. But how important is it? Lotsy (1916) proposed a dominant role for hybridisation in evolution:

> '*Crossing (…) is the cause of the origin of new types, heredity perpetuates them, selection is the cause* – not of their origin as was formerly supposed – *but of their extinction*'
> Lotsy 1916, pp134

Even though '*a complete list of natural hybrids and hybrid swarms in plants would run from* Achillea *to* Zinnia' (Grant 1981, pp197), Lotsy's theory of '*Evolution by means of hybridisation*' has received little support, not even among botanists, although in plants the numerous examples of extensive introgression and hybrid speciation suggest that hybridisation at least has to be considered as '*a third major evolutionary process*' besides mutation and selection (Stebbins 1950, pp251). In animals, the general view is that hybridisation is less important (but see Dowling & Secor 1997; Seehausen 2004). This may have to do with the fact that animals are less prone to form polyploids (Grant 1981).

However, in the next paragraph, it will become clear that polyploidy and allopolyploid hybrid speciation may not be as rare in animals as was previously thought.

Given this background on hybridisation and the opposing views regarding the potential evolutionary significance of the process, it becomes necessary to actually test whether hybrids form an evolutionary dead-end or not, and what role is played by reproductive isolation. If hybrids do not become isolated from their parents, introgressive hybridisation may shape the variation of existing lineages and potentially lead to combinations of traits that would have been impossible otherwise. If hybrids do become reproductively isolated, hybridisation may lead to speciation, and isolation could be promoted by ecological divergence, chromosomal rearrangements and allopolyploidisation. The latter probably provides the most straightforward means of hybrid speciation, and is therefore discussed in more detail in the next section.

Polyploidisation

Polyploidisation is considered to play an important role in evolution, especially in plants, but also in animals (Otto & Whitton 2000). In higher plants, it has been estimated that approximately 2-4% of speciation events in angiosperms involve polyloidisation (Otto & Whitton 2000) and that up to 70% of all angiosperms have a history of polyploidy (Masterson 1994, and reviewed in Soltis *et al.* 2004). Polyploid incidence appears to be much rarer in animals, although there are many examples of ancient and recent polyploidisation in insects, fish and amphibians (Otto & Whitton 2000). Polyploidy was long considered a difficult evolutionary transition for animals due to disruption of the sex determination system (Muller 1925) or due to disruption of the dosage compensation between genders (Orr 1990). This dominant view may have inhibited researchers from discovering polyploidy in animals (Mable 2004b). Recently a tetraploid mammal was discovered (Gallardo *et al.* 1999) and evidence has accumulated that the vertebrates underwent at least one ancient polyploidisation event (reviewed in Wolfe 2001).

Polyploid classification

Polyploids are normally subdivided in (i) autopolyploids and (ii) allopolyploids (Kihara and Ono 1926, as cited in Ramsey & Schemske 1998). Stebbins (1947) introduced the term (iii) segmental allopolyploidy for intermediate cases.

(i) Autopolyploids

Autopolyploids have their origin within a species and the copies of each chromosome are therefore homologous (e.g., $A_1A_2A_3A_4$). Each chromosome may then pair randomly with any of its homologues (random bivalent pairing), or, alternatively, multivalent pairing may occur during meiosis. The latter will lead to polysomic inheritance (Soltis & Soltis 1993), i.e. all possible allelic combinations are produced in equal frequencies (Muller 1914).

(ii) Allopolyploids

Allotetraploids originate from different species and are associated with hybridisation. The copies of each chromosome therefore consist of two homeologous sets of homologous chromosomes (e.g., $A_1A_2B_1B_2$). If each chromosome preferentially pairs with its homologue during meiosis, this will lead to disomic inheritance (Soltis & Soltis 1993) and fixed heterozygosities.

(iii) Segmental allopolyploids

Stebbins (1947) recognized that autotetraploidy and allotetraploidy are the extreme ends of a range, and introduced the term segmental allopolyploidy for intermediate cases. According to Stebbins' definition, segmental allopolyploids may show disomic inheritance for some loci, and tetrasomic inheritance for other loci. There is no reason however why the inheritance could not be intermediate between disomic and tetrasomic for a given locus (Grant 1981). This can be realised if the homeologous chromosomes are similar enough to allow occasional non-homologous pairings, but divergent enough to make pairing completely random. Stebbins (1950), and later Sybenga (1996) suggested that such an intermediate condition would not be stable. Either crossing-over and intergenomic recombination would quickly homogenise the chromosomes in later generations, or lowered viability of intergenomically recombined gametes would promote selection for mechanisms that prevent non-homologous pairing.

Polyploid formation

Polyploids can arise by somatic doubling in the zygote or early embryo, or through gametic nonreduction (failure of cell wall formation during meiosis). The latter is considered the most common way of polyploidisation (Karpechenko 1927; Harlan & deWet 1975; Bretagnolle & Thompson 1995). Unreduced gametes are formed at low frequency by many plants (reviewed in Bretagnolle & Thompson 1995), and environmental conditions (e.g., extreme temperature fluctuations) can increase their frequency (Ramsey & Schemske 1998). Since the chance of fusion of two unreduced gametes is rather low, polyploidisation may require an intermediate triploid generation (Harlan & deWet 1975). Such triploids are often partially sterile, but may occasionally produce viable unreduced (i.e., triploid) gametes, that can fuse with normal haploid gametes to form a tetraploid.

Polyploid establishment and further evolution

The successful establishment of newly formed polyploids in sexual species is difficult due to the minority cytotype exclusion principle (Levin 1975). Even though the consensus today is that polyploids are formed multiple times (Soltis & Soltis 1993; 2000), newly formed polyploids are most likely outnumbered by their diploid relatives and will therefore lack compatible partners. Evolution of self-compatibility would provide one way to overcome this barrier to establishment. However, an extensive review did not show a strong association between self-compatibility and polyploidy (Mable 2004a). Several other aspects may contribute to the successful establishment of newly formed polyploids.

First, a perennial or asexual habit will help survival in the initial stages of polyploid establishment (Stebbins 1950). Particularly if newly formed polyploids can survive and spread asexually for several generations, this may compensate for the rarity of their formation. Especially in small populations, this means that the minority cytotype disadvantage may not be so severe. Second, diploid ancestors may not be completely incompatible as partners, especially if they produce unreduced gametes (Marks 1966; Otto & Whitton 2000; Mable 2004b). If unreduced gametes led to the formation of the polyploid in the first place, it is not unlikely that they also provide a bridge between diploids and tetraploids. Third, polyploids are theoretically less affected by inbreeding depression than diploids, since deleterious recessive alleles may be effectively masked at the gamete and polyploid stages (Yadegari & Drews 2004). Fourth, polyploids may occupy a niche different from that of their ancestor, thereby escaping competition with their ancestors (e.g., Segraves

& Thompson 1999; Hardy *et al.* 2000; Husband & Schemske 2000; Johnson *et al.* 2003), and avoiding the effects of the minority cytotype disadvantage. Gene redundancy may add to the diversification of polyploids from their ancestors since the presence of duplicates of all genes offers great potential for sub- or neo-functionalisation (e.g., Adams & Wendel 2005; Moore & Purugganan 2005). Fifth, polyploids may have a selective advantage over diploids. It was suggested that polyploids may be better colonisers or have greater ecological adaptability, for example to cope with extreme climatic fluctuations (e.g., reviewed by Stebbins (1950) and Ramsey & Schemske (2002)). A greater genetic variability has been suggested as a factor underlying these selective advantages (Stebbins 1984; 1985; Soltis & Soltis 2000). In allopolyploids, such increased genetic variance can be attributed to their hybrid origin and the fixed heterozygosity (Soltis *et al.* 2004). In autopolyploids, continuous formation and gene exchange with diploid ancestors could be a source of variation of newly formed autopolyploids. However, none of these mechanisms could lead to levels of variation beyond that of diploid ancestors. Hybridisation after polyploidisation may provide an important additional source of variation for autotetraploids. Particularly closely related polyploid species from which diploid counterparts are normally reproductively isolated are candidates for such hybridisation events (Grant 1981). There is growing evidence that autopolyploidy is much more common than previously thought. A few studies have reported higher levels of diversity in outcrossing autotetraploids than in their diploid relatives (Soltis & Soltis 1989; Brown & Young 2000; Hardy & Vekemans 2001).

The previous paragraphs were mainly intended to provide some theoretical background to understand the work presented in the rest of this thesis. I intended to show that, although the success of polyploids is largely attributed to the hybrid origin and fixed heterozygosity of allopolyploids, autopolyploids may play a role in evolution as well. Autopolyploids do not have fixed heterozygosity but can theoretically still have increased genetic variability. The question arises under what conditions autopolyploidy may constitute an important evolutionary driving force.

General questions

In the first section, I presented introgressive hybridisation as a potential outcome of hybridisation. Numerous studies have documented posthoc evidence of introgressive

hybridisation in natural hybrid swarms. Introgression can be bilateral - e.g., between *Iris fulva* and *I. hexagona* (Arnold *et al.* 1991), between *Gossypium barbadense* and between *G. hirsutum* (Brubaker *et al.* 1993) and between *Rorippa amphibia* and *R. sylvestris* (Bleeker & Hurka 2001) - or unilateral - e.g., between *Rorippa palustris and R. amphibia* (Bleeker & Hurka 2001) and between *Populus fremontii* and *P. angustifolia* (Keim *et al.* 1989). What conditions favour bilateral or unilateral introgression? Is the direction of introgression dependent on the genetic compatibilities of the hybridising genomes? Do ecological factors play a role in determining the direction of introgression? Do cases of unilateral introgression imply that some genomes are more 'susceptible' to introgression than others (Martinsen *et al.* 2001)?

In the section on polyploidy, I referred to Stebbins' (1984; 1985) hypothesis that an increased genetic variance may underlie the evolutionary success of polyploids. Allopolyploids inherently possess an increased genetic variance owing to their hybrid origin. How could autopolyploids attain genetic variation? Could autopolyploidy be a more important evolutionary mechanism than previously thought? According to Stebbins (1947), autopolyploids and allopolyploids are the extremes of a range. He defined intermediate cases as segmental allopolyploids, that are characterised by a combination of auto- and allotetraploid characteristics (i.e. disomic and tetrasomic inheritance) on different genomic segments. Are individual chromosomal segments exclusively inherited in either a disomic or polysomic fashion? Or is intermediate inheritance possible? Would such a condition be unstable as suggested by Stebbins (1950) and Sybenga (1996)?

The study species: *Rorippa amphibia* and *R. sylvestris*

The genus *Rorippa* (Scop.) has a circumpolar, holarctic distribution. Most species were originally described by Linnaeus within the genus *Sisymbrium*. Scopoli (1760) introduced the generic name *Rorippa*, for which *R. sylvestris* should be considered the holotype (reviewed by Jonsell 1968, pp145). *Rorippa* belongs to the tribe Cardamineae within the Brassicaceae family (Al-Shehbaz *et al.* 2006). This tribe is part of the same phylogenetic lineage (I) as the genomics model species *Arabidopsis thaliana* (Beilstein *et al.* 2006), which provides excellent opportunities for comparative genomics. The native European lowland species have their closest relatives in western Asia and Siberia (Bleeker *et al.* 2002) and are represented by

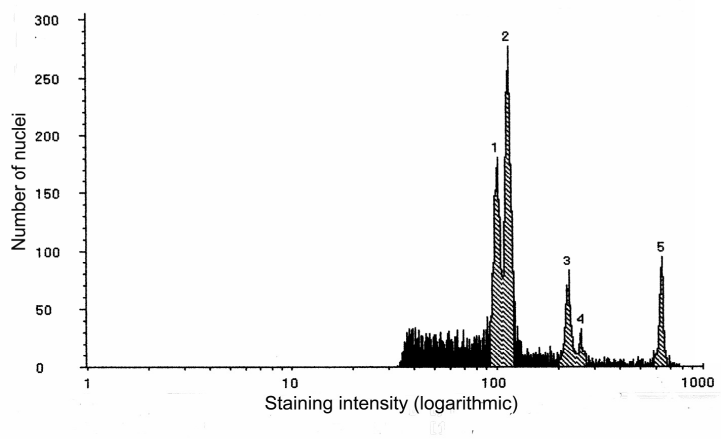

Figure 1.1. Histogram of genome sizes (inferred from DAPI-stained nuclei using flow cytometry) of a mixed sample of tetraploid *Rorippa amphibia* and *R. sylvestris* leaves. Peak 1 represents tetraploid *R. sylvestris*, peak 2 tetraploid *R. amphibia*. Peak 3 and 4 are due to endoreplication of *R. sylvestris* and *R. amphibia*, respectively. Peak 5 represents an external standard (trout erythrocytes).

Rorippa palustris (Linnaeus) Besser, *R. austriaca* (Crantz) Besser, *R. amphibia* (Linnaeus) Besser and *R. sylvestris* (Linnaeus) Besser. The latter two species are the focus of this thesis. They commonly occur along all major rivers in Europe, with exception of the far north and Mediterranean (Jonsell 1968; Jalas & Suominen 1994, personal observations). The species often occur in sympatry, particularly along rivers that are not canalised and have wide floodplains with fluctuating water levels. Their specific distribution within river floodplains suggests that the species are locally adapted to micro-habitats with different flooding regimes (Blom 1999). *R. amphibia* occurs in sites with more stagnant water (ponds, small lakes, swamps). It often has an emergent habit, or is associated with dense vegetation (e.g., reed beds or wet to moist grassland). *R. sylvestris* occurs in more ephemeral sites that can be flooded for longer or shorter periods throughout the year, but can also dry out completely in summer (Jonsell 1968; Blom 1999, personal observations). Both species are self incompatible perennials (i.e., they are obligate outcrossers) and can regenerate vegetatively from root and stem fragments, albeit that *R. sylvestris* does so more vigorously. This makes *R. sylvestris* a noxious weed in agricultural fields, gardens, and along roadsides far beyond its natural riverside habitat (Jonsell 1968, personal observations).

Morphologically, the species can be distinguished easily. *R. amphibia* has large leaves with big endlobes, while *R. sylvestris* has smaller, more 'pinnatisect' (i.e., almost pinnate) leaves (Jonsell 1968). Both species have polyploid forms. *R. amphibia* has diploid and tetraploid forms, *R. sylvestris* has tetraploid and hexaploid forms (Jonsell 1968). Moreover, based on flow cytometry, *R. amphibia* appears to have a ~16% larger genome (Figure 1.1).

Figure 1.2. Sample locations of diploid and tetraploid *Rorippa amphibia* (top), and tetraploid and hexaploid *R. sylvestris* (bottom).

Within species, there is some overlap in geographic distribution among the cytotypes. Diploid *R. amphibia* have been found in southern Europe and tetraploids in central and northwestern Europe (Figure 1.2 and Jonsell 1968) and are indistinguishable on the basis of morphology (Jonsell 1968). There are contact zones in England, France and southern Germany (Figure 1.2) and triploids have been found in England and France (data not shown). The morphological resemblance of diploid and tetraploid *R. amphibia* and the absence of any similar diploid relatives (Bleeker *et al.* 2002), suggest an autotetraploid origin of tetraploids. Tetraploid *R. sylvestris* occur throughout the species range, but are more abundant in central and eastern Europe (Jonsell 1968, personal observations). Hexaploid *R. sylvestris* has been mainly found in northern Europe (Netherlands, northern Germany, England and Scandinavia, Figure 1.2, Jonsell 1968), but the observation of hexaploids in Portugal (Figure 1.2) suggest an Atlantic distribution. However, sampling was too limited to draw definite conclusions. Pentaploids have been found occasionally throughout the hexaploid range (except in Portugal). Plants from agricultural fields and gardens in the Netherlands were often pentaploid or hexaploid (data not shown), confirming Jonsell's (1968) notion that hexaploids are more weedy than tetraploids.

At the tetraploid level *R. amphibia* and *R. sylvestris* are interfertile. The hybrid *Rorippa* x *anceps* (Wahlenberg) Reichenbach can easily be generated by hand pollination. First generation (F1) hybrids have an intermediate leaf morphology, are fertile and backcross easily with both parents (Jonsell 1968). Occasional putative hybrids have been described from many localities throughout Europe (Jonsell 1968). Natural hybrids were found around Lake Mälären in Sweden (Jonsell 1968), and more recently along the rivers Elbe in Germany (Bleeker & Hurka 2001), Vistula in Poland and Dnepr in Ukraine (personal observations). In these locations, a range of intermediate morphologies suggested that backcrossing had led to the formation of natural hybrid swarms. Molecular markers (AFLP, chloroplast *trn*L/F spacer sequences) have confirmed the morphological indications of introgressive hybridisation along the Elbe (Bleeker & Hurka 2001; Bleeker & Matthies 2005).

This thesis

The presence of different ploidy levels and the evidence of hybridisation in the genus *Rorippa* give rise to several questions related to polyploidy. Are polyploids in *R. amphibia* and *R. sylvestris* indeed autotetraploid? Do hybrids between tetraploids behave as allotetraploids showing disomic inheritance? Or are the species so similar that hybrids show tetrasomic inheritance? Or do hybrids behave as segmental allopolyploids in the classical sense, with some loci showing disomic and others tetrasomic inheritance? Or is inheritance intermediate for some or all loci? These questions have been addressed in Chapter 2, in which we analysed the segregation of microsatellite markers in the progeny of crosses involving natural tetraploids and artificial F1 hybrids. A technical note on the development of primers for these neutral markers is presented in Chapter 6.

Traditional chi-square based approaches could only test whether disomic or tetrasomic inheritance could be rejected. To distinguish between models of inheritance, we developed a new likelihood-based approach that incorporates the possibility of intermediate inheritance, i.e., intermediate between disomic (~allotetraploid) and tetrasomic (autotetraploid). Intermediate inheritance may have important repercussions for the calculation of population genetic parameters and linkage mapping in tetraploids. Currently available methods assume either disomic or tetrasomic inheritance, and may not be appropriate if these assumptions are violated.

The fact that tetraploids in *R. amphibia* are widespread and have a more northern distribution than diploids raises the question what underlies the apparent success of tetraploids in this species. Could an increased genetic diversity underlie this pattern? In Chapter 3 we compare microsatellite variation of a sample of diploid and tetraploid *R. amphibia* from different localities throughout the north-western part of their distribution ranges. We specifically test whether levels of variation deviate from the predictions of neutral theory (i.e., larger genetic variation in tetraploids). Are levels of variation lower or higher than expected assuming an equilibrium between mutation and drift? If tetraploids have a recent origin, in what way could they have attained genetic variation?

The latter question is addressed by a crossing experiment that is discussed in Chapter 4. Is gene flow with conspecific diploids a likely source of variation in tetraploids? And if so, is such gene flow more likely through recurrent formation, or through triploid intermediates or unreduced gametes? Is introgressive hybridisation through hybridisation with tetraploid *R. sylvestris* and backcrossing a likely source of variation? Is backcrossing equally likely with both species? We specifically tested to what extent reproductive barriers exist between diploids and tetraploids of *R. amphibia*, between tetraploid *R. amphibia* and *R. sylvestris* and if there are any barriers to backcrossing. We expected to find postzygotic isolation between diploids and tetraploids due to genomic imbalances. We screened the ploidy of a subset of the progeny of all crosses, allowing for the identification of unreduced gametes. These could not only provide a source of new polyploid lineages, but may also provide a means of overcoming reproductive barriers between diploids and tetraploids. Based on the presence of hybrid swarms under natural conditions, we expected to find no barriers to crossing between the species and to backcrossing. The absence of such barriers may suggest that introgression is bidirectional. But could the ecological position of hybrids with respect to that of the parents limit the possibility of backcrossing to one or both parents? We address this question in Chapter 5. *R. amphibia* and *R. sylvestris* have a different ecological position with respect to flooding. We quantified the reactions of *R. amphibia*, *R. sylvestris* and F1 hybrids in coping with different flooding regimes. In the first place, this may reveal the most likely habitats for hybrids. Is hybrid trait expression intermediate or extreme relative to that of the parents, so that it could be conducive for ecological divergence of hybrids? Do hybrids resemble one of the parents, so that unidirectional introgression becomes more likely? In the second place, with the possibilities for comparative genomics in mind, this provides a first step towards the use of *Rorippa* as a model system to unravel the genetic basis of flooding adaptation and species divergence.

References

Adams, K.L. & Wendel, J.F. (2005) Polyploidy and genome evolution in plants. *Current Opinion in Plant Biology*, **8**, 135-141.

Al-Shehbaz, I.A., Beilstein, M.A. & Kellogg, E.A. (2006) Systematics and phylogeny of the Brassicaceae (Cruciferae): an overview. *Plant Systematics and Evolution*, **259**, 89-120.

Anderson, E. (1948) Hybridization of the habitat. *Evolution*, **2**, 1-9.

Anderson, E. (1949) *Introgressive hybridization*. John Wiley and Sons, Inc.; Chapman & Hall, Ltd, New York, London.

Anderson, E. & Hubricht, L. (1938) Hybridization in *Tradescantia*. III The evidence for introgressive hybridization. *American Journal of Botany*, **25**, 396-402.

Arnold, M.L. (1992) Natural hybridization as an evolutionary process. *Annual Review of Ecology and Systematics*, **23**, 237-261.

Arnold, M.L. (1997) *Natural hybridization and evolution*. Oxford University Press, Oxford, UK.

Arnold, M.L., Buckner, C.M. & Robinson, J.J. (1991) Pollen-mediated introgression and hybrid speciation in Louisiana Irises. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 1398-1402.

Arnold, M.L., Bulger, M.R., Burke, J.M., Hempel, A.L. & Williams, J.H. (1999) Natural hybridization: How low can you go and still be important? *Ecology*, **80**, 371-381.

Arnold, M.L. & Hodges, S.A. (1995) Are natural hybrids fit or unfit relative to their parents. *Trends in Ecology & Evolution*, **10**, 67-71.

Beilstein, M.A., Al-Shehbaz, I.A. & Kellogg, E.A. (2006) Brassicaceae phylogeny and trichome evolution. *American Journal of Botany*, **93**, 607-619.

Bleeker, W. & Hurka, H. (2001) Introgressive hybridization in *Rorippa* (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. *Molecular Ecology*, **10**, 2013-2022.

Bleeker, W. & Matthies, A. (2005) Hybrid zones between invasive *Rorippa austriaca* and native *R-sylvestris* (Brassicaceae) in Germany: ploidy levels and patterns of fitness in the field. *Heredity*, **94**, 664-670.

Bleeker, W., Weber-Sparenberg, C. & Hurka, H. (2002) Chloroplast DNA variation and biogeography in the genus *Rorippa* Scop. (Brassicaceae). *Plant Biology*, **4**, 104-111.

Blom, C.W.P.M. (1999) Adaptations to flooding stress: From plant community to molecule. *Plant Biology*, **1**, 261-273.

Bretagnolle, F. & Thompson, J.D. (1995) Tansley Review No-78 - Gametes with the somatic chromosome-number - mechanisms of their formation and role in the evolution of autopolyploid plants. *New Phytologist*, **129**, 1-22.

Brown, A.H.D. & Young, A.G. (2000) Genetic diversity in tetraploid populations of the endangered daisy Rutidosis leptorrhynchoides and implications for its conservation. *Heredity*, **85**, 122-129.

Brubaker, C.L., Koontz, J.A. & Wendel, J.F. (1993) Bidirectional cytoplasmic and nuclear introgression in the new-world cottons, *Gossypium barbadense* and *G. hirsutum* (Malvaceae). *American Journal of Botany*, **80**, 1203-1208.

Buerkle, C.A., Morris, R.J., Asmussen, M.A. & Rieseberg, L.H. (2000) The likelihood of homoploid hybrid speciation. *Heredity*, **84**, 441-451.

Dobzhansky, T. (1937) *Genetics and the origin of species*. Columbia University Press, New York.

Dobzhansky, T. & Pavlovsky, O. (1958) Interracial hybridization and breakdown of coadapted gene complexes in *Drosophila paulistorum* and *Drosophila willistoni*. *Proceedings of the National Academy of Sciences of the United States of America*, **44**, 622-629.

Dowling, T.E. & Secor, C.L. (1997) The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics*, **28**, 593-619.

Gallardo, M.H., Bickham, J.W., Honeycutt, R.L., Ojeda, R.A. & Kohler, N. (1999) Discovery of tetraploidy in a mammal. *Nature*, **401**, 341-341.

Grant, V. (1981) *Plant Speciation*. Columbia University Press, New York.

Hardy, O.J., Vanderhoeven, S., De Loose, M. & Meerts, P. (2000) Ecological, morphological and allozymic differentiation between diploid and tetraploid knapweeds (*Centaurea jacea*) from a contact zone in the Belgian Ardennes. *New Phytologist*, **146**, 281-290.

Hardy, O.J. & Vekemans, X. (2001) Patterns of allozyme variation in diploid and tetraploid *Centaurea jacea* at different spatial scales. *Evolution*, **55**, 943-954.

Harlan, J. & deWet, J.M.J. (1975) On Ö. Winge and a prayer: the origins of polyploidy. *The Botanical Review*, **41**, 361-390.

Hoskin, C.J., Higgie, M., McDonald, K.R. & Moritz, C. (2005) Reinforcement drives rapid allopatric speciation. *Nature*, **437**, 1353-1356.

Husband, B.C. & Schemske, D.W. (2000) Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. *Journal of Ecology*, **88**, 689-701.

Jalas, J. & Suominen, J. (1994) *Atlas Flora Europaeae, Volume 12*., Helsinki.

Johnson, M.T.J., Husband, B.C. & Burton, T.L. (2003) Habitat differentiation between diploid and tetraploid *Galax urceolata* (Diapensiaceae). *International Journal of Plant Sciences*, **164**, 703-710.

Jonsell, B. (1968) Studies in the North-West European species of *Rorippa s. str. Symbolae Botanicae Upsalienses*, **19**, 1-221.

Karpechenko, G.D. (1927) The production of polyploid gametes in hybrids. *Hereditas*, **9**, 349-368.

Keim, P., Paige, K.N., Whitham, T.G. & Lark, K.G. (1989) Genetic analysis of an interspecific hybrid swarm of *Populus* - occurrence of unidirectional introgression. *Genetics*, **123**, 557-565.

Levin, D.A. (1975) Minority cytotype exclusion in local plant populations. *Taxon*, **24**, 35-43.

Lotsy, J.P. (1916) *Evolution by means of hybridization*. Martinus Nijhoff, The Hague.

Mable, B.K. (2004a) Polyploidy and self-compatibility: is there an association? *New Phytologist*, **162**, 803-811.

Mable, B.K. (2004b) 'Why polyploidy is rarer in animals than in plants': myths and mechanisms. *Biol J Linn Soc*, **82**, 453-466.

Marks, G.E. (1966) The origin and significance of intraspecific polyploidy: experimental evidence from *Solanum chacoense*. *Evolution*, **20**, 552-557.

Marshall, J.L., Arnold, M.L. & Howard, D.J. (2002) Reinforcement: the road not taken. *Trends in Ecology & Evolution*, **17**, 558-563.

Martinsen, G.D., Whitham, T.G., Turek, R.J. & Keim, P. (2001) Hybrid populations selectively filter gene introgression between species. *Evolution*, **55**, 1325-1335.

Masterson, J. (1994) Stomatal size in fossil plants - evidence for polyploidy in majority of angiosperms. *Science*, **264**, 421-424.

Moore, R.C. & Purugganan, M.D. (2005) The evolutionary dynamics of plant duplicate genes. *Current Opinion in Plant Biology*, **8**, 122-128.

Muller, H.J. (1914) A new mode of segregation in Gregory's tetraploid Primulas. *American Naturalist*, **48**, 508-512.

Muller, H.J. (1925) Why polyploidy is rarer in animals than in plants. *American Naturalist*, **59**, 346-353.

Orr, H.A. (1990) Why polyploidy is rarer in animals than in plants revisited. *American Naturalist*, **136**, 759-770.

Otto, S.P. & Whitton, J. (2000) Polyploid incidence and evolution. *Annual Review of Genetics*, **34**, 401-437.

Ramsey, J. & Schemske, D.W. (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics*, **29**, 467-501.

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

Rieseberg, L.H. (1997) Hybrid origins of plant species. *Annual Review of Ecology and Systematics*, **28**, 359-389.

Rieseberg, L.H., Archer, M.A. & Wayne, R.K. (1999) Transgressive segregation, adaptation and speciation. *Heredity*, **83**, 363-372.

Rieseberg, L.H. & Carney, S.E. (1998) Plant hybridization. *New Phytologist*, **140**, 599-624.

Rieseberg, L.H. & Wendel, J.F. (1993) Introgression and its consequences in plants. *Hybrid zones and the evolutionary process*. (eds R.G. Harrison). Oxford University Press, New York.

Schemske, D.W. (2000) Understanding the origin of species. *Evolution*, **54**, 1069-1073.

Seehausen, O. (2004) Hybridization and adaptive radiation. *Trends in Ecology & Evolution*, **19**, 198-207.

Segraves, K.A. & Thompson, J.N. (1999) Plant polyploidy and pollination: Floral traits and insect visits to diploid and tetraploid *Heuchera grossulariifolia*. *Evolution*, **53**, 1114-1127.

Servedio, M.R. & Kirkpatrick, M. (1997) The effects of gene flow on reinforcement. *Evolution*, **51**, 1764-1772.

Servedio, M.R. & Noor, M.A.F. (2003) The role of reinforcement in speciation: Theory and data. *Annual Review of Ecology Evolution and Systematics*, **34**, 339-364.

Soltis, D.E. & Soltis, P.S. (1989) Genetic consequences of autopolyploidy in *Tolmiea* (Saxifragaceae). *Evolution*, **43**, 586-594.

Soltis, D.E. & Soltis, P.S. (1993) Molecular data and the dynamic nature of polyploidy. *Critical Reviews in Plant Sciences*, **12**, 243-273.

Soltis, D.E., Soltis, P.S. & Tate, J.A. (2004) Advances in the study of polyploidy since Plant speciation. *New Phytologist*, **161**, 173-191.

Soltis, P.S. & Soltis, D.E. (2000) The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 7051-7057.

Stebbins, G.L. (1947) Types of polyploids: their classification and significance. *Advances in Genetics*, **1**, 403-429.

Stebbins, G.L. (1950) *Variation and evolution in plants*. Columbia University Press, New York & London.

Stebbins, G.L. (1984) Polyploidy and the distribution of the arctic-alpine flora: new evidence and a new approach. *Botanica Helvetica*, **94**, 1-13.

Stebbins, G.L. (1985) Polyploidy, hybridization, and the invasion of new habitats. *Annals of the Missouri Botanical Garden*, **72**, 824-832.

Sybenga, J. (1996) Chromosome pairing affinity and quadrivalent formation in polyploids: Do segmental allopolyploids exist? *Genome*, **39**, 1176-1184.

Welch, M.E. & Rieseberg, L.H. (2002) Habitat divergence between a homoploid hybrid sunflower species, Helianthus paradoxus (Asteraceae), and its progenitors. *American Journal of Botany*, **89**, 472-478.

Wolfe, K.H. (2001) Yesterday's polyploids and the mystery of diploidization. *Nature Reviews Genetics*, **2**, 333-341.

Yadegari, R. & Drews, G.N. (2004) Female gametophyte development. *Plant Cell*, **16**, S133-S141.

CHAPTER 2

Segregation models for disomic, tetrasomic and intermediate inheritance in tetraploids: a general procedure applied to Rorippa (Yellow Cress) microsatellite data

Marc Stift, Camillo Berenos, Peter Kuperus and Peter H. van Tienderen

Abstract

Traditionally, the mode of inheritance in tetraploids is discussed in terms of its two extreme forms: disomic inheritance in allotetraploids, and tetrasomic inheritance in autotetraploids. The latter can be due to the formation of quadrivalents, or random pairing of chromosomes into bivalents, followed by segregation into reduced gametes. The possibility of mixed, or intermediate, inheritance models had been suggested, but methods to test this have not been developed. Particularly in species that are similar enough to allow successful hybridisation, but divergent enough to earn their species status, intermediate inheritance models could apply to newly formed hybrids. We present a simple likelihood-based approach that is able to incorporate disomic, tetrasomic and intermediate inheritance models. Our model shows that inheritance of microsatellite markers in natural tetraploids of *R. amphibia* and *R. sylvestris* is tetrasomic, corroborating their autotetraploid origin as suggested previously. However, in F1 hybrids inheritance was intermediate to disomic and tetrasomic inheritance. In meiosis, chromosomes paired preferentially with the homolog from the same parental species, but not strictly so. Furthermore, the occurrence of double reduction gametes indicated that quadrivalent formation occurs. We also tested the general applicability of our model using published segregation data. In one case, an intermediate inheritance model gave a better fit to the data than the tetrasomic model advocated by the authors. The existence of inheritance intermediate to disomic and tetrasomic has important implications for linkage mapping and population genetics and hence breeding programs of tetraploids. Methods that have been developed for either disomic or tetrasomic tetraploids may not be generally applicable, particularly in systems where hybridisation is common.

Introduction

Polyploidy is considered to be a major evolutionary force in both plants and animals (Otto & Whitton 2000; Soltis & Soltis 2000). In higher plants, it has been estimated that approximately 2-4% of speciation events in angiosperms involve polyploidisation (Otto & Whitton 2000) and that up to 70% of all angiosperms have a history of polyploidy (Masterson 1994), and reviewed in (Soltis *et al.* 2004). Polyploid incidence appears to be much rarer in animals, although there are many examples of recent and ancient polyploidisation events in insects, fish and amphibians (Otto & Whitton 2000, also see Mable 2004). Recent genome analyses indicate that many extant diploids are actually ancient polyploids (Wolfe & Shields 1997; Wolfe 2001; McLysaght *et al.* 2002; Bowers *et al.* 2003). A common mechanism of polyploidisation is through fusion of unreduced gametes (Karpechenko 1927; Harlan & deWet 1975; Bretagnolle & Thompson 1995) from the same or from different species, termed autotetraploidy and allotetraploidy respectively (Kihara and Ono 1926, as cited in Ramsey & Schemske 1998). However, Stebbins (1947) already recognised that autopolyploidy and allopolyploidy are the extreme ends of a range, and introduced the term segmental allopolyploidy for intermediate cases.

In extreme autotetraploids, each chromosome has four homologous versions (denoted $A_1A_2A_3A_4$). Each chromosome may then pair randomly with any of its homologues (random bivalent pairing), or, alternatively, quadrivalent pairing may occur during meiosis. Both processes lead to tetrasomic inheritance, i.e., all possible allelic combinations are produced in equal frequencies (Muller 1914), which is generally considered indicative for autotetraploidy (Soltis & Soltis 1993). However, if quadrivalent pairing takes place at meiosis, tetrasomic inheritance may also be associated with double reduction, the phenomenon by which recombined distal parts of chromatids end up in the same gamete (Mather 1935). Double reduction thus leads to homozygous gametes being produced by fully heterozygous individuals. Approaches have been developed to account for these complexities of tetrasomic inheritance in population genetic analyses (Moody *et al.* 1993; Ronfort *et al.* 1998; Luo, Zhang, Zhang *et al.* 2006) and linkage mapping (e.g., Luo *et al.* 2004; Luo, Zhang, Leach *et al.* 2006).

In extreme allotetraploids, there are two homeologous sets consisting of two homologous chromosomes each (denoted $A_1A_2B_1B_2$). If a chromosome exclusively pairs with its homologue this leads to disomic inheritance, which is generally considered indicative for allotetraploidy (Soltis & Soltis 1993; Ramsey & Schemske 2002). This often surfaces as fixed heterozygosity in genetic analyses. Recombination only occurs between the chromosomes that originate from either one of the parental species, but not between chromosomes derived from the different parental species. Variation can be analyzed with the standard population genetic and linkage mapping tools developed for diploid organisms (Soltis & Soltis 1993; Cao *et al.* 2005).

Irrespective of the origin of an individual (auto- or allotetraploid), over time inheritance may shift from disomic to tetrasomic (or vice versa). In autotetraploids, a shift from tetrasomic to disomic inheritance may take place through (cyto)genetic diploidisation, a process in which the four initially homologous chromosomes differentiate into two sets of preferentially pairing chromosomes (Sybenga 1969; Soltis & Soltis 1993; Wolfe 2001; Ramsey & Schemske 2002). In allotetraploids, a shift from disomic to tetrasomic inheritance can take place when pairing is not always strictly preferential in meiosis (Sybenga 1996). This may lead to crossing over between homeologous chromosomes (e.g., Udall *et al.* 2005) and thus intergenomic recombination, a process that could theoretically homogenise the initially homeologous chromosomes (Sybenga 1996).

It may take many generations with intermediate pairing preferences before genetic diploidisation or homogenisation is complete. It seems likely that fertile interspecific hybrids could show a preference of pairing with a homologous chromosome that is not absolute, since their parents are usually related and therefore are expected to possess some degree of chromosomal homology, but on the other hand have diverged enough to earn their species status. Thus, particularly in systems where hybridisation is common, individuals characterised by intermediate pairing preferences (i.e., characterised by inheritance intermediate between disomic and tetrasomic) may not be exceptional (e.g., Ramsey & Schemske 2002). The exact mode of inheritance greatly affects the segregation of variation in the offspring of such plants, and is therefore of great interest, both from an evolutionary perspective and for breeding purposes. Moreover, neither the standard nor the tetrasomic-specific methods for population genetics and linkage mapping (see above) may be appropriate for tetraploids with intermediate inheritance.

In cytogenetic studies, models have been developed that incorporate the possibility of intermediate pairing preferences, based on meiotic configurations in meiosis I (reviewed in Sybenga 1994; Jackson & Jackson 1996; Wu *et al.* 2001). In segregation studies, normally only the completely disomic and tetrasomic inheritance models have been considered, thereby discounting the possibility of intermediate pairing preferences. Several studies suggested intermediate pairing preferences as an explanation for inheritance patterns intermediate to disomic and tetrasomic, but lacked a method to evaluate this hypothesis statistically (Hickok 1978a, b; Danzmann & Bogart 1982, 1983; Marsden *et al.* 1987; Allendorf & Danzmann 1997). In this paper, we propose a likelihood-based approach to statistically evaluate whether disomic, tetrasomic and intermediate inheritance models best explain the segregation of genetic markers.

We apply this framework to the perennial tetraploids *Rorippa amphibia* and *R. sylvestris* and their hybrid *R. x anceps*. The species form a polyploid complex with mainly diploids and tetraploids in *R. amphibia*, and mainly tetraploids and hexaploids in *R. sylvestris* (Jonsell 1968). The natural hybrids are mostly tetraploids (unpublished data). In *R. amphibia*, diploids are indistinguishable from tetraploids with respect to leaf morphology (Jonsell 1968) and other diploid close relatives are absent (Bleeker *et al.* 2002). This suggests an autotetraploid origin and the expectation to find tetrasomic inheritance. Diploids are absent in *R. sylvestris*, impeding speculations on the origin of tetraploids in this species and thus about the mode of inheritance.

We also study the mode of inheritance in artificial hybrids *R. x anceps* to evaluate whether the cytological divergence between the two species leads to a mostly disomic, tetrasomic or intermediate pattern of inheritance. At the tetraploid level, the species can be crossed easily and F1 hybrids readily backcross with both parental species. We intend to use the increased segregation variance of hybrids (Lexer *et al.* 2003) for mapping of traits associated with flooding tolerance (Chapter 5). The choice of linkage mapping tools depends on the exact mode of inheritance of the parental species and the hybrids (Cao *et al.* 2005; Luo, Zhang, Leach *et al.* 2006). We expect that intermediate inheritance models could very well apply to the *Rorippa* F1 hybrids, as their parents are closely related (also given the occurrence of fertile backcrossing hybrids in nature), while at the same time genomic differences exist that underlie the parental species' distinct morphologies (Jonsell 1968) and habitat preference

(Chapter 5). Moreover, the two species differ in DNA content by about 16 % (Chapter 1, Figure 1.1).

Despite the importance of exact knowledge of the mode of inheritance of tetraploids for evolutionary, genetic and linkage analysis (Ronfort *et al.* 1998; Cao *et al.* 2005; Luo, Zhang, Leach *et al.* 2006; Luo, Zhang, Zhang *et al.* 2006) this is – to our knowledge – the first approach that accounts for the possibility of inheritance intermediate to disomic and tetrasomic and for double reduction. Therefore, we also tested the applicability of our approach by re-analysing some published tetraploid segregation datasets for which only the extreme (i.e., disomic and/or tetrasomic) inheritance models had been tested and compared.

Materials and methods

Plant material and crosses

During the growing seasons of 2003-2005, root and shoot fragments of tetraploid *Rorippa amphibia* (denoted AAAA) and *Rorippa sylvestris* (denoted SSSS) were collected from several locations throughout Europe and grown in a greenhouse environment (Table 1). In the summer of 2004, we made reciprocal crosses between two independent wild collected pairs of AAAA and SSSS (Table 1) to create first generation (F1) hybrids. From each of the four resulting F1 hybrid seed families, we germinated approximately 50 seeds on filter paper moistened with 2ml of a 3µM gibberellic acid solution. Seedlings were transferred to soil and further grown in a common greenhouse environment. In the summer of 2005, one individual of each of the four F1 hybrid seedling families was backcrossed with an unrelated, wild-collected plant (Table 1) to create first generation backcrosses[*] (BC1). From each of the four resulting BC1 seed families, we again germinated approximately 50 seeds on 2ml of a 3µM gibberellic acid solution. Seedlings were transferred to soil and further grown in a common greenhouse environment.

[*] Technically, this should be referred to as a testcross, as it did not involve the same parental genotype.

Table 1. Characteristics of the tetraploid genotypes of *R. amphibia* (AAAA), *R. sylvestris* (SSSS) and F1 hybrids that were used to generate F1 and BC1 offspring. Plant codes, crossing partners, and for wild collected plants the specifics of origin (river, closest town, country) and the WGS84 coordinates of the exact sampling location are given. For F1 hybrids, the parents are indicated.

Plant code	Origin	Latitude Longitude		Crossed with
AAAA1	Vecht, Dalfsen Netherlands	North: East:	52°30'09" 06°15'36"	SSSS1
AAAA2	Zwarte Water, Zwartsluis, Netherlands	North: East:	52°37'30" 06°04'48"	SSSS2
AAAA3	Lake Balaton, Balatongyörök, Hungary	North: East:	46°46'13" 17°22'04"	AASS2 SSAA2
SSSS1	Waal, Millingerwaard, Netherlands	North East:	51°52'48" 06°00'17"	AAAA1
SSSS2	Stour, Child Okeford, United Kingdom	North: West:	50°54'05" 02°15'01"	AAAA2
SSSS3	Elbe, Darchau, Germany	North: East:	53°14'01" 10°54'18"	AASS1 SSAA1
AASS1	F1 hybrid AAAA1 x SSSS1			SSSS3
SSAA1	F1 hybrid SSSS1 x AAAA1			SSSS3
AASS2	F1 hybrid AAAA2 x SSSS2			AAAA3
SSAA2	F1 hybrid SSSS2 x AAAA2			AAAA3

DNA extraction and analysis of microsatellite loci

DNA was extracted from fresh leaves using a modified CTAB protocol (Doyle & Doyle 1987). We genotyped the wild collected plants and the backcrossed F1 hybrids for 12 microsatellite loci (Stift *et al.* 2006, Chapter 6). Based on this initial screening, we selected the most informative loci for each cross. Thus, ideally, each parent possessed four different alleles (i.e., fully heterozygous) and shared no alleles with its crossing partner. We genotyped each of the four F1 and the four BC1 offspring families for the selected loci. Offspring with genotypes that could only be explained by mutation or contamination (i.e., alleles observed that were not present in the parents) or non-disjunctions (i.e., more than two alleles observed from one of the parents) were excluded from the analyses. Such anomalous genotypes were found for two loci - RS44 (5 times) and RS101 (8 times) – and never constituted more than 4% of the offspring within one family.

Testing for reciprocal differences

From the genotypes observed in the offspring of the experimental crosses, we reconstructed the parental gamete frequencies. In cases where the two parents had alleles in common, we worked with the observed genotype frequencies in the offspring. We used the likelihood G-test for contingency tables (Sokal & Rohlf 1995) to test whether the observed parental gamete frequencies differed between the reciprocal crosses.

Gamete formation model

Consider a tetraploid where each chromosome is marked by a different allele (e.g., ABCD). Under complete tetrasomic inheritance, assuming no double reduction, gametes carrying the allelic combinations AB, AC, AD, CD, BD and BC will occur in equal proportions (1/6) (Muller 1914). The maximum frequency of double reduction (α) is 1/6, which can be reached if quadrivalents are always formed at meiosis, orientation is adjacent and one effective cross-over occurs between the locus and its centromere (Mather 1935). Under this scenario, the allelic combinations AB, AC, AD, CD, BD and BC will still occur in equal proportions ($1/6 - 1/6\alpha$), and there will be double reduction gametes (AA, BB, CC and DD) each at an expected frequency of $1/4\alpha$.

Preferential (bivalent) pairing in meiosis leads to expected gamete frequencies characteristic of disomic inheritance. If alleles A and B mark homologous chromosomes that pair exclusively with each other, allele A and B will never end up in the same gamete, and

likewise for C and D marked chromosomes. This AB/CD pattern of pairing thus produces gametes with the allelic combinations AC, AD, BC and BD in equal proportions (1/4). Double reduction is not possible with bivalent formation. The other possible pairings, namely of AC/BD and AD/BC, result in gametes AB, AD, BC, CD and AB, AC, BD, CD, respectively. The expected proportions of all possible gametes produced by an individual ABCD is calculated by the formulas:

$$
\begin{aligned}
p(AA) &= \tfrac{1}{4}\,\beta\tau \\
p(BB) &= \tfrac{1}{4}\,\beta\tau \\
p(CC) &= \tfrac{1}{4}\,\beta\tau \\
p(DD) &= \tfrac{1}{4}\,\beta\tau \\
p(AB) &= \tfrac{1}{6}\,\tau - \tfrac{1}{6}\,\beta\tau + (1-\tau)(\tfrac{1}{4}\,\delta 2 + \tfrac{1}{4}\,\delta 3) \\
p(AC) &= \tfrac{1}{6}\,\tau - \tfrac{1}{6}\,\beta\tau + (1-\tau)(\tfrac{1}{4}\,\delta 1 + \tfrac{1}{4}\,\delta 3) \\
p(AD) &= \tfrac{1}{6}\,\tau - \tfrac{1}{6}\,\beta\tau + (1-\tau)(\tfrac{1}{4}\,\delta 1 + \tfrac{1}{4}\,\delta 2) \\
p(CD) &= \tfrac{1}{6}\,\tau - \tfrac{1}{6}\,\beta\tau + (1-\tau)(\tfrac{1}{4}\,\delta 2 + \tfrac{1}{4}\,\delta 3) \\
p(BD) &= \tfrac{1}{6}\,\tau - \tfrac{1}{6}\,\beta\tau + (1-\tau)(\tfrac{1}{4}\,\delta 1 + \tfrac{1}{4}\,\delta 3) \\
p(BC) &= \tfrac{1}{6}\,\tau - \tfrac{1}{6}\,\beta\tau + (1-\tau)(\tfrac{1}{4}\,\delta 1 + \tfrac{1}{4}\,\delta 2)
\end{aligned}
$$

Or in matrix notation:

$$
\begin{pmatrix} p(AA) \\ p(BB) \\ p(CC) \\ p(DD) \\ p(AB) \\ p(AC) \\ p(AD) \\ p(CD) \\ p(BD) \\ p(BC) \end{pmatrix} = \begin{pmatrix} \mathbf{0} \\ \mathbf{0} \\ \mathbf{0} \\ \mathbf{0} \\ \mathbf{1/6} \\ \mathbf{1/6} \\ \mathbf{1/6} \\ \mathbf{1/6} \\ \mathbf{1/6} \\ \mathbf{1/6} \end{pmatrix} \tau + \begin{pmatrix} \mathbf{1/4} \\ \mathbf{1/4} \\ \mathbf{1/4} \\ \mathbf{1/4} \\ \mathbf{-1/6} \\ \mathbf{-1/6} \\ \mathbf{-1/6} \\ \mathbf{-1/6} \\ \mathbf{-1/6} \\ \mathbf{-1/6} \end{pmatrix} \beta\tau + (1-\tau) \begin{pmatrix} 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 0 \\ \mathbf{0} & \mathbf{1/4} & \mathbf{1/4} \\ \mathbf{1/4} & \mathbf{0} & \mathbf{1/4} \\ \mathbf{1/4} & \mathbf{1/4} & \mathbf{0} \\ \mathbf{0} & \mathbf{1/4} & \mathbf{1/4} \\ \mathbf{1/4} & \mathbf{0} & \mathbf{1/4} \\ \mathbf{1/4} & \mathbf{1/4} & \mathbf{0} \end{pmatrix} \begin{pmatrix} \delta 1 \\ \delta 2 \\ \delta 3 \end{pmatrix}
$$

These equations define a set of non-linear equations with four unknown parameters. The 'tetrasomic' parameter (τ) indicates the proportion of gametes formed by random meiotic chromosome associations (i.e., random bivalent or quadrivalent pairing) and can take values from zero (full disomic) to 1 (full tetrasomic). In the latter case, the entire last (disomic) part of the equation cancels out. If $\tau<1$, the expected gamete proportions depend on the setting of three 'disomic' parameters (δ1, δ2 and δ3) that indicate the respective degree of preferential

pairing of AB/CD, AC/BD and AD/BC marked chromosomes (respectively) in the non-random meiotic chromosome associations. Each can take values from 0 (no pairing) to 1 (obligate pairing) with the constraints that $\delta1 + \delta2 + \delta3 = 1$ and $\delta1 \times \delta2 \times \delta3 = 0$ (i.e., one of the disomic parameters must be zero). The latter constraint guarantees that random bivalent or quadrivalent meiotic configurations are exclusively expressed in the parameter τ. Without such a constraint, there would be an alternative solution for each parameter setting with $\tau > 0$ (e.g., $\tau=1$ would be equivalent to $\tau=0$ with $\delta1=\delta2=\delta3=1/3$). Finally, the 'double reduction' parameter β represents the frequency of double reductions relative to the total frequency of random (quadrivalent or random bivalent) meiotic associations, from which the frequency of double reduction as used in the literature (α) can be calculated as $\alpha=\beta\tau$.

Parameter estimation

Given observed gamete frequencies, the parameter values can be estimated with standard iterative methods for non-linear regression models available in many statistical packages. This results in parameters that give the best fit (e.g., lowest G) – at least if the procedure converges at the global optimum for the fit. We used the constrained nonlinear regression function as implemented in SPSS, using the sequential quadratic programming algorithm with a user-defined binomial loss function that represents the deviance for each gamete class:

$$(OBS / N)\ln(PRED / N) + (1 - OBS / N)\ln(1 - PRED / N)$$

in which OBS is the observed class frequency, PRED is the predicted class frequency and N is the total number of observations.

Application of gamete formation model

For plant-locus combinations of the type ABCD or AABC with no alleles shared with the crossing partner, we deduced the parental gamete frequencies from the observed offspring genotypes. Then we used SPSS (constrained nonlinear regression, see specifications above) to obtain the parameter values that gave the best model fit for the following situations:

1. the full tetrasomic null model ($\tau=1$), in which only the double reduction rate ($\beta\tau$) was estimated;
2. three constrained intermediate models, in which the proportion of random segregations (τ) and the DR rate ($\beta\tau$) were estimated, while the disomic parameters were fixed at either $\delta1=1$, $\delta2=1$, $\delta3=1$, respectively;

3. three unconstrained intermediate models, in which τ, βτ, and two of the disomic parameters were estimated, while the third was set to zero.

For partially informative cross-locus combinations (of type ABCD or AABC) with some alleles shared with the crossing partner for that particular locus it is not possible to unambiguously reconstruct the parental gamete frequencies from the observed offspring genotypes. Therefore, we worked the other way around and calculated the expected offspring genotype frequencies from the expected gamete frequencies of the parents under the following parameters setting. We assumed full tetrasomic inheritance for one parent (i.e., $\tau=1$) and let the τ of the other parent increase from 0 (~full disomic) to 1 (full tetrasomic) in steps of 0.01, at $\delta1=1$, $\delta2=1$, $\delta3=1$, respectively. For each of these parameter settings and their expected offspring genotype frequencies we calculated the fit (G-test statistic) to the observed frequencies and identified the parameter settings that gave the best fit (lowest G). This procedure was executed as a (more tedious) spreadsheet algorithm (Microsoft Excel) scanning the parameter space, rather than as a non-linear regression problem.

To evaluate whether intermediate models ($0 < \tau < 1$) provided a significantly better fit than the tetrasomic null model ($\tau = 1$), we calculated the difference between the G-values of the models. Such a difference follows a chi-square distribution in which the degrees of freedom (df) corresponds to the difference in degrees of freedom between the two models compared (Sokal & Rohlf 1995).

Evaluation of model performance on tetraploid segregation data from the literature

We selected some specific crosses and loci from the literature to test the general applicability of our gamete formation model (specified in Table 6). We specifically included cases in which tetrasomic inheritance could not be statistically rejected in the original study, but for which we suspected that intermediate inheritance models might apply. Also, we included a case where the observed patterns were clearly disomic (Pairon & Jacquemart 2005).

Results

Reciprocal differences

There were no significant differences between observed female and male gamete frequencies of the same individual (data not shown). Therefore, in subsequent analyses the reciprocal data were pooled.

Inheritance in natural tetraploids

For the wild collected tetraploid *Rorippa sylvestris* (genotypes SSSS1, SSSS2 and SSSS3), the estimated value of τ of the best fitting intermediate inheritance models varied from $\tau = 0.64$ to $\tau = 1$ for the different loci (Table 2). In none of these cases the fit was significantly better than the null model of full tetrasomic inheritance $\tau = 1$ (Table 2). The likelihood of intermediate models typically increased asymptotically upon approaching $\tau = 0$, flattened out around the minimum and increased again towards τ=1 (Figure 1). For the wild collected tetraploid *R. amphibia* (genotypes AAAA1, AAAA2 and AAAA3), the estimated value of τ of the best fitting intermediate model varied from τ=0.59 to τ=0.96 for the different loci (Table 2). Only for locus RA12 (plant AAAA1) an intermediate model provided a

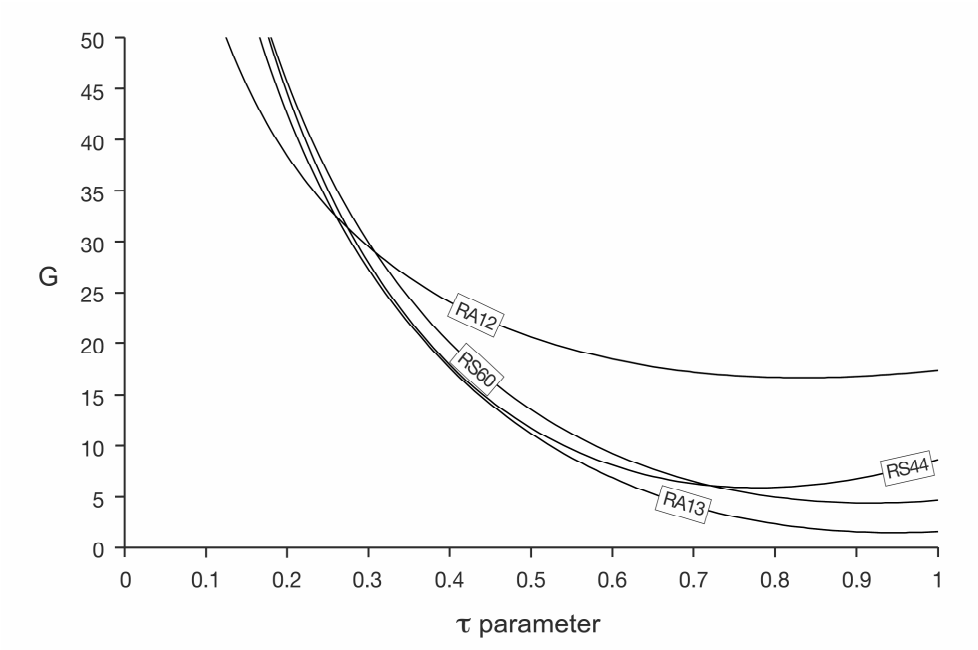

Figure 1. Fit (G-test statistic) of observed segregation of microsatellite loci in a tetraploid *R. sylvestris* (SSSS2) to inheritance models ranging from complete disomic (τ=0) to complete tetrasomic inheritance (τ=1). The 'disomic' coefficient of the most likely disomic model was constrained to 1. Boxes specify locus names.

Table 2. Fitting inheritance models on segregation of microsatellite loci in progeny of crosses involving wild collected tetraploid *R. sylvestris* (SSSS) and *R. amphibia* (AAAA). Comparison of fit (G-test statistic) of a tetrasomic model of inheritance (null model) and the best fitting intermediate model. For intermediate models, τ indicates the proportion of random meiotic parings (~tetrasomy), if τ<1, the alleles that preferentially pair are indicated. The degrees of freedom of the model comparison is always one. Bold G values are significant at the indicated level (*: p<0.05)

				Null model (τ=1)	Best intermediate model			Model comparison
Plant	Locus	Genotype	n	G	pairing alleles	τ	G	$G_{[1]}$
SSSS1	RS10	A A B C	99	6.73	-	1.00	6.73	0.00
	RS44	A C I N	100	5.23	AN/CI	0.93	4.95	0.28
	RS46	B B C C	99	1.81	BC/BC	0.64	0.23	1.58
	RS64	A A B C	99	2.12	AA/BC	0.91	1.71	0.41
SSSS2	RA12	D E E F	102	17.41	DF/EE	0.83	16.71	0.71
	RA13	A B B D	98	1.59	AD/BB	0.95	1.49	0.10
	RS44	B D E M	108	8.77	BD/EM	0.78	5.98	2.79
	RS60	A A C E	106	4.66	AA/CE	0.92	4.39	0.27
SSSS3	RS44	A G H J	114	8.33	AJ/GH	0.88	7.43	0.90
	RS44	A G H J	115	3.10	AG/HJ	0.86	1.94	1.16
	RS60	B D D F	116	8.77	-	1.00	8.77	0.00
	RS89	B B C E	115	40.14	BC/BE	0.95	40.12	0.02
	RS101	C D E K	108	6.13	CK/DE	0.78	3.27	2.86
	RS101	C D E K	113	59.96	CD/EK	0.92	59.67	0.29
AAAA1	RA12	A B E G	100	16.68	AB/EG	0.70	12.22	**4.46***
	RS44	F K L L	100	5.00	FL/KL	0.59	3.00	2.00
AAAA2	RA12	C E F H	102	17.41	CF/EH	0.83	16.68	0.74
	RS30	B D F -	104	7.12	BF/D-	0.84	5.72	1.40
	RS44	L L N O	108	1.65	LL/NO	0.89	0.97	0.68
AAAA3	RS89	A D D G	121	52.36	AD/DG	0.96	52.34	0.02
	RS101	G H I I	101	13.11	GH/II	0.71	10.70	2.41

Table 3. Fitting inheritance models on segregation of microsatellite loci in progeny of crosses involving first generation hybrids *R. amphibia x R. sylvestris* (AASS) and *R. sylvestris* x *R. amphibia* (SSAA). Comparison of fit (G-test statistic) of a tetrasomic model of inheritance (null model) and the best fitting intermediate model. For intermediate models, τ indicates the proportion of random meiotic parings (~tetrasomy), if τ<1, the alleles that preferentially pair are indicated. The degrees of freedom of the model comparison is always one. Bold G values are significant at the indicated level (*: p<0.05; **: p<0.005; ***: p<0.0005)

Plant	Locus	Origin alleles		n	Null model (τ=1)	Best intermediate model			Model comparison
		AAAA	SSSS		G	pairing alleles	τ	G	$G_{[1]}$
AASS1	RS44	FL	CN	113	34.98	FL/CN	0.53	21.10	**13.88*** **
	RS60	FF	CE	116	25.45	FF/CE	0.48	8.77	**16.68*** **
	RS101	J	FHH	109	7.11	FH/HJ	0.72	6.05	1.06
AASS2	RS44	LO	BE	121	14.09	LO/BE	0.69	8.17	**5.92***
	RS60	CF	AE	121	8.19	AC/EF	0.74	4.01	**4.18***
	RS89	CG	EF	121	52.36	CG/EF	0.58	40.79	**11.57****
SSAA1	RS44	LL	IN	115	37.13	LL/IN	0.29	1.18	**35.95*** **
	RS89	AH	DE	115	40.14	AH/DE	0.42	18.68	**21.46*** **
	RS101	GJ	EF	112	60.00	EF/GJ	0.58	50.30	**9.70****
SSAA2	RS44	NO	BE	101	7.54	BO/EN	0.74	4.03	3.51
	RS46	BE	BD	100	3.76	BE/BD	0.96	3.74	0.02
	RS101	GG	AD	101	13.11	AG/DG	0.94	13.07	0.04

significantly better fit than the full tetrasomic null model (Table 2) and included 30% preferential pairing (i.e., τ=0.70) of the chromosomes marked by alleles A-B and E-G (i.e., δ1=1).

Inheritance in artificial F1 hybrids

For the artificial F1 hybrids (genotypes AASS1, AASS2, SSAA1 and SSAA2), the estimated value of τ of the best fitting (constrained) intermediate models varied from 0.29 to 1 for the different loci (Table 3). In nine of the 12 cases the fit was significantly better than the null

model of tetrasomic inheritance $\tau = 1$ (Table 3). In seven of these nine cases the preferential pairing involved chromosomes that originated from the same parental species, i.e., preferential pairing of the homologous chromosomes (Table 3). In the two other cases, the preferential pairing involved chromosomes that originated from different parental species, i.e., preferential pairing of homeologous chromosomes (AASS2, locus RS60; SSAA2, locus RS44). An unconstrained intermediate inheritance model never provided a significant improvement in fit over a constrained intermediate model (data not shown). The likelihood of intermediate models typically increased asymptotically upon approaching $\tau = 0$, reached a clear minimum and increased again towards $\tau=1$ (Figure 2).

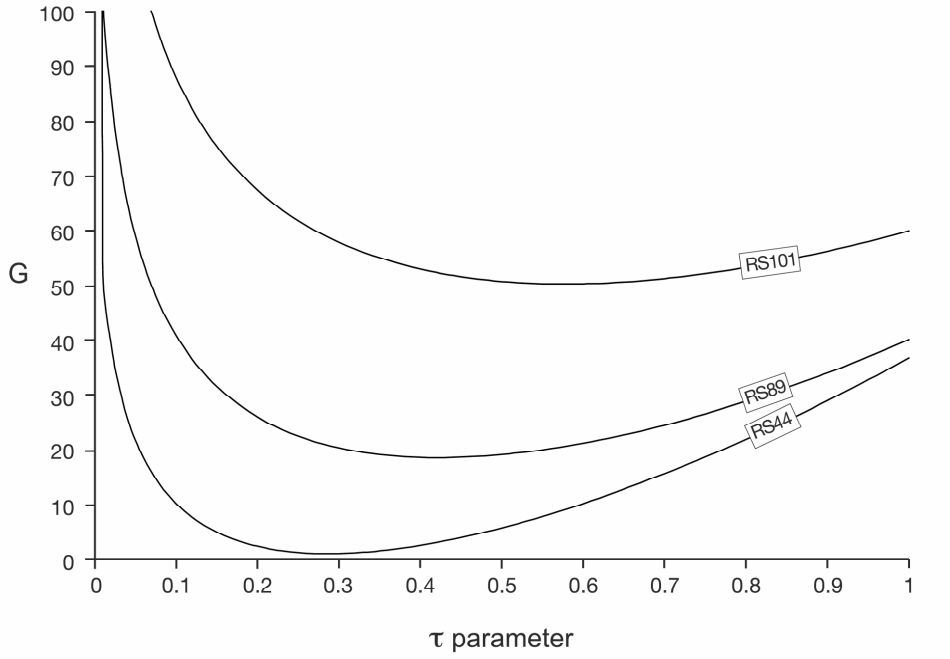

Figure 2. Fit (G-test statistic) of observed segregation of microsatellite loci in a tetraploid F1 hybrid *R. sylvestris x R. amphibia* (SSAA1) to inheritance models ranging from complete disomic ($\tau=0$) to complete tetrasomic inheritance ($\tau=1$). The 'disomic' coefficient of the most likely disomic model was constrained to 1. Boxes specify locus names.

Double reduction

For loci RA13, RS10, RS64 no double reduction (DR) gametes were detected. For locus RS30 null alleles prevented DR gamete identification. For all other loci we detected DR gametes (Table 4). With only 1 observed DR in more than 1000 offspring analysed, the DR rate for locus RS44 was the lowest. With 16 observed DR in 641 offspring, locus RS101 had the highest DR rate (Table 4). The total number of DR was higher in female meioses (17 *vs.* 10). In four cases the parental genotypes allowed iterative estimation of the DR parameter

($\beta\tau$). For the cases involving full heterozygotes (i.e., plants of type ABCD), the observed and estimated DR rates were equal. For the two remaining genotypes (of type AABC), the estimated DR rates were higher than the observed rate (Table 5).

Table 4. Overview of the observed number and rate of double reduction gametes (DR) per locus per type, for female and male gametes.

Locus	Parental type	DR in female meiosis	DR in male meiosis	n	Observed rate
RA12	AABC	1	2	304	0.010
RA13	AABC	0	0	98	0.000
RS10	AABC	0	0	99	0.000
RS30	ABC-	No information due to null allele			
RS44	AABC	0	0	323	0.000
RS44	ABCD	1	0	772	0.001
RS46	AABC	1	0	199	0.005
RS60	AABC	4	0	338	0.012
RS60	ABCD	0	0	121	0.000
RS64	AABC	0	0	99	0.000
RS89	AABC	0	1	236	0.004
RS89	ABCD	0	1	236	0.004
RS101	AABC	5	1	311	0.019
RS101	ABCD	5	5	333	0.030
	Total	17	10		

Evaluation of model performance on tetraploid segregation data from the literature

For the allozyme inheritance data of tetraploid *Centaurea jacea* (Hardy *et al.* 2001), the estimated values of τ of the best fitting intermediate inheritance models varied from $\tau = 0.71$ to $\tau = 0.98$ (Table 6). In one case the fit was significantly better than the null model (and author's conclusions) of full tetrasomic inheritance $\tau = 1$ (Table 6), and included 29% preferential pairing (i.e., τ=0.71) of the chromosomes marked by alleles A-C and B-D (i.e., δ2=1). For the allozyme inheritance data of tetraploid *Tolmiea menziesii* (Soltis & Soltis 1988), the estimated value of τ of the best fitting intermediate model varied from τ=0.27 to τ=0.96 (Table 6). In no case the fit was significantly better than the null model (and author's

Table 5. Observed number of double reduction (DR) gametes, observed DR rate and estimated DR rate per plant per locus.

Plant	Locus	Genotype	N	Observed DR freq.	Observed DR rate	Estimated DR rate
SSSS3	RS44	A G H J	114	1	0.0088	0.0088
SSSS3	RS101	C D E K	108	3	0.028	0.028
AASS1	RS101	F H H J	109	4	0.037	0.068
SSAA2	RS46	B B D E	100	1	0.010	0.016

conclusions) of full tetrasomic inheritance. For the microsatellite inheritance data of tetraploid *Prunus serotina* (Pairon & Jacquemart 2005), in agreement with the author's conclusions, the fit of disomic inheritance was better than any intermediate model and significantly better than the tetrasomic null model (Table 6). The likelihood of the disomic model increased from $\tau = 0$ to $\tau = 1$ in an almost linear fashion (Figure 3).

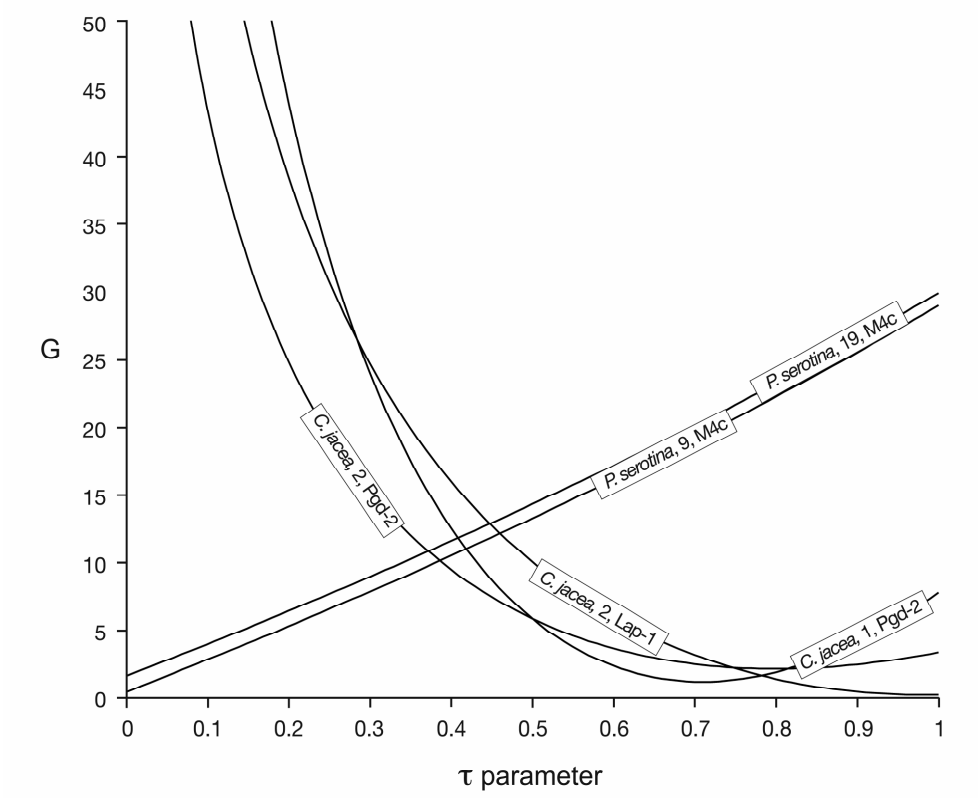

Figure 3. Fit (G-test statistic) of observed segregation of microsatellite loci in tetraploid *Centaurea jacea* (Hardy *et al.* 2001) and *Prunus serotina* (Pairon & Jacquemart 2005) to inheritance models ranging from complete disomic (τ=0) to complete tetrasomic inheritance (τ=1). The 'disomic' coefficient of the most likely disomic model was constrained to 1. Boxes specify species, individual/cross and locus name as used in the original publication.

Table 6. Fitting inheritance models on published tetraploid segregation ratios. Comparison of fit (G-test statistic) of a tetrasomic model of inheritance (null model) and the best fitting intermediate model. For intermediate models, τ indicates the proportion of random meiotic parings (~tetrasomy), if τ<1, the alleles that preferentially pair are indicated. The degrees of freedom of the model comparison is always one. Bold G values are significant at the indicated level (*: p<0.05; ***: p<0.0005

Species, marker	Locus Geno-type	n	Data source	Null model (τ =1)	Best disomic model			Model comparison
				G	pairing alleles	τ	G	G [1]
Centaurea jacea, Allozyme	Pgd-2 abcd	148	Hardy et al 2001: Table 3 (Cross 1)	7.81	AC/BD	0.71	1.22	**6.59***
	Pgd-2 abcd	60	Hardy et al 2001: Table 3 (Cross 2)	3.39	AC/BD	0.80	2.16	1.23
	Lap-1 bbcd	64	Hardy et al 2001: Table 4 (Cross 2)	0.26	BB/CD	0.98	0.26	0.01
Tolmiea menziesii, Allozyme	Fe-1 aacc	37	Soltis and Soltis 1988: Table 2 (1398-24 x 1398-20)	6.78	AC	0.42	3.31	3.46
	Fe-1 bbcc	37	Soltis and Soltis 1988: Table 2 (1398-24 x 1398-20)	6.78	BC	0.27	5.67	1.11
	Fe-1 abcc	72	Soltis and Soltis 1988: Table 2 (VANC x 1352-1)	8.29	AB/CC	0.47	6.22	2.07
	Pgi-2 bbce	369	Soltis and Soltis 1988: Table 2 (1347-5 x 1347-3)	3.43	BB/CE	0.96	3.33	0.10
Prunus serotina Microsat	M4c abcd*	36	Pairon and Jacque-mart 2005: Table 3	29.88	AB/CD	0.00	1.60	28.28***
	M4c eefg*	36	Pairon and Jacquemart 2005: Table 3	28.97	EE/FG	0.00	0.44	28.53***

Discussion

In this paper, we propose a likelihood-based approach to estimate the parameters of a general tetraploid inheritance model that best fits observed segregation data. The model incorporates full disomic inheritance, tetrasomic inheritance and the whole range of intermediate inheritance. In addition, it estimates the rate of double reduction.

We applied the approach to establish whether the perennial tetraploids *Rorippa amphibia* and *R. sylvestris* most likely have an auto- or allotetraploid origin and to pave the road for future studies of population genetics and linkage mapping in these species. We analysed the segregation of microsatellites for six tetraploid plants (three of both *R. amphibia*, and *R. sylvestris*). Only for one of the loci analysed an intermediate inheritance model provided a significantly better explanation for the observed progeny ratios than a tetrasomic null model. This provides strong evidence for an autotetraploid origin of both *Rorippa amphibia* and *R. sylvestris*. This is in concordance with the morphological resemblance of diploid and tetraploid *R. amphibia* (Jonsell 1968). In contrast, for most of the loci analysed for artificial F1 hybrids between *R. amphibia* and *R. sylvestris*, an intermediate model (i.e., including some degree of preferential pairing) explained the observed segregation ratios significantly better than the disomic and tetrasomic null models. To our knowledge, this provides the first example of an approach incorporating intermediate pairing preferences in the context of segregation in tetraploids. Previous attempts to calculate pairing preferences were all based on meiotic configurations (e.g., Jackson & Jackson 1996; Wu *et al.* 2001). This builds on the assumption that a quadrivalent frequency of less than 2/3 is always the consequence of preferential pairing (Sybenga 1994), which appears to be violated in many cases (Ramsey & Schemske 2002). Induced autotetraploid *Arabidopsis thaliana* lines had quadrivalent frequencies beyond the expected theoretical maximum of 2/3, whereas established lines were often cytogenetically diploidised, in that they formed a relatively high number of bivalents (Santos *et al.* 2003). Tetrasomic inheritance was associated with exclusively bivalent pairing in *Lotus corniculatus* (Fjellstrom *et al.* 2001), *Vaccinium darrowi and V. corymbosum* (Qu *et al.* 1998) and in colchicine induced autotetraploid *Brassica oleracea* (Jenczewski *et al.* 2002).

In *Rorippa* hybrids, chromosomes derived from the same parental species paired more frequently than heterospecific chromosomes. This gives rise to the question whether the observed intermediate pairing affinities in the F1 hybrids are stable, or whether recombination between homologous and homoeologous chromosomes will homogenise the genome and result in a shift to tetrasomic segregation in future generations (Stebbins 1950; Sybenga 1996).

Loci that were analysed in more individuals did not always follow the same model of inheritance, and the model of inheritance was also not always consistent across loci analysed for the same individual. This underscores that segregation in tetraploid hybrids is not always predictable. Our approach also estimates the double reduction rate. Double reduction can occur if the recombined chromosomes move to the same pole (i.e., adjacent orientation). This is very rare in diploids, but more common in triploids and tetraploids. It requires multivalent formation and further depends on the frequency of crossing over and the distance of the locus from the centromere. Double reduction can play a role in the purging of deleterious mutations through gametophytic selection (Butruille & Boiteux 2000). We observed a large variation in the DR rate among individuals. Although the sample sizes presented in this paper are probably not sufficient for an accurate estimation of the DR rate, this suggests that double reduction may be individual specific. Our data further suggested a higher prevalence of double reduction in female meioses. In two cases (both full heterozygotes of type ABCD), the observed DR rates and those estimated by our model were exactly the same. In two other cases (both partial heterozygotes of type AABC), the DR rate estimated by the model was higher than the observed rate. This pattern makes sense since not all double reductions can be observed in partial heterozygotes.

Many of the papers on inheritance patterns in tetraploids used chi-square or other goodness-of-fit approaches to test whether observed segregation ratios fit either a disomic or tetrasomic model of inheritance. Four outcomes are possible in such an approach. First, neither disomic, nor tetrasomic inheritance may be rejected, in which case power/sampling size appears to be insufficient. Second, tetrasomic inheritance may be rejected, and one of the possible disomic models not (e.g., Pairon & Jacquemart 2005). This type of outcome is regarded as evidence for disomic inheritance. Third, disomic inheritance may be rejected, and tetrasomic not (e.g., Quiros 1982; Marsden *et al.* 1987; Soltis & Soltis 1988; Hardy *et al.* 2001). This type of outcome is normally regarded as evidence for tetrasomic inheritance resulting from random

bivalent or quadrivalent pairing in meiosis. However, this may overestimate the importance of tetrasomic inheritance, since it disregards the possibility of intermediate inheritance models. Fourth, both disomic and tetrasomic inheritance may be rejected. This can only be explained by the existence of intermediate pairing preferences in meiosis, resulting in an inheritance intermediate to disomic and tetrasomic (e.g., Hickok 1978b; Danzmann & Bogart 1982; 1983). This type of outcome raises the question what is the relative importance of disomic and tetrasomic inheritance (or preferential vs. random chromosome pairing) for a particular tetraploid genome. Our approach allows addressing this question, and showed that intermediate inheritance (with 29% disomic inheritance) is more likely than 100% tetrasomic inheritance better fits segregation of allozyme locus Pgd-2 in tetraploid *Centaurea jacea* (Hardy *et al.* 2001). This may reflect different scenarios regarding the history of the chromosomes on which this locus is located. First, the chromosomes may have been homologous at the time of the polyploidisation event (i.e., an autopolyploidisation event), and now differentiating into two homoeologous sets (i.e., diploidising), so that inheritance is shifting to disomic inheritance. Second, the chromosomes may have been similar, but not completely homologous at the time of the polyploidisation event (i.e., allopolyploidisation). This could mean that recombination is homogenizing the chromosomes and that the intermediate inheritance is shifting to tetrasomic inheritance. In *Tolmiea menziesii* (Soltis & Soltis 1988), our approach showed that the likelihood surface was extremely flat for segregation of locus Fe-1 in several crosses for which only very few individuals had been analysed. This means that the intermediate models (including 73-53% disomic inheritance) could not be distinguished from full tetrasomic inheritance. Larger sample sizes would be needed to elucidate whether locus Fe-1 has an exceptional inheritance, or that the observed patterns simply reflect random noise. All other loci supported tetrasomic inheritance (Soltis & Soltis 1988).

Summarising, our approach showed that intermediate inheritance models provided a significantly better fit than the tetrasomic model of inheritance in first generation hybrids between *R. amphibia* and *R. sylvestris*, and in tetraploids of *Centaurea jacea*. The existence of inheritance patterns intermediate to disomic and tetrasomic inheritance has important repercussions for population genetics and mapping in tetraploids. In *Rorippa*, and in any system where hybridisation plays a role, any wild collected tetraploid individual may exhibit different pairing preferences, depending on the locus under study and the ancestry of the individual. This means that the methods that have been developed for linkage mapping and

population genetics of tetrasomic tetraploids (Ronfort *et al.* 1998; Cao *et al.* 2005; Luo, Zhang, Leach *et al.* 2006; Luo, Zhang, Zhang *et al.* 2006) may not be generally applicable in these systems. In the case of *Rorippa*, an assumption of tetrasomic inheritance may be legitimate only if the individuals under study have been collected from locations where hybridisation is known to be absent.

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REFERENCES

Bleeker, W., Weber-Sparenberg, C. & Hurka, H. (2002) Chloroplast DNA variation and biogeography in the genus Rorippa Scop. (Brassicaceae). *Plant Biology*, **4**, 104-111.

Bowers, J.E., Chapman, B.A., Rong, J.K. & Paterson, A.H. (2003) Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. *Nature*, **422**, 433-438.

Bretagnolle, F. & Thompson, J.D. (1995) Tansley Review No-78 - Gametes with the somatic chromosome number - Mechanisms of their formation and role in the evolution of autopolyploid Plants. *New Phytologist*, **129**, 1-22.

Butruille, D.V. & Boiteux, L.S. (2000) Selection-mutation balance in polysomic tetraploids: Impact of double reduction and gametophytic selection on the frequency and subchromosomal localization of deleterious mutations. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 6608-6613.

Cao, D.C., Craig, B.A. & Doerge, R.W. (2005) A model selection-based interval-mapping method for autopolyploids. *Genetics*, **169**, 2371-2382.

Danzmann, R.G. & Bogart, J.P. (1982) Evidence for a polymorphism in gametic segregation using a Malate Dehydrogenase Locus in the tetraploid treefrog *Hyla versicolor*. *Genetics*, **100**, 287-306.

Danzmann, R.G. & Bogart, J.P. (1983) Further evidence for a polymorphism in gametic segregation in the tetraploid treefrog *Hyla versicolor* using a Glutamate Oxaloacetic Transminase locus. *Genetics*, **103**, 753-769.

Doyle, J.J. & Doyle, J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **19**, 11-15.

Fjellstrom, R.G., Beuselinck, P.R. & Steiner, J.J. (2001) RFLP marker analysis supports tetrasonic inheritance in *Lotus corniculatus* L. *Theoretical and Applied Genetics*, **102**, 718-725.

Hardy, O.J., De Loose, M., Vekemans, X. & Meerts, P. (2001) Allozyme segregation and inter-cytotype reproductive barriers in the polyploid complex *Centaurea jacea*. *Heredity*, **87**, 136-145.

Harlan, J. & deWet, J.M.J. (1975) On Ö. Winge and a prayer: the origins of polyploidy. *The Botanical Review*, **41**, 361-390.

Hickok, L.G. (1978a) Homoeologous chromosome pairing and restricted segregation in the fern Ceratopteris. *American Journal of Botany*, **65**, 516-521.

Hickok, L.G. (1978b) Homoeologous chromosome pairing: frequency differences in inbred and intraspecific hybrid polyploid ferns. *Science*, **202**, 982-984.

Jackson, R.C. & Jackson, J.W. (1996) Gene segregation in autotetraploids: Prediction from meiotic configurations. *American Journal of Botany*, **83**, 673-678.

Jenczewski, E., Eber, F., Manzanares-Dauleux, M.J. & Chevre, A.M. (2002) A strict diploid-like pairing regime is associated with tetrasomic segregation in induced autotetraploids of kale. *Plant Breeding*, **121**, 177-179.

Jonsell, B. (1968) Studies in the North-West European species of *Rorippa s. str*. *Symbolae Botanicae Upsalienses*, **19**, 1-221.

Karpechenko, G.D. (1927) The production of polyploid gametes in hybrids. *Hereditas*, **9**, 349-368.

Lexer, C., Randell, R.A. & Rieseberg, L.H. (2003) Experimental hybridization as a tool for studying selection in the wild. *Ecology*, **84**, 1688-1699.

Luo, Z.W., Zhang, R.M. & Kearsey, M.J. (2004) Theoretical basis for genetic linkage analysis in autotetraploid species. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 7040-7045.

Luo, Z.W., Zhang, Z., Leach, L., Zhang, R.M., Bradshaw, J.E. & Kearsey, M.J. (2006) Constructing genetic linkage maps under a tetrasomic model. *Genetics*, **172**, 2635-2645.

Luo, Z.W., Zhang, Z., Zhang, R.M., Pandey, M., Gailing, O., Hattemer, H.H. & Finkeldey, R. (2006) Modeling population genetic data in autotetraploid species. *Genetics*, **172**, 639-646.

Mable, B.K. (2004) 'Why polyploidy is rarer in animals than in plants': myths and mechanisms. *Biol J Linn Soc*, **82**, 453-466.

Marsden, J.E., Schwager, S.J. & May, B. (1987) Single-locus inheritance in the tetraploid treefrog Hyla versicolor with an analysis of expected progeny ratios in tetraploid oirganisms. *Genetics*, **116**, 299-311.

Masterson, J. (1994) Stomatal Size in Fossil Plants - Evidence for Polyploidy in Majority of Angiosperms. *Science*, **264**, 421-424.

Mather, K. (1935) Reductional and equational separation of the chromosomes in bivalents and multivalents. *Journal of Genetics*, **30**, 53-78.

McLysaght, A., Hokamp, K. & Wolfe, K.H. (2002) Extensive genomic duplication during early chordate evolution. *Nature Genetics*, **31**, 200-204.

Moody, M.E., Mueller, L.D. & Soltis, D.E. (1993) Genetic variation and random drift in autotetraploid populations. *Genetics*, **134**, 649-657.

Muller, H.J. (1914) A new mode of segregation in Gregory's tetraploid Primulas. *American Naturalist*, **48**, 508-512.

Otto, S.P. & Whitton, J. (2000) Polyploid incidence and evolution. *Annual Review of Genetics*, **34**, 401-437.

Pairon, M.C. & Jacquemart, A.L. (2005) Disomic segregation of microsatellites in the tetraploid *Prunus serotina* Ehrh. (Rosaceae). *Journal of the American Society for Horticultural Science*, **130**, 729-734.

Qu, L.P., Hancock, J.F. & Whallon, J.H. (1998) Evolution in an autopolyploid group displaying predominantly bivalent pairing at meiosis: Genomic similarity of diploid *Vaccinium darrowi* and autotetraploid *V-corymbosum* (Ericaceae). *American Journal of Botany*, **85**, 698-703.

Quiros, C.F. (1982) Tetrasomic segregation for multiple alleles in Alfalfa. *Genetics*, **101**, 117-127.

Ramsey, J. & Schemske, D.W. (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics*, **29**, 467-501.

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

Ronfort, J.L., Jenczewski, E., Bataillon, T. & Rousset, F. (1998) Analysis of population structure in autotetraploid species. *Genetics*, **150**, 921-930.

Santos, J.L., Alfaro, D., Sanchez-Moran, E., Armstrong, S.J., Franklin, F.C.H. & Jones, G.H. (2003) Partial diploidization of meiosis in autotetraploid *Arabidopsis thaliana*. *Genetics*, **165**, 1533-1540.

Sokal, R.R. & Rohlf, F.J. (1995) *Biometry. The principles and Practice of Statistics in Biological Research - Third Edition*. W. H. Freeman and Company, New York.

Soltis, D.E. & Soltis, P.S. (1988) Electrophoretic evidence for tetrasomic segregation in *Tolmiea menziesii* (Saxifragaceae). *Heredity*, **60**, 375-382.

Soltis, D.E. & Soltis, P.S. (1993) Molecular data and the dynamic nature of polyploidy. *Critical Reviews in Plant Sciences*, **12**, 243-273.

Soltis, D.E., Soltis, P.S. & Tate, J.A. (2004) Advances in the study of polyploidy since Plant Speciation. *New Phytologist*, **161**, 173-191.

Soltis, P.S. & Soltis, D.E. (2000) The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 7051-7057.

Stebbins, G.L. (1947) Types of polyploids: their classification and significance. *Advances in Genetics*, **1**, 403-429.

Stebbins, G.L. (1950) *Variation and evolution in plants*. Columbia University Press, New York & London.

Stift, M., Kuperus, P. & Van Tienderen, P.H. (2006) Development of highly conserved primers for 12 new polymorphic microsatellite loci for the genus *Rorippa* Scop. (Brassicaceae), yellow-cress. *Molecular Ecology Notes*, **6**, 1129-1131.

Sybenga, J. (1969) Allopolyploidization of autopolyploids I. Possibilities and limitations. *Euphytica*, **18**, 355-371.

Sybenga, J. (1994) Preferential pairing estimates from multivalent frequencies in tetraploids. *Genome*, **37**, 1045-1055.

Sybenga, J. (1996) Chromosome pairing affinity and quadrivalent formation in polyploids: Do segmental allopolyploids exist? *Genome*, **39**, 1176-1184.

Udall, J.A., Quijada, P.A. & Osborn, T.C. (2005) Detection of chromosomal rearrangements derived from homeologous recombination in four mapping populations of *Brassica napus* L. *Genetics*, **169**, 967-979.

Wolfe, K.H. (2001) Yesterday's polyploids and the mystery of diploidization. *Nature Reviews Genetics*, **2**, 333-341.

Wolfe, K.H. & Shields, D.C. (1997) Molecular evidence for an ancient duplication of the entire yeast genome. *Nature*, **387**, 708-713.

Wu, R.L., Gallo-Meagher, M., Littell, R.C. & Zeng, Z.B. (2001) A general polyploid model for analyzing gene segregation in outcrossing tetraploid species. *Genetics*, **159**, 869-882.

CHAPTER 3

Genetic diversity in diploid versus tetraploid *Rorippa amphibia* (Brassicaceae)

Pieternella C. Luttikhuizen, Marc Stift, Peter Kuperus and Peter H. van Tienderen

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Abstract

The frequency of polyploidy increases with latitude in the northern hemisphere, especially in deglaciated, recently colonized areas. The cause or causes of this pattern are largely unknown, but a greater genetic diversity of individual polyploid plants due to a doubled genome and/or a hybrid origin is seen as a likely factor underlying selective advantages related to life in extreme climates and/or colonization ability. A history of colonization in itself, as well as a recent origin, and possibly a limited number of polyploidization events would all predict less genetic diversity in polyploids than in diploids. The null hypothesis of higher gene diversity in polyploids has to date hardly been quantified and is here tested in self incompatible *Rorippa amphibia* (Brassicaceae). The species occurs in diploid and tetraploid forms and displays clear geographic polyploidy in Europe. On the basis of eight microsatellite loci it can be concluded that the level of gene diversity is higher in tetraploids than in diploids, to an extent that is expected under neutral evolution when taking into account the larger effective population size in the doubled cytotype. There is thus no evidence for reduced genetic diversity in the tetraploids. Furthermore, the cytotypes are structured geographically to a comparable extent and differentiation between cytotypes is modest. The evidence presented here may mean that the tetraploids' origin is not recent, has not been affected by bottlenecks, and/or that tetraploids were formed multiple times, while an effect of introgression may also play a role.

Introduction

The frequency of polyploidy in plants in the northern hemisphere increases with latitude (Vandel 1940; Stebbins 1984, 1985; Little et al. 1997; Abbott and Brochmann 2003, Brochmann et al. 2004). Several hypotheses have been put forward to explain this pattern. It was suggested that polyploids may be better adapted to extreme climates than diploids (Hagerup 1931), better able to deal with climatic fluctuations and associated frequent recolonization (Manton 1950; Löve & Löve 1957; Johnson & Packer 1965; Johnson et al. 1965), or have greater ecological adaptability (Stebbins 1950). There is also evidence to show that harsh conditions enhance the formation of unreduced gametes and, hence, polyploid formation (Ramsey and Schemske 1998). When looking at the distribution of cytotypes in more detail, it appears that polyploids are common in recently deglaciated areas rather than in northern areas *per se* (Stebbins 1984, 1985; Abbott and Brochmann 2003, Brochmann et al. 2004).

A greater genetic diversity of individual polyploid plants that are of hybrid origin has been suggested as a factor underlying these selective advantages (Stebbins 1984, 1985). Hybridization is here used in a broad sense; not only between species but also between races or ecotypes. At the population level, however, recently formed polyploids might harbour lower genetic diversity if polyploid formation is a rare and/or recent event (Wagner 1970; but see Soltis and Soltis 1993, 1999; Abbott et al. 2007). Since newly formed polyploids then start out with limited genetic diversity, it would thus take a considerable amount of time to reach an equilibrium between mutation and drift, and ultimately higher levels of genetic diversity. A recent history of colonization, which is likely given the climatic oscillations that the northern hemisphere has undergone during the Pleistocene, would contribute to this effect (Hewitt 2004). Empirical data for other species underscore the likelihood that genetic diversity decreases with latitude, most probably as a result of range contractions and expansions during the Pleistocene glacials and interglacials (e.g., Gabrielsen et al. 1997; Broyles 1998; Nason et al. 2002; Persson et al. 2004).

Lowered population-level genetic diversity can be important for the functioning of populations in general (Reed and Frankham 2003) and in particular when self-incompatibility is involved (e.g., Fischer et al 2003, Mateu-Andrés and Segarra-Moragues 2004, Bleeker 2004, Willi et al. 2005). A population bottleneck experienced during formation of the new polyploid race could result in a small number of alleles at S-loci and lead to mate limitation and reduced seed set. In some plant families, polyploidization is accompanied by the loss of self incompatibility (Lewis 1954; Baker 1959; Mable 2004). However, in the Brassicaceae this is not the case and many polyploid species in this family are self incompatible (e.g. East 1940; Bateman 1955; Jonsell 1968; Grime et al. 1988; Mable et al 2004; Luttikhuizen et al. unpublished results). In such polyploid self incompatible species low genetic diversity might lead to increased mate limitation due to low numbers of S-alleles in the population. Whether this prediction holds depends on many factors associated with the fact that S-alleles are not selectively neutral and may be under the influence of dominance interactions (see Mable et al. 2003; 2004 and references therein).

While the consensus today is that in most polyploid taxa, polyploidization happened more than once (Soltis and Soltis 1993, 1999), the expected and actual amount of genetic diversity within polyploid populations have only rarely been quantified. For allotetraploid species it is hard if not impossible to define an expected level of diversity because of disomic inheritance and fixed heterozygosity. For species with different breeding systems for different cytotypes (in general: self incompatible diploids and self compatible polyploids) it is not possible, either, to formulate accurate predictions. In contrast, outcrossing autotetraploids form excellent candidates for such quantifications. A number of studies reported higher levels of diversity in outcrossing autotetraploids than in their diploid relatives (Soltis and Soltis 1989; Brown and Young 2000; Hardy and Vekemans 2001). This is in concordance with expectations under a neutral model of evolution (assuming population sizes and histories are equal and drift-mutation equilibrium has been reached) simply because each individual harbors four instead of two independent alleles per locus (Moody et al. 1993). However, earlier studies have not made explicit whether the level observed is as high as expected, although Hardy and Vekemans (2001) report that the overall genetic diversity is slightly lower in autotetraploid *Centaurea jacea* than expected solely on the basis of the doubled nuclear genome.

Here, we compare genetic diversity in diploids and tetraploids of the species *Rorippa amphibia* (L.) (BRASSICACEAE), which mainly inhabits ditches and river floodplains in Europe. It occurs in diploid and tetraploid forms, with the tetraploids having a more northerly distribution (Jonsell 1968). Diploids are found in southern Europe and tetraploids in central and northwestern Europe, with contact zones in, among others, England, France and southern Germany. Both cytotypes are self incompatible and highly clonal (Jonsell 1968; Bleeker 2004). The polyploids in *R. amphibia* are considered to be autotetraploids: the cytotypes are indistinguishable on the basis of morphology (Jonsell 1968); there are no similar diploid relatives known (Bleeker et al. 2002) and the tetraploids have tetrasomic inheritance (Stift et al. in prep.). The more northerly distribution of the tetraploids probably means that they have been exposed to repeated glacial cycle-related episodes of being pushed southwards and subsequent recolonizations of the north (Comes and Kadereit 1998; Hewitt 2004), as is seen in other species (e.g., Gabrielsen et al. 1997; Broyles 1998; Nason et al. 2002; Persson et al. 2004). Such periodic range compressions and expansions throughout the Pleistocene may not have happened, or to a lesser extent, to the diploids.

Here we test the null hypothesis that tetraploid *R. amphibia* have a level of genetic diversity that is expected under a neutral model of evolution, under the assumption of equilibrium between mutation and genetic drift, as compared to their diploid counterparts. If so, this would imply that observed levels of diversity are higher in the tetraploids than in the diploids because effective population size is larger in tetraploids, as each individual harbours twice the number of gene copies. The alternative hypothesis is that the tetraploids display lowered genetic diversity due to a putatively recent origin, a possibly limited number of occasions of origin, and repeated range compressions and expansions during the Pleistocene glacial cycles. These events would each result in lower genetic diversity compared to the diploids because, respectively, equilibrium between drift and mutation would not have been reached yet, the initial level of genetic diversity would have been low, and population bottlenecks associated with recent colonization would have led to loss of genetic diversity more in the north (tetraploids) than in the south (diploids). We test the null hypothesis using eight polymorphic microsatellite DNA markers (Stift et al. 2006).

Materials and methods

Plant material

We analyzed a collection of *Rorippa amphibia* from a set of populations throughout the geographical range where both cytotypes occur, with no apparent differences in habitats or population characteristics that might interfere with the comparison of cytotypes. Plant cuttings were collected at 22 field locations throughout Europe (Fig. 1, Table 1) during spring and summer of the years 2002-2005. Cuttings were grown in the greenhouse for DNA extraction. The ploidy level of each specimen was determined using flow cytometry using DAPI staining (ParTec, Münster, Germany). Only diploids and tetraploids were found among the plants used for the present study, although a small number of triploids have been encountered during our broader geographic surveys.

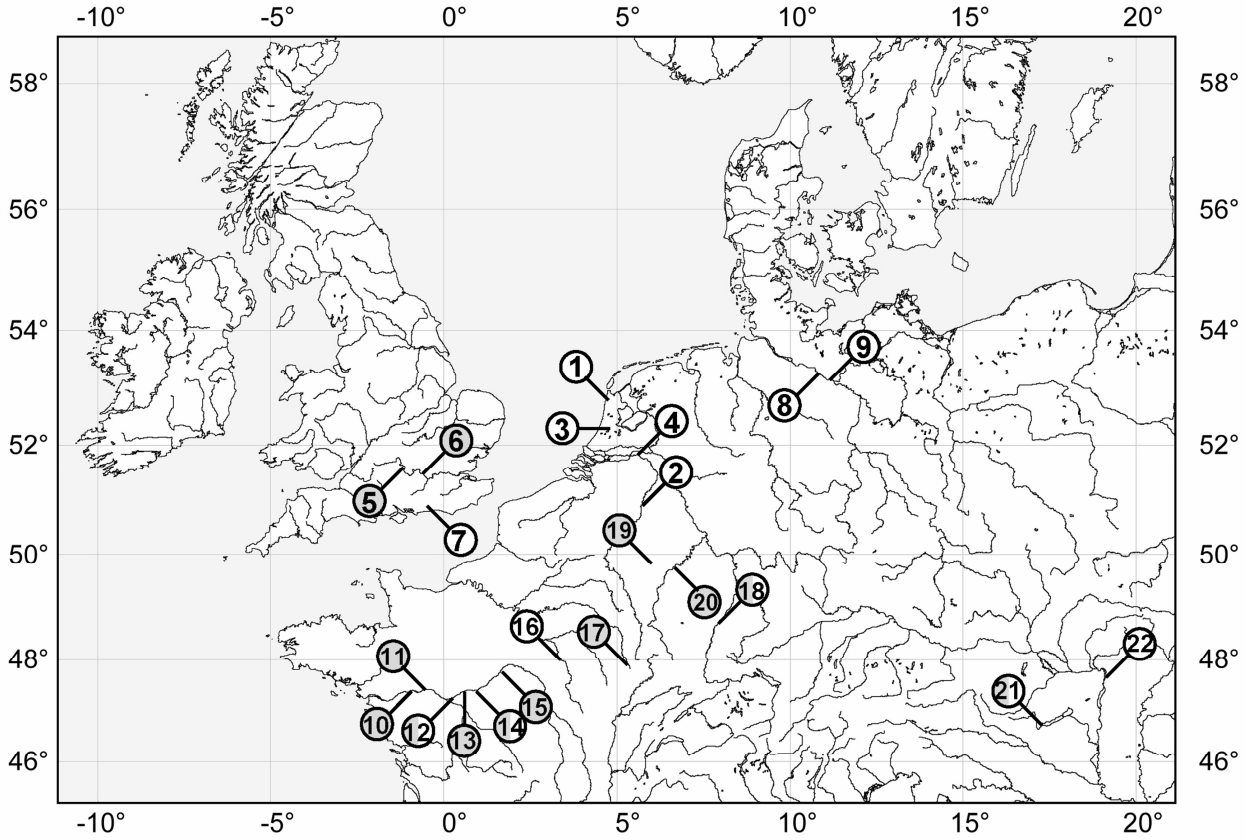

Figure 1. *Rorippa amphibia* sampling locations: 1) Zwanewater, The Netherlands; 2) Itteren-Borgharen, The Netherlands; 3) Amstel, The Netherlands; 4) Millingerwaard, The Netherlands; 5) Sonning, United Kingdom; 6) Coate Water, United Kindom; 7) Bury, United Kingdom; 8) Darchau, Germany; 9) Hirzacker, Germany; 10) Saint Florent, France; 11) Rochefort-sur-Loire, France; 12) Berthenay-1, France; 13) Berthenay-2, France; 14) Berthenay-3, France; 15) Mesnil, France; 16) l'Armancon, France; 17) Lac de Villegusien, France; 18) Kehl, Germany; 19) Vianden, Luxemburg; 20) Wasserbillig, Luxemburg; 21) Balatongyörök, Hungary; 22) Szentendre, Hungary. Filled symbols designate populations of diploids, open symbols of tetraploids and half-filled symbols are populations where both cytotypes were found. For coordinates see Table 1 heading.

Table 1. *Rorippa amphibia* samples and characteristics of sampling locations: number of cuttings analysed, number of multilocus genotypes detected among these cuttings, cytotypes detected within samples (in sample 18: one diploid and three tetraploids) and cytotypes known from the locations for three *Rorippa* species (own unpublished data); 2x=diploid; 3x=triploid; 4x=tetraploid; 5x=pentaploid; 6x=hexaploid.

							cytotypes known from location		
	sampling site	drainage system	location	no. cuttings	no. genotypes	cytotypes in study	*Rorippa amphibia*	*Rorippa sylvestris*	*Rorippa austriaca*
1	Zwanewater	Zwane-water	52°48'33"N 04°41'25"E	4	2	4x	4x		
2	Itteren-Borgharen	Maas (Rhine)	50°53'15"N 05°41'08"E	4	4	4x	4x	4x,5x,6x	
3	Amstel	Amstel	52°20'00"N 04°54'00"E	4	2	4x	4x		
4	Millingerwaard	Waal (Rhine)	51°51'58"N 05°59'41"E	4	4	4x	4x		
5	Sonning	Thames	51°20'54"N 00°50'36"W	4	2	2x	2x		
6	Coate Water	Thames	51°30'13"N 01°40'31"W	2	1	2x	2x		
7	Bury	Arun	50°50'28"N 00°30'23"W	4	3	4x	4x	4x	
8	Darchau	Elbe	53°14'01"N 10°54'18"E	4	4	4x	4x	4x	
9	Hirzacker	Elbe	53°09'16"N 11°02'47"E	4	3	4x	4x	4x,5x	
10	Saint Florent	Loire	47°22'26"N 00°59'15"W	4	4	2x	2x	4x	
11	Rochefort-sur-Loire	Loire	47°21'41"N 00°42'16"W	4	4	2x	2x	4x,5x	
12	Berthenay-1	Loire	47°15'44"N 00°17'15"E	4	4	2x	2x,4x	4x,5x	
13	Berthenay-2	Loire	47°17'41"N 00°21'01"E	4	3	2x	2x	5x	
14	Berthenay-3	Loire	47°21'35"N 00°30'07"E	4	4	2x	2x,4x	4x,5x,6x	
15	Mesnil	Loire	47°49'43"N 02°15'38"E	4	4	2x	2x	4x,6x	2x
16	l'Armancon	Seine	47°59'35"N 03°44'01"E	4	4	4x	4x	4x	
17	Lac de Villegusien	Seine	47°44'23"N 05°18'31"E	4	4	2x	2x		
18	Kehl	Rhine	48°36'52"N 07°49'42"E	4	4	2x,4x	2x,3x,4x	4x,5x	2x
19	Vianden	Rhine	49°56'00"N 06°12'00"E	4	4	2x	2x		
20	Wasserbillig	Rhine	49°43'00"N 06°30'00"E	4	3	2x	2x	4x,6x	
21	Balatongyörök	Donau	46°46'13"N 17°22'04"E	4	4	4x	2x,4x		
22	Szentendre	Donau	47°44'58"N 19°05'11"E	4	4	4x	4x	4x	

Per field location, four field collected plants were used in the study, except for Coate Water, where only two plants were found and collected, resulting in a total of 86 plants. The statistical sampling design was a setup with many locations per cytotype and few plants per location, because the goal was to quantitatively test the level of genetic diversity between cytotypes. For this purpose, such a design is more powerful than a design with few locations with many plants per location (Pons and Chaouche 1995; Pons and Petit 1995). The latter design would estimate within-population diversity with greater precision but would be less suitable for comparing levels of variation between cytotypes. In addition, clonal taxa such as *Rorippa amphibia* are frequently found in populations that vary considerably in number of individuals or ramets, but even large populations can be composed of a limited numbers of genets (Bleeker 2004). Thus, a sampling design of many genotypes per location for few locations would require a selection of extremely large populations for each cytotype. This would have resulted in unnecessary complications in the interpretation (e.g.: are the few large populations random populations for both cytotypes?; does genetic diversity differ between small and large populations in ways other than due to population size?).

Molecular procedures

DNA was extracted from fresh leaf material following a modified CTAB procedure (Doyle and Doyle 1990). Eight polymorphic microsatellite loci were amplified and run on a 6.5% KB+ polyacrylamide gel (Licor 4200) using fluorescently labelled forward primers (Table 2; Stift et al. 2006). Each PCR reaction contained 1x PCR buffer (HT Biotechnology: 100mM Tris-HCl, pH 9.0, 15 mM MgCl2, 500 mM KCl, 1% Triton X-100, 0.1% (w/v) stabiliser), 0.1mM of each dNTP, 0.5 mg/ml Bovine Serum Albumin, 0.15 µM of each primer, 0.04 units/µl Taq DNA polymerase (HT Biotechnology) and approximately 20 ng DNA. Amplifications were carried out in a PTC 100 thermocycler (MJ Research) with the following temperature program: 2 min at 94°C, 32 cycles of 30s at 94°C, 30s at primer specific annealing temperature (Table 2) and 30s at 72°C, and a final extension of 3 min at 72°C.

Genotypes were scored visually from the gels, with the most common band named ‘100’. Bands with higher mobility were given smaller numbers. For the tetraploids, genotype was scored by taking band intensity into account (Landergott et al. 2006). The PCR based dosage effects allowed unambiguous scoring, which has been confirmed by comparing observed and expected genotype frequencies among progenies of tetraploid *R. amphibia* in the greenhouse (Stift et al. in prep.).

Table 2. *Rorippa amphibia*, details for eight microsattelite loci: primer DNA sequences (*f* = forward primer, *r* = reverse primer); locus-specific annealing temperatures (T_A); overall number of alleles ($n_{alleles}$) detected per cytotype (2x = diploids, 4x = tetraploids).

			$n_{alleles}$		
locus	primer sequences (5'->3')	T_A (ºC)	2x	4x	total
Ra12	*f*: GTTCTCGTGTTTCCATGTTG	60	8	13	13
	r: AATTGGGCTTGAGTTTGTTC				
Rs101	*f*: AGCTACTTTGTGTGGAGTG	55	5	11	12
	r: TTTATTACACAAGTGTCACATG				
Rs64	*f*: GGTCTCGATGTAGCCCTTG	62	2	3	4
	r: TGACCACCGCGTAATAGATG				
Ra01	*f*: CACACAAAGCACAAAATGAGAG	62	2	5	5
	r: GCTACAGTCGGTGAAGAGGAG				
Rs60	*f*: CCAATCTTAACTGCACACACA	55	1	4	4
	r: GCAGTATATTTGTTTTGCACACT				
Rs46	*f*: GTGTGCTTCGATGTTGGAG	58	1	6	6
	r: GGAGACACAACAGGAACATAAAC				
Rs30	*f*: CTCATATGGGTCAAGGTCTTC	60	6	8	8
	r: GAAGTTCTCTTCGGGTTCAG				
Ra13	*f*: ATGCCTTTAGAGTTCGACCAG	62	6	8	8
	r: GTTGTTAGGAGCACCAATGAG				

Statistical analysis

Clonality among the samples was detected by looking for identical genotypes using the software GENOTYPE (Meirmans and Van Tienderen 2004). Only unique genotypes were subsequently used in the analyses, assuming that identical genotypes represented clone members.

Total gene diversities were calculated according to Nei (1973) and compared between diploids (H_{Tdip}) and tetraploids (H_{Ttet}). A test for total gene diversity in diploids versus tetraploids was conducted using a non-parametric Wilcoxon signed ranks test across loci. The expectation was that H_{Ttet} should be higher due to a larger effective population size for populations of tetraploids. However, H_{Ttet} could also be higher due to the larger number of alleles sampled in tetraploids than in diploids. Therefore, we also calculated heterozygosities, for which a theoretical framework has been developed that accounts for this problem. Individual intrapopulation heterozygosities were not analysed, as the sampling design is optimal for comparing global levels of variation between the cytotypes, but is unsuitable for

such comparisons. Expected heterozygosities were calculated per locus per location as $H_e = (1 - \sum_i p_i^2)$, where p_i is the frequency of the i-th allele, and averaged over locations. Observed heterozygosity was calculated for the diploids as the proportion of heterozygotes H_o (zygotic heterozygosity), and for the tetraploids as H_o', gametic heterozygosity (Moody et al. 1993). Gametic heterozygosity is the frequency of heterozygous (diploid) gametes within the gamete pool that is inferred to have given rise to the (tetraploid) zygote genotype frequencies. Under panmixis the expectation is that $H_e = H_o$ for diploids and, given tetrasomic inheritance and assuming no double reduction, that $H_e = H_o'$ for tetraploids (Moody et al. 1993). The prediction was that heterozygosity would be higher for tetraploids to a level expected under a neutral model of evolution (following Moody et al. 1993). Tests between cytotypes and observed versus expected heterozygosity within cytotype were conducted using non-parametric Wilcoxon signed ranks tests across loci.

Results

One population sampled contained both tetraploid (n=3) and diploid (n=1) cytotypes. All other samples consisted of only one cytotype; 10 of these contained tetraploids and 11 contained diploids. Several instances of identical multilocus genotypes occurred, every time for plants collected at the same location. Because a substantial fraction of clones in a random sample is expected in this highly clonal species (see Bleeker 2004) and because identical multilocus genotypes only occurred within locations, it is assumed that the identity is due to clonality. Although we cannot exclude the possibility that some of the identical multilocus genotypes actually represent independent individuals, this is unlikely given the high overall average pairwise difference between individuals (4.1 alleles for diploids, 12.9 for tetraploids). The total number of genotypes found among 86 plants was 75 (38 diploid and 37 tetraploid). Table 1 shows the cytotypes and numbers of genotypes detected per sample.

The overall level of polymorphism averaged 7.5 (standard deviation SD=3.5) alleles per locus, with 3.9 (SD=2.7) alleles per locus for the diploids and 7.3 (SD=3.5) alleles per locus for the tetraploids. Levels of polymorphism per locus across cytotypes ranged from 4 alleles (loci Rs64 and Rs101) to 13 alleles for locus Ra12 (for full details per locus and per cytotype see Table 2). The only locus that yielded no bands in a few cases (eight individuals) was Rs30. This may be taken as evidence of null alleles (see also Stift et al. 2006).

Table 3. Total gene diversities (H_{Tdip} and H_{Ttet})and expected (H_e) and observed (H_o, H_o') heterozygosities, averaged over populations, for eight microsatellite loci in *Rorippa amphibia*, comparing diploids and tetraploids; SD = standard deviation.

	diploids			tetraploids		
locus	H_{Tdip}	H_e	H_o	H_{Ttet}	H_e	H_o'
Ra12	0.777	0.56	0.74	0.875	0.69	0.79
Rs101	0.463	0.33	0.44	0.792	0.63	0.66
Rs64	0.0206	0.017	0.021	0.221	0.16	0.19
Ra01	0.0206	0.017	0.021	0.229	0.20	0.26
Rs60	0	0.00	0.00	0.139	0.11	0.10
Rs46	0	0.00	0.00	0.219	0.17	0.16
Rs30	0.628	0.36	0.24	0.770	0.49	0.28
Ra13	0.317	0.44	0.55	0.667	0.55	0.55
average	0.317	0.21	0.25	0.489	0.38	0.37
(SD)	(0.338)	(0.23)	(0.056)	(0.313)	(0.24)	(0.12)

Some alleles present in the tetraploids were not observed in the diploids. This was found in the two most variable loci in particular, Ra12 and Rs101 (Fig. 2). In both these loci the tetraploids contained a class of several alleles that were smaller in fragment length than the alleles shared between cytotypes. The other loci had a unimodal allele size distribution, apart from locus Rs30, of which the distribution was bimodal for the tetraploids. However, in contrast to loci Ra12 and Rs101, both allele size classes for locus Rs30 were shared between cytotypes.

Total gene diversities were significantly higher for tetraploid *R. amphibia* than for diploids ($p<0.05$, Wilcoxon signed ranks test, $Z=-2.521$, $n=8$), with H_{Ttet}=0.489 (SD=0.313) and H_{Tdip}=0.317 (SD=0.338). In fact, as can be seen in Table 3, H_{Ttet} was higher than H_{Tdip} for all eight microsatelliteloci examined. The difference was also significant when the two loci that were monomorphic for the diploids were excluded: H_{Ttet}=0.592 (SD=0.292) and H_{Tdip}=0.422 (SD=0.326) (Wilcoxon signed ranks test, $Z=-2.201$, $n=6$).

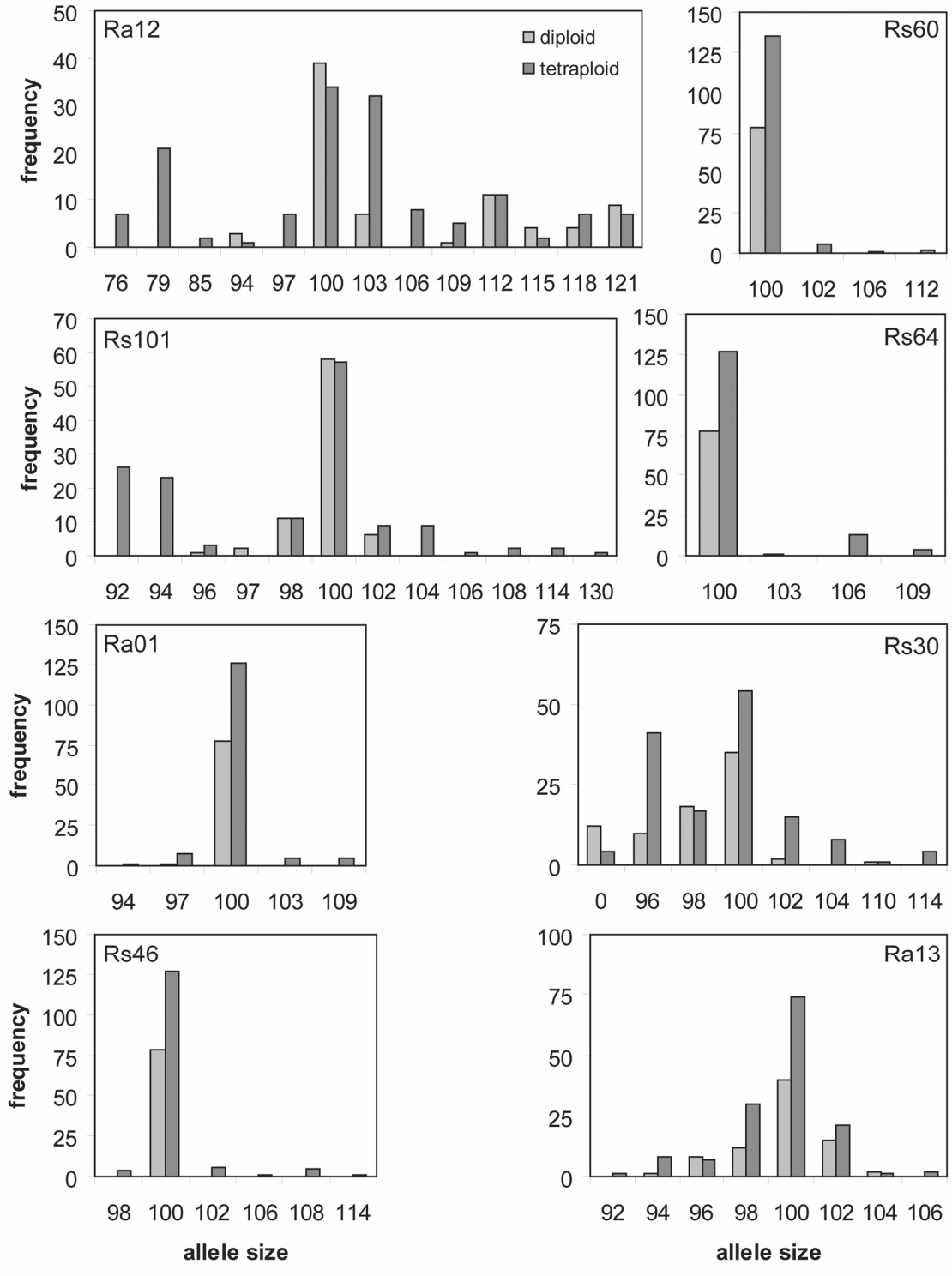

Figure 2. Overall allele frequencies for eight microsatellite loci for diploid and tetraploid *Rorippa amphibia*. Locus names are in panel corners. Allele size symbol '0' in locus Rs30 indicates missing data (i.e., individuals with no amplified product; a putative null allele).

Observed heterozygosities did not differ significantly from expected heterozygosities, as inferred from comparisons across loci (n.s., Wilcoxon signed ranks tests, Z=-1.156 for diploids and Z=-0.840 for tetraploids, n=8). Furthermore, the average level of intrapopulation variation differed between cytotypes: in accordance with expectations under a neutral model of evolution, both expected ($p<0.05$, Wilcoxon signed ranks test, Z=-2.524, n=8) and observed ($p<0.05$, Wilcoxon signed ranks test, Z=-2.521, n=8) heterozygosities were higher for tetraploids than for diploids. Over eight loci, expected heterozygosity averaged H_e=0.21 (SD 0.23) and H_e=0.38 (SD 0.24) over eight loci for diploids and tetraploids, respectively, with observed heterozygosities of H_o=0.25 (SD 0.056) and H_o'=0.37 (SD 0.12), respectively, again averaged over eight loci (see Table 3 for results per locus).

Figure 3 shows the theoretical relationship between heterozygosity for a diploid and a tetraploid if all other aspects would be equal (e.g., mutation rate, population size), with tetrasomic inheritance in the tetraploid and without double reduction. The relationship between the two is then expected to be $H_{tet}=H_{dip}/(1+H_{dip})$, where H_{tet} is heterozygosity in the tetraploid and H_{dip} in the diploid (Moody et al. 1993). The data points resulting from this study can also be seen in Fig. 3. The observations do not differ from the theoretical relationship based on either a Wilcoxon signed ranks test or on a sign test (significance level p=0.05). This pattern did not change when loci Rs60 and Rs46 (monomorphic in the diploids), were excluded (analyses not shown).

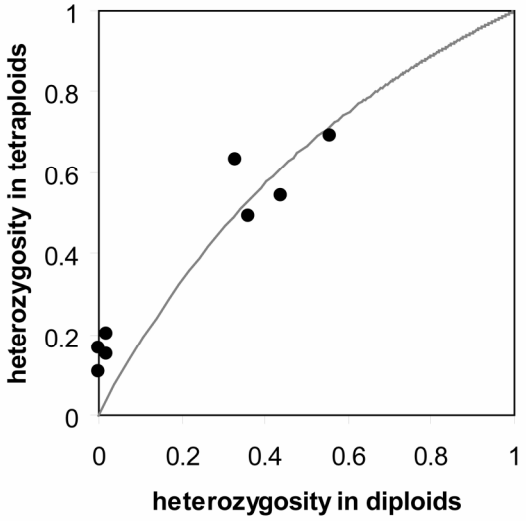

Figure 3. Observed heterozygosity (dots) for eight microsatellite loci in diploid versus tetraploid *Rorippa amphibia*. Line indicates theoretical relationship between heterozygosity in diploids and tetraploids if mutation rate and population size are the same and given that drift-mutation equilibrium has been reached.

Discussion

Microsatellite DNA marker diversity, estimated as total gene diversity (Nei, 1973), was found to be higher in tetraploids of the plant species Ror*ippa amphibia* than in diploids, in accordance with our null hypothesis. In addition, heterozygosity was higher in the tetraploids, to a level expected from a neutral model of evolution due to larger effective population size of tetraploids (Moody et al. 1993). This can be concluded on the basis of eight polymorphic microsatellite loci scored for a total of 86 plant cuttings collected throughout the European distribution range of the species. These findings have important repercussions for our understanding of the origin, spread and functioning of tetraploid *R. amphibia* that are most probably directly derived from diploid *R. amphibia* (Jonsell 1968; Bleeker et al. 2002), and of genetic diversity within populations of polyploids in general.

Our findings lend support to the notion that polyploids harbor higher levels of genetic diversity, which might make them more capable of dealing with harsh or novel environments (Hagerup 1931; Manton 1950; Stebbins 1950; Löve & Löve 1957; Johnson & Packer 1965; Johnson et al. 1965), a possible explanation of why they are found more frequently at higher latitudes than diploids (Vandel 1940; Stebbins 1984, 1985; Little et al. 1997; Abbott and Brochmann 2003, Brochmann et al. 2004).

Our results can be explained by three not mutually exclusive scenarios that may underlie the higher levels of variation in the tetraploids: a) tetraploids have accumulated variation from diploids (through interbreeding or multiple polyploidization events) or from related tetraploid species (through introgressive hybridisation); b) tetraploids and diploids are both of a sufficient age to allow mutation/selection balance to have reached an equilibrium; and c) tetraploids, in spite of their more northerly distribution, were not more strongly affected than diploids by reductions in population size and subsequent northward migrations, i.e. going through population size bottlenecks, due to periodic Pleistocene glaciations and associated climate change and range contractions (Comes and Kadereit 1998; Hewitt 2004).

We first discuss the scenario of accumulation of variation from diploids. The low level of genetic differentiation in terms of gene diversity that can be attributed to the between-cytotype component (3.9% between cytotypes, versus 23.9% between populations within

cytotypes) might be interpreted as an indication of this. Occasionally, some triploids have been encountered during our geographic surveys, e.g. at Kehl (own unpublished data, see Table 1) and at other locations not included in this study, e.g. upstream in the Thames valley near Cricklade (data not shown). These triploids, which are highly similar to the other *R. amphibia* cytotypes in morphology and clonality (own unpublished data), could be first-generation hybrids between diploid and tetraploid *R. amphibia*. Although greenhouse hand pollinations have shown that the diploid and tetraploid cytotypes do not readily interbreed, triploid and tetraploid offspring are somtimes formed (Stift et al. in prep.). Triploids carrying ripe seed were never encountered in the field and probably suffer from triploid block (Woodell and Valentine 1961), although it is a possibility that some viable pollen of variable ploidy level is formed that may occasionally fertilize ovules of diploids or tetraploids, thus constituting gene flow between cytotypes (Ramsey and Schemske 1998). Triploids in the field that merely survive by clonality obviously do not contribute to the accumulation of variation in tetraploids, while on the other hand clonality would prolong the life of genets and enhance the effect of a triploid bridge.

A more likely pathway of gene flow between cytotypes is multiple and continuous formation of tetraploids from diploids. In fact, multiple polyploidization events have been inferred for many species and are now considered to be the rule rather than the exception (Soltis and Soltis 1993, 1999).

Some of the variation in the tetraploids may also stem from hybridization with *R. sylvestris* and/or *R. austriaca*. The former is a native species of the area, while the latter is a recent invasive addition to the flora of the region (Bleeker and Hurka 2001). These three *Rorippa* species hybridize readily under greenhouse conditions and, in addition, introgression in the field is known from some locations but not others (Jonsell 1968; Bleeker and Hurka 2001). Our preliminary analysis of the most variable loci Ra12 and Rs101 in eight *R. sylvestris* genotypes suggest that the tetraploid-specific alleles (Fig. 2) do not result from hybridization with *R. sylvestris*, as the *R. sylvestris* alleles do not correspond with the additional alleles from *R. amphibia* tetraploids. However, this conclusion is based on a numerically as well as geographically limited sample only and should be investigated in more detail in future, as well as the possibility of introgression from *R. austriaca*. A possible alternative is that the tetraploid-specific alleles arose by mutation within the cytotype, or that they were once also

present within the diploids but have been lost. The locations where tetraploid-specific alleles were found to occur are widespread: locations 1-4, 8, 9, 16, 18, 21, and 22 (see Table 1 and Fig. 1). These alleles thus appear to only be absent from the Thames and Loire drainage systems.

The second scenario for the high tetraploid genetic diversity is that they may not be much younger than the diploids if polyploidization occurred early in the origin of the species. Polyploidization may have happened at least early enough for equilibrium between polyploid formation ('immigration' of new alleles), drift and mutation to have been reached or well approached. In fact, the geographical separation of the cytotypes in this and other species (Vandel 1940; Stebbins 1984, 1985; Little et al. 1997; Brochmann et al. 2004) does not corroborate the idea of continuing polyploid formation. In order to explain the geographical pattern we may need to invoke selection differences to explain the absence of one cytotype from either part of the distribution range. Although the cytotype minority exclusion principle (Levin 1975) could explain part of this problem, the fact that independent river systems show the same geographic pattern in *R. amphibia* (Fig. 1) remains to be explained, as does the general pattern of geographic polyploidy in other taxa.

The third scenario is that *R. amphibia* tetraploids may not have undergone bottlenecks during expansion northwards, or at least not more so than diploids. Perhaps both cytotypes have been compressed to the same extent, with perhaps competition between the two at the contact zone adding to a more southerly distribution during glacial maxima for the diploids. Microsatellite allele size distributions may be used as indicators of population bottlenecks, with unimodal allele size distributions being indicative of recent population bottlenecks, while multimodal distributions point to more stable population sizes (Kimmel et al. 1998; King et al. 2000). In the diploids, seven out of eight loci examined have a unimodal distributions and only one a bimodal pattern, while in the tetraploids five loci are unimodal and three bimodal (Fig. 2). Although some of the loci are probably not variable enough to be informative in this respect (King et al. 2000), the difference between the cytotypes is indeed in the more variable loci and therefore nevertheless conspicuous, suggesting that, if anything, the diploids rather than the tetraploids have undergone bottlenecks. However, the possibility must be taken into account that some alleles result from introgression, obscuring any patterns in allele size distributions resulting from stepwise mutations.

The fact that autotetraploid *R. amphibia* have levels of genetic variation in comparison to their diploid relatives to a level that may be expected from a neutral, equilibrium model of evolution, has some important evolutionary consequences. It implies that the intra-individual level of heterozygosity will be higher in tetraploids than in diploids as a result of comparable population-level genetic variation. This implies that we may expect effects of ploidy level, in general (Reed and Frankham 2003), and specifically as a consequence of self incompatibility (Mable et al. 2004). If for neutral loci tetraploid individuals are more heterozygous than diploids, they could also carry more (expressed) alleles at the S-locus. This would reduce the overall fraction of incompatible sexual partners in the population in this self incompatible tetraploid. It is possible that the accumulation of genetic variation in tetraploids is stimulated by the fact that assimilation of new variants at the S-locus is beneficial as it leads to a direct increase in the number of compatible partners. If so, different patterns of variation between cytotypes are expected for diploid/polyploid complexes that are self-incompatible or self-compatible. The evolutionary dynamics of S-alleles in tetraploids versus diploids and its consequences are currently under investigation, both theoretically and empirically.

This paper shows that genetic diversity in tetraploid *Rorippa amphibia* is higher than in diploids, to a level in accordance with neutral expectations. The present example adds to the growing set of plant species for which it has been shown that polyploids harbour considerable amounts of molecular genetic variation, just as their diploid counterparts do. Our findings imply that Stebbins' (1984, 1985) hypothesis, i.e., that higher genetic diversity of polyploids may underlie the greater ecological versatility that is required for colonizing taxa or taxa in harsher environments, may hold in this outcrossing autotetraploid.

References

Abbott RJ, Brochmann C (2003) History and evolution of the arctic flora: in the footsteps of Eric Hultén. *Molecular Ecology* **12**, 299-313.

Abbott RJ, Ireland HE, Rogers HJ (2007) Population decline despite high genetic diversity in the new allopolyploid species *Senecio cambrensis*(Asteraceae). *Molecular Ecology* **16**: 1023-1033.

Baker HG (1959) Reproductive methods as factors in speciation in flowering plants. *Cold Spring Harbor Symposia on Quantitative Biology* **24**, 177-191.

Bateman AJ (1955) Self-incompatibility systems in angiosperms. III. Cruciferae. *Heredity* **9**, 53-68.

Bleeker W, Hurka H (2001) Introgressive hybridization in *Rorippa* (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. *Molecular Ecology* **10**, 2013-2022.

Bleeker W, Weber-Sparenberg C, Hurka H (2002) Chloroplast DNA variation and biogeography in the genus *Rorippa* Scop. (Brassicaceae). *Plant Biology* **4**, 104-111.

Bleeker W (2004) Genetic variation and self-incompatibility within and outside a *Rorippa* hybrid zone (Brassicaceae). *Plant Systematics and Evolution* **246**, 35-44.

Brochmann C, Brysting AK, Alsos IG, et al. (2004) Polyploidy in arctic plants. *Biological Journal of the Linnean Society* **82**, 521-536.

Brown AHD, Young AG (2000) Genetic diversity in tetraploid populations of the endangered daisy *Rutidosis leptorrhynchoides* and implications for its conservation. *Heredity* **85**, 122-129.

Broyles SB (1998) Postglacial migration and the loss of allozyme variation in northern populations of *Asclepias exaltata* (Asclepiadaceae). *American Journal of Botany* **85**, 1091-1097.

Comes HP, Kadereit JW (1998) The effect of quaternary climatic changes on plant distribution and evolution. *Trends in Plant Science* **3**, 432-438.

East EM (1940) The distribution of self-sterility in the flowering plants. *Proceedings of the American Philosophical Society* **82**, 449-518.

Fischer M, Hock M, Paschke M (2003) Low genetic variation reduces cross-compatibility and offspring fitness in populations of a narrow endemic plant with a self-incompatibility system. *Conservation Genetics* **4**, 325-336.

Gabrielsen TM, Bachmann K, Jakobsen KS, Brochmann C (1997) Glacial survival does not matter: RAPD phylogeography of Nordic *Saxifraga oppositifolia*. *Molecular Ecology* **6**, 831-842.

Grime JP, Hodgson JG, Hunt R (1988) *Comparative plant ecology.* Unwin Hyman, London.

Hagerup O (1931) Über Polyploidie in Beziehung zu Klima, Ökologie und Phylogenie. *Hereditas* **16**, 19-40.

Hardy OJ, Vekemans X (2001) Patterns of allozyme variation in diploid and tetraploid *Centaurea jacea* at different spatial scales. *Evolution* **55**, 943-954.

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **359**, 183-195.

Johnson AW, Packer JG, Reese G (1965) Polyploidy, distribution, and environment. In: *The quaternary of the United States* (eds. Wright HEJ, Frey DG), pp. 497-507. Princeton University Press, Princeton, NJ.

Johnson AW, Packer JG (1965) Polyploidy and environment in arctic Alaska. *Science* **148**, 237-239.

Jonsell B (1968) *Studies in the north-west European species of* Rorippa *s. str.* A.-B. Lundequistska Bokhandeln, Uppsala.

Kimmel M, Chakraborty R, King JP, et al. (1998) Signatures of population expansion in microsatellite repeat data. *Genetics* **148**, 1921-1930.

King JP, Kimmel M, Chakraborty R (2000) A power analysis of microsatellite-based statistics for inferring past population growth. *Molecular Biology and Evolution* **17**, 1859-1868.

Landergott U, Naciri Y, Schneller JJ, Holderegger R (2006) Allelic configuration and polysomic inheritance of highly variable microsatellites in tetraploid gynodioecious *Thymus praecox* agg. *Theoretical and Applied Genetics* **113**, 453-465.

Levin DA (1975) Minority cytotype exclusion in local plant populations. *Taxon* **24**, 35-43.

Lewis D (1954) Comparative incompatibility in angiosperms and fungi. *Advances in Genetics* **6**, 235-285.

Little TJ, Demelo R, Taylor DJ, Hebert PDN (1997) Genetic characterization of an arctic zooplankter: insights into geographic polyploidy. *Proceedings of the Royal Society of London B* **264**, 1363-1370.

Löve Á, Löve D (1957) Arctic polyploidy. *Proceedings of the Genetics Society of Canada* **2**, 23-27.

Mable BK, Schierup MH, Charlesworth D (2003) Estimating the number, frequency, and dominance of S-alleles in a natural population of *Arabidopsis lyrata* (Brassicaceae) with sporophytic control of self-incompatibility. *Heredity* **90**, 422-431.

Mable BK (2004) Polyploidy and self-incompatibility: is there an association? *New Phytologist* **162**, 803-811.

Mable BK, Beland J, Berardo CD (2004) Inheritance and dominance of self-incompatibility alleles in polyploid *Arabidopsis lyrata*. *Heredity* **93**, 476-486.

Manton I (1950) *Problems of cytology and evolution in the Pteridophyta.* Cambridge University Press, Cambridge.

Mateu-Andrés I, Segarra-Moragues JG (2004) Reproductive system in the iberian endangered endemic *Antirrhinum valentinum* FQ (Antirrhineae, scrophulariaceae): Consequences for species conservation. *International Journal of Plant Sciences* **165**, 773-778.

Meirmans PG, Tienderen PH van (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* **4**, 792-794.

Moody ME, Mueller LD, Soltis DE (1993) Genetic variation and random drift in autotetraploid populations. *Genetics* **134**, 649-657.

Nason JD, Hamrick JL, Fleming TH (2002) Historical vicariance and postglacial colonization effects on the evolution of genetic structure in *Lophocereus*, a Sonoran Desert columnar cactus. *Evolution* **56**, 2214-2226.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA* **70**, 3321-3323.

Persson H, Widen B, Andersson S, Svensson L (2004) Allozyme diversity and genetic structure of marginal and central populations of *Corylus avellana* L. (Betulaceae) in Europe. *Plant Systematics and Evolution* **244**, 157-179.

Pons O, Chaouche K (1995) Estimation, variance and optimal sampling of gene diversity. 2. Diploid locus. *Theoretical and Applied Genetics* **91**, 122-130.

Pons O, Petit RJ (1995) Estimation, variance and optimal sampling of gene diversity. 1. Haploid locus. *Theoretical and Applied Genetics* **90**, 462-470.

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Reviews in Ecology and Systematics* **29**, 467-501.

Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. *Conservation Biology* **17**, 230-237.

Soltis DE, Soltis PS (1989) Genetic consequences of autopolyploidy in *Tolmiea* (Saxifragaceae). *Evolution* **43**, 586-594.

Soltis DE, Soltis PS (1993) Molecular data and the dynamic nature of polyploidy. *Critical Reviews in Plant Sciences* **12**, 243-273.

Soltis DE, Soltis PS (1999) Polyploidy: recurrent formation and genome evolution. *Trends in Ecology and Evolution* **14**, 348-352.

Stebbins GL (1950) *Variation and evolution in plants.* Columbia University Press, New York.

Stebbins GL (1984) Polyploidy and the distribution of the arctic-alpine flora: new evidence and a new approach. *Botanica Helvetica* **94**, 1-13.

Stebbins GL (1985) Polyploidy, hybridization, and the invasion of new habitats. *Annals of the Missouri Botanial Garden* **72**, 824-832.

Stift M, Kuperus P, Tienderen PH van (2006) Development of highly conserved primers for 12 new polymorphic microsatellite loci for the genus *Rorippa* Scop. (Brassicaceae), yellow-cress. *Molecular Ecology Notes* **6**, 1129-1131.

Vandel A (1940) La parthenogenese geographique. IV. Polyploidie et distribution geographique. *Bulletin Biologique de la France et de la Belgique* **74**, 94-100.

Wagner WH, Jr. (1970) Biosystematics and evolutionary noise. *Taxon* **19**, 146.

Willi Y, Van Buskirk J, Fischer M (2005) A threefold genetic allee effect: Population size affects cross-compatibility, inbreeding depression and drift load in the self-incompatible Ranunculus reptans. *Genetics* **169**, 2255-2265.

Woodell SRJ, Valentine DH (1961) Studies in British Primulas IX. Seed incompatibility in diploid-autotetraploid crosses. *New Phytologist* **60**, 282-295.

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CHAPTER 4

Tetraploid *Rorippa sylvestris* and conspecific diploids as sources of genetic variation in tetraploid *Rorippa amphibia*?

Marc Stift, Rob Bregman, J. Gerard B. Oostermeijer and Peter H. van Tienderen

Abstract

Earlier results indicated that autotetraploid *R. amphibia* harbour more genetic variation than the conspecific diploids, in agreement with expectations from a neutral model of evolution assuming mutation-drift balance. Tetraploids may have attained this variation through a combination of new mutations, recurrent formation of tetraploids, ongoing gene exchange with diploids or introgression of genes from related, tetraploid species. To investigate the latter two processes, we crossed tetraploid *R. amphibia* with conspecific diploids, tetraploid *R. sylvestris* and F1 hybrids. The results indicate that *R. amphibia* diploids are strongly isolated from tetraploids, and that this isolation is postzygotic. However, crosses between diploids and tetraploids sometimes yielded viable progeny that was often tetraploid. This implies that unreduced gametes can not only give rise to new tetraploid lineages, but can also lead to occasional gene flow from diploids to tetraploids. Particularly in a scenario where other compatible partners are absent, gene flow through unreduced gametes from diploid *R. amphibia* could be a source of genetic variation.

We further show that there is hardly any crossing barrier between tetraploids of *R. amphibia* and *R. sylvestris*. We also demonstrate that the F1 hybrids readily backcross to the parental species, so that introgression of genes from tetraploid *R. sylvestris* can form a second source of variation in tetraploid *R. amphibia*.

Introduction

Hybridisation and polyploidisation are considered as major evolutionary forces in plant speciation (Stebbins 1950; Anderson & Stebbins 1954; Grant 1981; Arnold 1997; Rieseberg 1997; Otto & Whitton 2000; Soltis 2005). Often, hybridisation and polyploidisation go together (e.g., Stebbins 1985; Dufresne & Hebert 1994): Many hybrids between diploid species exhibit genomic imbalances (e.g., Lynch & Force 2000; Orr & Turelli 2001) that may be resolved by genome doubling, thus alleviating the postzygotic barrier between the species. Polyploids can be formed directly by somatic doubling or the fusion of unreduced gametes (Karpechenko 1927; Harlan & deWet 1975; Bretagnolle & Thompson 1995). Since the formation of unreduced gametes is a rare event, polyploidisation is often thought to occur through the formation of a 'triploid bridge' generation (Harlan & deWet 1975; deWet 1980; Bretagnolle & Thompson 1995; Ramsey & Schemske 1998).

The evolutionary success of polyploids may depend on their level of genetic variation (Stebbins 1985; Soltis & Soltis 2000). A neutral model of evolution predicts higher levels of variation in tetraploids than in diploids, simply because each tetraploid individual has four instead of two alleles per locus (Moody *et al.* 1993), hence tetraploids have a larger effective population size, reducing the impact of random drift. Newly formed allopolyploids inherit the genes of their parents and thus polyploids depend on the genetic variation present in their ancestors. Newly formed autopolyploids start out with lower variation initially, but may acquire increasing amounts of variation if autopolyploidisation events occur repeatedly (Soltis & Soltis 1993; Soltis & Soltis 2000). However, if the formation of new polyploid individuals is rare, other means of acquiring variantion after their initial formation could become important. Potential sources of variation are gene exchange with diploid ancestors, and hybridisation with closely related other polyploid species.

In this paper, we test whether gene exchange with conspecific diploid *Rorippa amphibia* (L.) Besser and tetraploid *R. sylvestris* (L.) Besser (Brassicaceae) is a likely source of genetic variation for tetraploid *R. amphibia*. *R. amphibia* inhabits ditches and river floodplains throughout Europe. It occurs in diploid and tetraploid forms, with the tetraploids having a more northern distribution (Jonsell 1968). Diploids are found in southern Europe and tetraploids in central and northwestern Europe, with known contact zones in England, France

and southern Germany. Triploids, however, have rarely been observed (Jonsell 1968). The diploid and tetraploid cytotypes are both self-incompatible and highly clonal (Jonsell 1968; Bleeker 2004). Tetraploids can hybridise with tetraploid *R. sylvestris*, sometimes forming hybrid swarms, e.g., along the river Elbe (Bleeker & Hurka 2001). The tetraploids in *R. amphibia* have tetrasomic inheritance (Chapter 2) and are morphologically indistinguishable from diploids (Jonsell 1968), which suggests an autotetraploid origin. Microsatellite variation has been shown to be higher in tetraploids than in diploids, following predictions of neutral theory (Chapter 3). This may indicate that mutation-drift equilibrium has been reached since the origin of the *R. amphibia* tetraploids. However, reaching such equilibrium requires considerable evolutionary time, and alternative explanations for the larger genetic variation of tetraploids, i.e., recurrent formation, ongoing gene flow from diploids or introgression from closely related (i.e. congeneric) tetraploid species, need to be considered. The acquisition of genetic variation, from whatever source, could be enhanced by the fact that the tetraploid *R. amphibia* remained self-incompatible, so that plants with new S-haplotypes always have a selective advantage.

We addressed these issues by experimentally crossing tetraploid *R. amphibia* with conspecific diploids and tetraploid *R. sylvestris*. For each cross, we counted the number of viable and aborted seeds, and the number of unfertilised ovules. In addition, we measured the seed mass and the germination rate from a subsample of each cross.

Given the occurrence of backcrossing hybrid swarms in nature (Bleeker & Hurka 2001), we expected that interspecific crosses (with tetraploid *R. sylvestris*) could be successful. Interploidal intraspecific crosses were expected to suffer from problems inherent to the formation of triploids, i.e., imbalance in the ratio of maternal vs. paternal genes in the developing endosperm, or general genetic imbalances in the coordinated expression of genes in triploids. The presence of such a postzygotic barrier with the diploid ancestor could explain the low incidence of triploids in nature. If so, the only way that tetraploids can pick up variation from the diploids is through unreduced gametes or new polyploidisation events.

Table 1. Overview of the sampling details of the wild collected genotypes that were used in this study. AA: diploid *Rorippa amphibia*; AAAA: tetraploid *R. amphibia*; SSSS: tetraploid *R. sylvestris*. Plants within the same set were crossed according to the setup outlined in Table 3.

Set		AA1	AA2	AAAA1	AAAA2	SSSS1	SSSS2
1	WGS84	N:49°56'00" E:06°12'00"	N:43°46'53" E:04°05'22"	N:48°53'59" E:12°37'19"	N:51°58'52" E:05°02'29"	N:53°21'57" E:10°39'12"	N:50°54'05" W:02°15'01"
	Location	Our, Vianden, Luxembourg	Vidourle, Sommières, France	Danube, Straubing, Austria	Lek, Lopikerkapel, Netherlands	Elbe, Barfurde, Germany	Stour, Child Okeford, United Kingdom
2	WGS84	N:47°44'23" E:05°18'31"	N:47°22'26" W:00°59'15"	N:50°54'43" E:05°42'26'	N:47°14'15" E:20°18'57"	N:46°57'24" E:20°06'54"	N:47°21'41" W:00°42'16"
	Location	L de Villegusien, Longeau, France	Loire, Berthenay, France	Herbricht, Meuse, Belgium	Tisza, Tiszapuspoki, Hungary	Tisza, Nygyed, Hungary	Loire, le Grand Aireau, France
3	WGS84	N:40º14'28" W:08º17'07"	N:47°22'26" W:00°59'15"	N:48°36'52" E:07°49'42"	N:46°46'13" E:17°22'04"	N:47°23'36" W:00°52'08"	N:47°14'15" E:20°18'57"
	Location	Mondego, Rebordosa, Portugal	Loire, Ile Batailleuse, France	Rhine, Kehl, Germany	Lake Balaton, Balatongyörök, Hungary	Loire, Montjean, France	Tisza, Tiszapuspoki, Hungary
4	WGS84	N:47°17'41" E:00°21'01"	N:49°43'00" E:06°30'00"	N:47°50'43" E:16°48'57"	N:50°54'43" E:05°42'26'	N:50°41'26" E:34°38'50"	N:47°17'41" E:00°21'01"
	Location	Loire, Bréhémont, France	Moselle, Wasserbillig, Luxembourg	Neusiedler See, Podersdorf, Austria	Herbricht, Meuse, Belgium	Obolon, Vorozhba, Ukraine	Loire, Bréhémont, France
5	WGS84	N:45º29'33" E:04º49'09"	N:45º03'27" E:11º08'16"	N:51°58'52" E:05°02'29"	N:50°31'47" E:30°32'32"	N:49°43'00" E:06°30'00"	N:50°31'47" E:30°32'32"
	Location	Rhone, Anpuis, France	Po, Ostiglia, Italy	Lek, Lopikerkapel, Netherlands	Dnepr, Kiev, Ukraine	Moselle, Wasserbillig, Luxembourg	Dnepr, Kiev, Ukraine
6	WGS84	N:46°46'13" E:17°22'04"	N:48°09'07" E:07°36'01"	N:46°07'33" E:18°49'29"	N:50°31'47" E:30°32'32"	N:53°14'01" E:10°54'18"	N:56°19'55" E:43°01'09"
	Location	Lake Balaton, Balatongyörök, Hungary	Rhine, Marckolsheim, Germany	Danube, Dunafalva, Hungary	Dnepr, Kiev, Ukraine	Elbe, Darchau, Germany	Volga, Nizhniy Novgorod, Russia
7	WGS84	N:51°30'13" W:01°40'31"	N:49°37'38" E:06°25'25"	N:53°14'01" E:10°54'18"	N:52°26'10" E:04°53'24"	N:46°11'58" E:20°27'51"	N:50°00'44" E:08°03'43"
	Location	Thames, Coate Water, United Kingdom	Moselle, Ahn, Germany	Elbe, Darchau, Germany	't Twiske, Oostzaan, Netherlands	Maros, Mako, Hungary	Rhine, Ruedesheim, Germany

Materials & methods

Collection of plants, ploidy determination and formation of F1 hybrids

During the growing seasons of 2003-2005, rhizomes of *Rorippa amphibia* and *Rorippa sylvestris* were collected from several locations throughout Europe and grown in a greenhouse environment (Table 1). Ploidy levels were determined using a flow cytometer (Partec PA, Münster, Germany). We chopped freshly collected young leaves (approximately 0.5 cm^2) in 50 µL of DAPI staining buffer (05-5001, Partec) and filtered the samples through a 50 µm mesh filter. After 10 minutes of staining in an additional 1000 µL of DAPI staining buffer, we added trout erythrocytes (05-7302, Partec) as an external reference and analysed the sample. In the summer of 2004, we performed reciprocal crosses between tetraploid *R. amphibia* and *R. sylvestris* to generate first generation (F1) hybrids. The parents of the F1 hybrids used in this experiment are indicated in Table 2.

Crossing experiment

We created seven sets of plants, each consisting of two diploid *R. amphibia* (denoted AA in figures and tables), two tetraploid *R. amphibia* (denoted AAAA) and two tetraploid *R. sylvestris* (denoted SSSS). Five of the sets also included two pairs of reciprocal siblings of F1 hybrids *R. amphibia x R. sylvestris* (denoted AASS) and *R. sylvestris x R. amphibia* (denoted SSAA). Plants selected for crossing were placed in a temperature-controlled greenhouse compartment. Temperatures never exceeded 26°C. Plants were watered regularly, so that the soil never dried out completely. The origins of the plants contained in each set are given in Table 1 and 2. During the summer of 2005, we crossed the plants according to the scheme outlined in Table 3. Pollinations were performed by removing anthers that had just dehisced from donor flowers with fine forceps, and rubbing them over the receptive stigma of the target flower. Flower stems were marked with a touch of water-based paint with a colour identifying the father. Each cross was replicated at least ten times using different flowers. At least half of the replications were performed on a different day. Ripe fruits were harvested individually just before dehiscence, i.e., when the carpels started to separate from the false septum, and the fruit started to turn yellow or brown. Of each fruit, we counted the number of seeds, distinguishing viable seeds (i.e., containing an embryo and a regularly developed endosperm) from aborted seeds (i.e., without an embryo or with a poorly developed

Table 2. Overview of the sampling details of the parents (AAAA: tetraploid *R. amphibia*; SSSS: tetraploid *R. sylvestris*) of the hybrids that were used in this study (AASS: tetraploid *R. amphibia x R. sylvestris*; SSAA: tetraploid *R. sylvestris x R. amphibia*). Plants within the same set were crossed according to the setup outlined in Table 3.

		AASS1 and SSAA1		AASS2 and SSAA2	
Set		AAAA parent	SSSS parent	AAAA parent	SSSS parent
1	WGS84	N:53°14'01" E:10°54'18"	N:47°23'36" W:00°52'08"	N:52°21'52" E:04°56'56"	N:47°21'52" W:01°00'58"
	Location	Elbe, Darchau, Germany	Loire, Montjean, France	Flevopark, Amsterdam, Netherlands	Loire, St. Florent, France
3	WGS84	N:52°01'25" E:06°08'42"	N:51°52'02" E:05°59'18"	N:52°37'30" E:06°04'48"	N:50°54'05" W:02°15'01"
	Location	IJssel, Doesburg, Netherlands	Waal, Millingerwaard, Netherlands	Zwarte Water, Zwartsluis, Netherlands	Stour, Child Okeford, United Kingdom
4	WGS84	N:50°54'43" E:05°42'26'	N:53°09'16" E:11°02'47"	N:53°14'01" E:10°54'18"	N:47°49'43" E:02°15'38"
	Location	Herbricht, Meuse, Belgium	Elbe, Hitzacker, Germany	Elbe, Darchau, Germany	Loire, Mesnil, France
5	WGS84	N:53°13'16" E:10°55'53"	N:47°21'41" W:00°42'16"	N:51°14'36" E:06°00'31"	N:49°52'07" E:06°16'04"
	Location	Elbe, Vockfey, Germany	Loire, le Grand Aireau, France	Meuse, Swalmen, Netherlands	Sure, Reisdorf, Luxembourg
6	WGS84	N:52°30'09" E:06°15'36"	N:51°52'48" E:06°00'17"	N:52°37'30" E:06°04'48"	N:50°54'05" W:02°15'01"
	Location	Vecht, Doesburg, Netherlands	Waal, Kekerdom, Netherlands	Zwarte Water, Zwartsluis, Netherlands	Stour, Child Okeford, United Kingdom

endosperm). In addition, we counted the remaining funicules to determine the initial number of ovules. Viable and aborted seeds were stored in separate paper bags and stored in a refrigerator at 7-8 ^{o}C for later use.

Seed biomass and germination assays

We determined the average seed biomass for each cross that had yielded at least 50 seeds, by taking a random sample of 25 seeds from all seeds produced. After weighing them on a microbalance (Sartorius M500P), we put the seeds in petri dishes on filter paper moistened with 2ml of a 3µM gibberellic acid solution to break any dormancy. The dishes were sealed with Parafilm and placed in a growth cabinet (Sanyo MLR-350) at 16h light (36 µE), 23°C,

and 8h dark, 15°C. Only seeds that developed green cotyledons and a root were considered to have germinated. Seedlings were transferred to soil and further grown under standard greenhouse conditions.

Screening of progeny ploidy levels

To identify potential cases of gametic non-reduction, we established the ploidy of the progeny of each cross by flow cytometry. We pooled similar-sized leaf samples of the same seedling batch (never more than 10 samples together). When exceptional ploidy levels were detected in the pool, we took new individual leaf samples and identified which seedling(s) deviated.

Table 3. Crossing scheme showing the crosses performed within one set from the maternal perspective. A set contained two diploid *R. amphibia* (AA), two tetraploid *R. amphibia* (AAAA), two tetraploid *R. sylvestris* (SSSS), and two pairs of reciprocal F1 hybrids *R. amphibia x R. sylvestris* (AASS) and *R. sylvestris x R. amphibia* (SSAA).

Mother		Father				
Type	Code	Diploid *R. amphibia*	Tetraploid *R. amphibia*	Tetraploid *R. sylvestris*	Tetraploid F1 hybrid father F1	
Diploid *R. amphibia*	AA1	AA2	AAAA1	SSSS1		
	AA2	AA1	AAAA2	SSSS2		
Tetraploid *R. amphibia*	AAAA1	AA1	AAAA2	SSSS1	AASS1	SSAA1
	AAAA2	AA2	AAAA1	SSSS2	AASS2	SSAA2
Tetraploid *R. sylvestris*	SSSS1	AA1	AAAA1	SSSS2	AASS1	SSAA1
	SSSS2	AA2	AAAA2	SSSS1	AASS2	SSAA2
Tetraploid F1 (AAAA x SSSS)	AASS1		AAAA1	SSSS1	AASS2	SSAA2
	SSAA1		AAAA1	SSSS1	AASS2	SSAA2
Tetraploid F1 (SSSS x AAAA)	AASS2		AAAA2	SSSS2	AASS1	SSAA1
	SSAA2		AAAA2	SSSS2	AASS1	SSAA1

Statistical analyses

All analyses were performed with SPSS v11.0 for Windows (SPSS Incorporated, Chicago, USA). To compare the seed set (viable and aborted) among all crosses we used ANOVA with cross as the main factor. The design included “set” as a random factor, and the number of

ovules as a covariate. Independent contrasts were designed from the perspective of the mother, testing the effect of different father types (e.g., mother: diploid *R. amphibia*; fathers contrasted: diploid *R. amphibia* vs. tetraploid *R. amphibia*). Effects were tested against the Mean Squares (MS) of the set*cross interaction. To compare the average seed biomass and germination rate we used ANOVA with maternal type and paternal type as the main factors. Since in most cases the interploidal crosses yielded too few seeds for the seed biomass determination and the germination assay, only crosses involving tetraploids were included in this analysis. Independent contrasts were designed to test the effect of maternal type on seed biomass.

Results

From the perspective of the diploid *R. amphibia* mother, pollen from diploid *R. amphibia* fathers yielded more viable seeds than from tetraploid *R. amphibia* or *R. sylvestris* fathers (Table 4). The latter paternal types yielded hardly any viable seeds (Figure 1) and significantly more aborted seeds than diploid fathers (Table 4). The number of aborted seeds from a cross with tetraploid *R. amphibia* was comparable to the number of viable seeds produced after a cross with diploid *R. amphibia* (Figure 1). Tetraploid *R. sylvestris* fathers yielded fewer aborted seeds than tetraploid *R. amphibia* fathers (Table 4).

From the perspective of the tetraploid *R. amphibia* mother, pollen from tetraploid *R. amphibia* and *R. sylvestris* yielded more seeds than from diploid fathers (Table 4).The latter cross resulted in significantly more aborted seeds (Table 4) and yielded hardly any viable seeds (Figure 1). There was no difference in the seed set using pollen from tetraploid *R. amphibia*, *R. sylvestris* or hybrids (Table 4). At the tetraploid level, intraspecific crosses, backcrosses and hybridizations all yielded similar numbers of seeds (Figure 2). The same pattern was found from the perspective of the tetraploid *R. sylvestris* mother (summarised in Figure 2 and Table 4).

From the perspective of the hybrid mothers, the paternal type neither had an effect on the number of viable seeds, nor on seed abortion (Table 4). Crosses with other F1 hybrids and backcrosses with tetraploid *R. amphibia* or *R. sylvestris* all yielded similar numbers of seeds (Figure 2).

Table 4. Overview of independent contrasts used in ANOVA for the number of viable seeds and the number of aborted seeds per fruit. For detailed explanation of the types of contrasts, see text. Father types in bold yielded significantly more viable seeds, underlined father types yielded significantly more aborted seeds. Contrast differences had two degrees of freedom (df) and were tested against the set*cross interaction (df=188, viable: $\sqrt{MS_{error}} = \sqrt{MS_{set*cross}} = 22.3$; aborted: $\sqrt{MS_{error}} = \sqrt{MS_{set*cross}} = 17.2$). The resulting F values are indicated, values in bold are significant at the indicated level (*: p<0.05; **: p<0.005; ***: p<0.0005).

				Viable seeds per fruit			Aborted seeds per fruit		
Mother type	Father types contrasted			Difference	F		Difference	F	
AA	**AA**	-	AAAA	25.0	**23.0**	***	-17.7	**20.1**	***
AA	**AA**	-	SSSS	23.5	**21.5**	***	-7.2	**4.54**	*
AA	AAAA	-	SSSS	-1.5	0.09		10.5	**7.02**	**
AAAA	**AAAA**	-	AA	14.7	**8.76**	***	-12.1	**12.2**	***
AAAA	AA	-	**SSSS**	-13.9	**6.30**	**	13.1	**11.1**	***
SSSS	AA	-	**AAAA**	-20.7	**11.9**	***	14.1	**7.85**	**
SSSS	**SSSS**	-	AA	22.3	**13.1**	***	-14.0	**8.50**	***
AAAA	AAAA	-	SSSS	0.83	0.32		1.01	0.14	
AAAA	AAAA	-	AASS+SSAA	-3.5	1.04		0.64	0.16	
SSSS	SSSS	-	AAAA	1.6	0.45		0.05	0.02	
SSSS	SSSS	-	AASS+SSAA	-2.5	0.93		-0.52	0.03	
AASS	AASS	-	SSAA	-4.5	0.59		1.9	0.18	
AASS	AAAA+SSSS	-	AASS+SSAA	-3.0	0.61		1.3	0.61	
SSAA	SSAA	-	AASS	-1.4	0.62		0.12	0.13	
SSAA	AAAA+SSSS	-	AASS+SSAA	-0.98	0.59		-1.4	0.58	

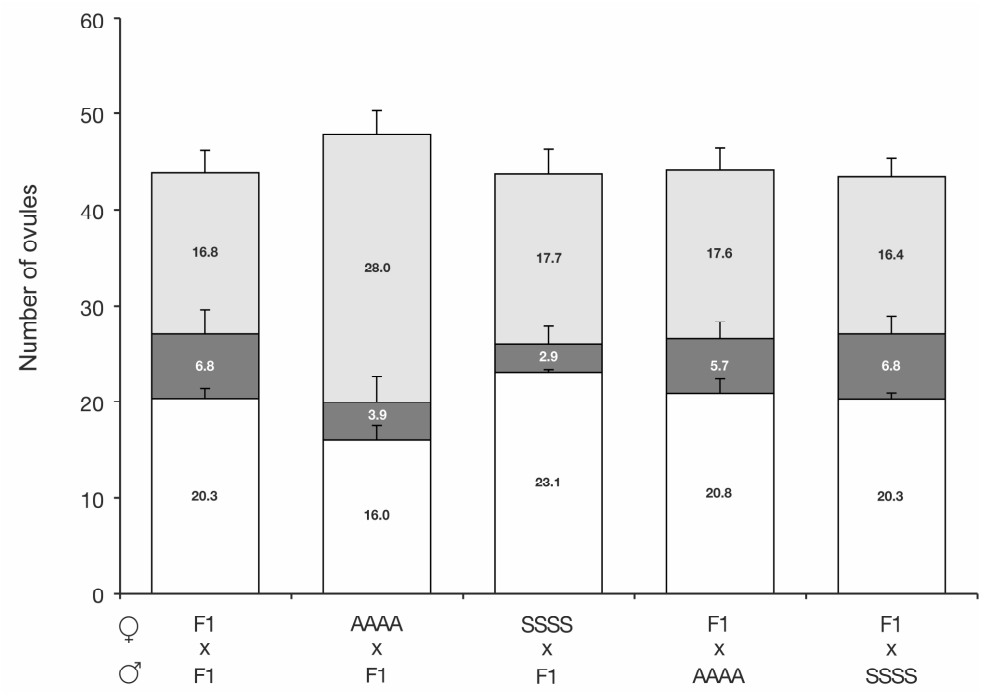

Figure 1. Mean number of viable seeds, aborted seeds and unfertilized ovules per fruit per cross type, error bars indicate the standard error. The first mentioned type is the maternal type: AA: diploid *R. amphibia*, AAAA: tetraploid *R. amphibia*; SSSS: tetraploid *R. sylvestris*.

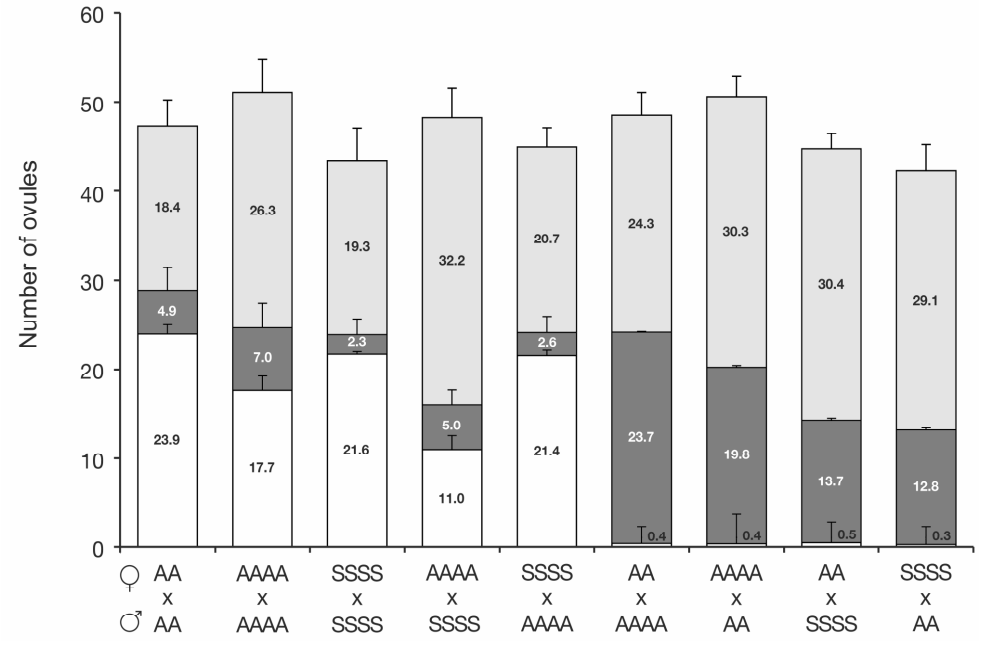

Figure 2. Mean number of viable seeds, aborted seeds and unfertilized ovules per fruit per cross type, error bars indicate the standard error. The type mentioned first is the maternal type: AAAA: tetraploid *R. amphibia*; SSSS: tetraploid; F1: pooled reciprocal first generation hybrids.

Table 5. Summary of ANOVA of seed mass (μg). F values and their associated p-values are given for the main factors. The main factor maternal type has been partitioned into three independent contrasts (AAAA: tetraploid *R. amphibia*; SSSS: tetraploid *R. sylvestris*; AASS: tetraploid F1 hybrid *R. amphibia x R. sylvestris*; SSAA: tetraploid F1 hybrid *R. sylvestris x R. amphibia*.

Factor		Degrees of freedom	Mean Squares	F	p-value
Maternal type		3	$1.567*10^7$	67.0	<0.0005
Contrasts:	AAAA vs SSSS	1	$4.660*10^7$	199	<0.0005
	AASS vs SSAA	1	$3.910*10^6$	1.671	0.198
	(AAAA+SSSS) vs. (AASS+SSAA)	1	$2.506*10^4$	0,107	0.744
Paternal type		3	$1.520*10^5$	0.650	0.584
Maternal type * paternal type		9	$4.126*10^4$	0.176	0.996
Error term		144	$2.340*10^5$		

At the tetraploid level, crosses between *R. sylvestris* plants yielded significantly more viable seeds than crosses between *R. amphibia* plants (Figure 1, ANOVA independent contrast: SSSS x SSSS vs. AAAA x AAAA, $\sqrt{MS_{set*cross}}=22.3$, $F_{[1,188]}=6.57$, $p<0.05$). However, the *R. amphibia* seeds had about twice the mass of tetraploid *R. sylvestris* seeds (Figure 3). The maternal type had a strong effect on seed mass, while the paternal type had no effect (Table 5). Hybrids had a seed mass intermediate to that of the parental species (Figure 3, Table 5). Germination rates (of seeds derived from crosses at the tetraploid level) were always high (95% on average) and not affected by the maternal or paternal type (data not shown).

Table 6. Overview per cross type of all progeny batches in which progeny derived from non-reduction gametes were detected.

Cross type	Non-reduction progeny in batch	Number of offspring in batch
AA x AA	1	19
AAAA x AAAA	1	24
AAAA x SSSS	1	24
AAAA x AASS	1	24
AAAA x SSAA	1	22
SSAA x SSSS	1	25
SSAA x SSAA	1	25
AASS x AASS	1	25
AA x AAAA	3	3
AA x SSSS	21	23
AAAA x AA	1	8
AAAA x AA	9	10
AAAA x AA	7	13
SSSS x AA	4	22
SSSS x AA	2	2

The flow cytometric screening of the progeny of crosses between tetraploids revealed a number of non-reductions that are specified in more detail in Table 6. In crosses between diploid *R. amphibia*, one case of non-reduction (i.e., a triploid in the progeny) was detected. In crosses between tetraploids seven cases of non-reduction (i.e., a hexaploid in the progeny) were detected. Of these, one resulted from a cross between tetraploid *R. amphibia*. Almost all of the (rare) progeny of crosses between a diploid *R. amphibia* mother and a tetraploid father were tetraploid. The (rare) progeny of crosses between tetraploid (*R. amphibia* or *R. sylvestris*) mothers and diploid *R. amphibia* fathers consisted of triploids (32) and tetraploids (23) in various ratios (Table 6).

Discussion

Our results indicated that interploidal crosses in general (i.e., crosses between diploids and tetraploids) yielded only very little viable seeds, irrespective of which ploidy type was used as the maternal plant. The number of aborted seeds produced accounted for the reduction in viable seeds. Apparently, the reproductive isolation between diploid and tetraploid *R. amphibia* has a postzygotic origin. This is a common observation in crosses between diploids and tetraploids (e.g. van Dijk & van Delden 1990; Hardy *et al.* 2001, and reviewed in Haig & Westoby 1991). The observed seed abortion may be due to deviation from the 2:1 maternal to paternal genomic ratio that is required for normal endosperm development (Nishiyama & Inomata 1966; Johnston *et al.* 1980). Seeds that result from the fusion of haploid ovules with diploid pollen (from a tetraploid donor) will have a ratio in the

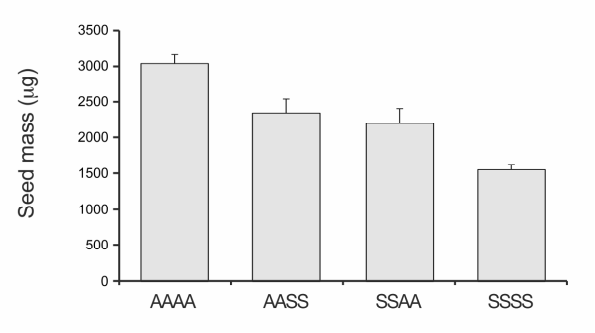

Figure 3. Mean seed mass (µg) of each maternal type averaged over all tetraploid paternal types, error bars indicate the standard error.

endosperm of 2m:2p, while the reciprocal cross will have a 4m:1p ratio, which both could lead to abnormal endosperm development (Johnston *et al.* 1980; Haig & Westoby 1991).

In crosses between diploid *R. amphibia* and tetraploid *R. sylvestris*, the increase in number of aborted seeds did not account completely for the reduction in viable seeds. This suggests that the crossing barrier also has a prezygotic component. Prezygotic barriers can be related to disturbed pollen-pistil interactions (Swanson *et al.* 2004). Our results did not indicate any prezygotic isolation between tetraploids of *R. amphibia* and *R. sylvestris*. However, we crossed plants by applying ample pollen from a single donor plant, thereby (deliberately) excluding pollen competition, pollen limitation, and premating isolation from our experiment. This means that some prezygotic barriers to crossing may have remained unnoticed. Differences in flower morphology and insect foraging greatly reduced the frequency of interploidal mating in *Chamerion angustifolium*, (Husband & Schemske 2000; Husband & Sabara 2004) and *Heuchera grossulariifolia* (Segraves & Thompson 1999). Pollen competition imposed an additional unilateral prezygotic barrier in *C. angustifolium*. On both diploid and tetraploid mother plants, pollen from tetraploids sired more seeds than pollen from diploids (Husband *et al.* 2002). Pollen competition and insect visitation have also proven to be important factors in determining the likelihood and direction of interspecific crossing (i.e., hybridisation) in several well studied systems: e.g *Iris* (Arnold *et al.* 1993; Emms *et al.* 1996; Carney & Arnold 1997), *Mimulus* (Diaz & Macnair 1999) and *Helianthus* (Rieseberg *et al.* 1995). Such factors remain to be studied in *Rorippa*.

The low success of interploidal crosses in *Rorippa* may suggest that gene exchange with diploids forms an unlikely source of variation for newly formed tetraploid *R. amphibia*. However, our assay of progeny ploidy levels revealed the occurrence of unreduced gametes. Unreduced gametes can contribute to tetraploid genetic variation in three ways.

First, fusion of (diploid) unreduced gametes from diploids with normal (diploid) gametes from tetraploids can lead to direct gene flow (Marks 1966). Our results indicated that the few seeds produced by interploidal crosses were more often tetraploid than triploid, and thus must

have involved unreduced gametes. In a natural setting, this phenomenon may be particularly relevant in situations where compatible partners are limited or absent, which can be expected to occur in the self-incompatible and highly clonal *R. amphibia*. For a tetraploid *R. amphibia* clone that is surrounded by diploids, unreduced gametes from diploids may well provide the only opportunity to produce viable sexual offspring.

Second, tetraploids may form repeatedly through fusion of two unreduced gametes (Harlan & deWet 1975). This may mean that newly formed tetraploids start out with more initial variation than under a scenario of a single origin, and may not suffer from any 'formation bottleneck' (Soltis & Soltis 1999). We detected only one triploid in the progeny of crosses between diploid *R. amphibia*, and not a single tetraploid. Such low numbers do not tell a lot, but it may be a first indication that in *Rorippa* the frequency of non-reduction is low and that the formation of new tetraploids via an intermediate triploid generation is more likely that by fusion of two unreduced gametes (Harlan & deWet 1975; deWet 1980; Bretagnolle & Thompson 1995). The frequency of non-reduction that we detected in this study may well underestimate the frequency of non-reduction under natural conditions. It has been shown that extreme temperatures may increase the frequency of non-reduction (reviewed in Ramsey & Schemske 1998).

We detected seven cases of non-reduction (hexaploids) in the progeny of tetraploid x tetraploid crosses. This supports the hypothesis that the hexaploids of *R. sylvestris* that occur in the wild (Jonsell 1968) have multiple origins and probably still form repeatedly. Our data provide no evidence that non-reduction is more likely in tetraploids than in diploids. Although the absolute number of unreduced gametes (hexaploids) in the offspring of tetraploid x tetraploid crosses (seven) was higher than in the progeny of diploid x diploid crosses (one), this probably merely reflects the much larger number of tetraploid x tetraploid crosses included in our design (each set contained two diploid x diploid crosses vs. 32 tetraploid x tetraploid crosses).

In contrast to the intraspecific reproductive isolation between diploid and tetraploid *R. amphibia*, our results show that there is hardly any interspecific reproductive isolation between tetraploids of *R. amphibia* and *R. sylvestris*, in agreement with the occurrence of hybrids in different river systems where they co-occur (Elbe: Bleeker & Hurka (2001), Wisla and Dnepr: personal observations). This means that introgression from other species forms a likely source of variation of newly formed tetraploid lineages. The direction of the introgression process could depend on reciprocal differences in the seed production of the

crosses. The number of seeds per fruit after pollination with the other species was lower in *R. amphibia* than in *R. sylvestris* mothers, while germination rates of the seeds were similar (95% for both species). This would bias the introgression process towards *R. sylvestris*, but on the other hand seeds produced by *R. amphibia* mothers were heavier, so seed number could be traded-off against seed weight and hence seedling survival or recruitment (Eriksson & Jakobsson 1999; Moles & Westoby 2006). In any case, strong directional introgression does not seem likely given the magnitude of these differences. Our data further show that backcrosses and crosses between F1 hybrids all have a normal seed set. This indicates that hybrids are by no means less fit than the parents and not reproductively isolated from the parental species. Hybrids are likely to overlap in habitat with the parents, given the experimental responses to flooding of hybrids and parents (Chapter 5). A lack of ecological divergence, and the absence of crossing barriers suggest that backcrossing and further introgression are likely consequences of hybridisation in the *Rorippa x anceps* hybrid complex.

It would be nice if the relative importance of the different processes in generating diversity in the tetraploids could be quantified using molecular data and sharing of alleles between the different species and cytotypes. Tetraploid *R. amphibia* possessed a class of microsatellite allele lengths that was absent in diploids (Chapter 3). A preliminary screen found now evidence that this class of alleles could was derived from hybridisation with *R. sylvestris*. However, only eight *R. sylvestris* genotypes were analysed, and clearly more work is needed.

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References

Anderson, E. & Stebbins, G.L. (1954) Hybridization as an evolutionary stimulus. *Evolution*, **8**, 378-388.

Arnold, M.L. (1997) *Natural hybridization and evolution*. Oxford University Press, Oxford, UK.

Arnold, M.L., Hamrick, J.L. & Bennett, B.D. (1993) Interspecific pollen competition and reproductive isolation in Iris. *Journal of Heredity*, **84**, 13-16.

Bleeker, W. (2004) Genetic variation and self-incompatibility within and outside a Rorippa hybrid zone (Brassicaceae). *Plant Systematics and Evolution*, **246**, 35-44.

Bleeker, W. & Hurka, H. (2001) Introgressive hybridization in *Rorippa* (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. *Molecular Ecology*, **10**, 2013-2022.

Bretagnolle, F. & Thompson, J.D. (1995) Tansley Review No-78 - Gametes with the somatic chromosome-number - mechanisms of their formation and role in the evolution of autopolyploid plants. *New Phytologist*, **129**, 1-22.

Carney, S.E. & Arnold, M.L. (1997) Differences in pollen-tube growth rate and reproductive isolation between Louisiana Irises. *Journal of Heredity*, **88**, 545-549.

deWet, J.M.J. (1980) Origins of polyploids. *Polyploidy: biological relevance*. (eds W.H. Lewis). Plenum Press, New York and London.

Diaz, A. & Macnair, M.R. (1999) Pollen tube competition as a mechanism of prezygotic reproductive isolation between *Mimulus nasutus* and its presumed progenitor *M-guttatus*. *New Phytologist*, **144**, 471-478.

Dufresne, F. & Hebert, P.D.N. (1994) Hybridization and Origins of Polyploidy. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **258**, 141-146.

Emms, S.K., Hodges, S.A. & Arnold, M.L. (1996) Pollen-tube competition, siring success, and consistent asymmetric hybridization in Louisiana Irises. *Evolution*, **50**, 2201-2206.

Eriksson, O. & Jakobsson, A. (1999) Recruitment trade-offs and the evolution of dispersal mechanisms in plants. *Evolutionary Ecology*, **13**, 411-423.

Grant, V. (1981) *Plant Speciation*. Columbia University Press, New York.

Haig, D. & Westoby, M. (1991) Genomic imprinting in endosperm - its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **333**, 1-13.

Harlan, J. & deWet, J.M.J. (1975) On Ö. Winge and a prayer: the origins of polyploidy. *The Botanical Review*, **41**, 361-390.

Husband, B.C. & Sabara, H.A. (2004) Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae). *New Phytologist*, **161**, 703-713.

Husband, B.C. & Schemske, D.W. (2000) Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. *Journal of Ecology*, **88**, 689-701.

Husband, B.C., Schemske, D.W., Burton, T.L. & Goodwillie, C. (2002) Pollen competition as a unilateral reproductive barrier between sympatric *Chamerion angustifolium*. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **269**, 2565-2571.

Johnston, S.A., den Nijs, T.P.M., Peloquin, S.J. & Hanneman, R.E. (1980) The significance of genic balance to endosperm development in interspecific crosses. *Theoretical and Applied Genetics*, **57**, 5-9.

Jonsell, B. (1968) Studies in the North-West European species of *Rorippa s. str. Symbolae Botanicae Upsalienses*, **19**, 1-221.

Karpechenko, G.D. (1927) The production of polyploid gametes in hybrids. *Hereditas*, **9**, 349-368.

Lynch, M. & Force, A.G. (2000) The origin of interspecific genomic incompatibility via gene duplication. *American Naturalist*, **156**, 590-605.

Marks, G.E. (1966) The origin and significance of intraspecific polyploidy: experimental evidence from *Solanum chacoense*. *Evolution*, **20**, 552-557.

Moles, A.T. & Westoby, M. (2006) Seed size and plant strategy across the whole life cycle. *Oikos*, **113**, 91-105.

Moody, M.E., Mueller, L.D. & Soltis, D.E. (1993) Genetic variation and random drift in autotetraploid populations. *Genetics*, **134**, 649-657.

Nishiyama, I. & Inomata, N. (1966) Embryological studies on cross incompatibility between 2x and 4x in *Brassica*. *Japanese Journal of Genetics*.

Orr, H.A. & Turelli, M. (2001) The evolution of postzygotic isolation: Accumulating Dobzhansky-Muller incompatibilities. *Evolution*, **55**, 1085-1094.

Otto, S.P. & Whitton, J. (2000) Polyploid incidence and evolution. *Annual Review of Genetics*, **34**, 401-437.

Ramsey, J. & Schemske, D.W. (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics*, **29**, 467-501.

Rieseberg, L.H. (1997) Hybrid origins of plant species. *Annual Review of Ecology and Systematics*, **28**, 359-389.

Rieseberg, L.H., Desrochers, A.M. & Youn, S.J. (1995) Interspecific pollen competition as a reproductive barrier between sympatric species of *Helianthus* (Asteraceae). *American Journal of Botany*, **82**, 515-519.

Segraves, K.A. & Thompson, J.N. (1999) Plant polyploidy and pollination: Floral traits and insect visits to diploid and tetraploid *Heuchera grossulariifolia*. *Evolution*, **53**, 1114-1127.

Soltis, D.E. & Soltis, P.S. (1993) Molecular data and the dynamic nature of polyploidy. *Critical Reviews in Plant Sciences*, **12**, 243-273.

Soltis, D.E. & Soltis, P.S. (1999) Polyploidy: recurrent formation and genome evolution. *Trends in Ecology & Evolution*, **14**, 348-352.

Soltis, P.S. (2005) Ancient and recent polyploidy in angiosperms. *New Phytologist*, **166**, 5-8.

Soltis, P.S. & Soltis, D.E. (2000) The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 7051-7057.

Stebbins, G.L. (1950) *Variation and evolution in plants*. Columbia University Press, New York & London.

Stebbins, G.L. (1985) Polyploidy, hybridization, and the invasion of new habitats. *Annals of the Missouri Botanical Garden*, **72**, 824-832.

Swanson, R., Edlund, A.F. & Preuss, D. (2004) Species specificity in pollen-pistil interactions. *Annual Review of Genetics*, **38**, 793-818.

CHAPTER 5

Flooding responses in *Rorippa amphibia*, *R. sylvestris* and their artificial F1 hybrids

Marc Stift, Pieternella C. Luttikhuizen, Eric J.W. Visser and Peter H. van Tienderen

Abstract

We studied the potential ecological divergence of *Rorippa amphibia*, *R. sylvestris* and their F1 hybrids with respect to three water treatments mimicking conditions in the parental habitats. The first goal was to identify potential species differences in responses associated with flooding tolerance. The second goal was to assess the extent to which hybrids differ in phenotype from the parents, and whether this could potentially lead to ecological divergence and reproductive isolation between parents and hybrids. Corresponding to the expectations given their natural habitats, waterlogging had hardly any effect on the growth of *R. amphibia*. *R. sylvestris* grew fastest under well-drained conditions, while growth was reduced upon waterlogging. Both species established shoot growth over two weeks of submergence. In *R. sylvestris*, this shoot growth was not associated with a loss of root biomass, suggesting that *R. sylvestris* is capable of underwater photosynthesis, whereas in *R. amphibia* carbohydrate reserves may have been depleted. Hybrids resembled *R. amphibia* in their growth response. Furthermore, *R. sylvestris* showed more pronounced leaf morphological changes than *R. amphibia*, suggesting a hyponastic response to submergence. Hybrids were intermediate in leaf morphology. In contrast to *R. sylvestris*, *R. amphibia* readily formed adventitious and above ground roots, which may be adaptive under waterlogged conditions. Hybrids resembled *R. amphibia* in this respect. The phenotypic responses of hybrids suggest that a large overlap in habitat use of parents and hybrids is likely. If so, the main evolutionary consequence of hybridisation in *Rorippa* will be the introgression of genes, as the hybrids are fully fertile. The mosaic of habitats of some natural floodplains appears to be conducive for hybridisation. Our study shows that the average hybrid trait ranges are not always intermediate to the parental trait ranges. This is perhaps surprising for growth related traits that are most likely determined by many genes, and for which additivity would be expected on average.

Introduction

Interspecific hybridisation is considered by many to be a process with important evolutionary consequences, both in plants and animals (Stebbins 1950; Anderson & Stebbins 1954; Arnold 1992; 1997; Dowling & Secor 1997; Rieseberg 1997; Seehausen 2004, but see also Schemske 2000). A first and crucial step in the process involves the creation of F1 hybrids. Their viability, fertility and ecological position combined with the availability of a suitable habitat determines whether hybrid formation is incidental and without further consequences, or a route to the formation of a permanent hybrid swarm or the origin of a new evolutionary lineage.

If hybrids are viable, fertile, and not (completely) reproductively isolated from their parents hybridisation may lead to the formation of hybrid swarms by repeated backcrossing (Barton & Hewitt 1985). Hybridisation may result in the introgression of genes (Anderson & Hubricht 1938; Anderson 1949; Stebbins 1950; Rieseberg & Wendel 1993; Arnold 2004): new allelic combinations that give rise to novel phenotypes (Arnold 1992, 1997; Rieseberg *et al.* 2000). The evolutionary consequence of this process will depend on the selective advantage that such novel combinations have in a potential hybrid habitat, and the proximity and availability of such a habitat (Anderson 1948; Stebbins 1950; Anderson & Stebbins 1954).

In many cases, however, hybrids will be reproductively isolated from the parents, preventing introgression. Depending on the fitness of hybrids, the process may stop at the F1 stage, or continue, potentially leading to hybrid speciation (Arnold 1992, 1997; Rieseberg 1997). There are several pocesses that may cause reproductive isolation. First, allopolyploid hybrids (Stebbins 1947; Winge 1917, as cited in Rieseberg 1997) become reproductively isolated from their parents instantaneously (see Arnold 1997; Ramsey & Schemske 1998; Soltis & Soltis 1999; Otto & Whitton 2000; Ramsey & Schemske 2002; Soltis *et al.* 2004 for extensive reviews on (allo)polyploid speciation). Second, homoploid hybrids may be reproductively isolated through chromosomal rearrangements and karyotypic or genic incompatibilities (but see Yatabe *et al* 2007). There are several examples of karyotypic divergence of hybrid species with respect to their parents (Rieseberg 1997; Buerkle *et al.* 2000), although this does not prove that the karyotypic differentiation has been the cause and

not the consequence of reproductive isolation (Coyne & Orr 1998). Third, ecological divergence (whether or not followed by secondary karyotypic divergence) can also lead to reproductive isolation of hybrids (Buerkle *et al.* 2000; Schwarzbach *et al.* 2001; Rosenthal *et al.* 2002; Welch & Rieseberg 2002; Johnston *et al.* 2004; Gross & Rieseberg 2005). This may arise when the hybrid phenotype allows them to grow in different habitats, or if hybrids differ in in phenology from the parents.

The parents in our study system are the self-incompatible perennials *Rorippa amphibia* and *R. sylvestris*, both commonly occurring members of the floodplain vegetation along all major rivers in Europe. The species' distributions within the floodplain suggest that they have become adapted to micro-habitats with different flooding regimes (Blom 1999). The former species occurs in sites with more stable water tables mostly as emergent plants, or in dense, constantly moist grassland vegetation. The latter prefers more open, ephemeral sites on riverbanks that can be flooded for longer periods, but can also dry out completely in summer (Jonsell 1968; Blom 1999).

Morphologically, the *Rorippa* species can be distinguished easily. *R. amphibia* has large leaves with big endlobes, while *R. sylvestris* has smaller, more pinnate leaves. Both can regenerate vegetatively from root and stem fragments, albeit that *R. sylvestris* does so more vigorously (Jonsell 1968).

Since sedimentation and erosion shape river floodplains into a mosaic of habitats, the species often grow in sympatry. The species are interfertile at the tetraploid level and the hybrid *Rorippa x anceps* (Wahlenb.) Rchb. can easily be generated and backcrossed with both parents in the greenhouse (Jonsell 1968, Chapter 4). In several locations along the rivers Elbe and Wisla (among others), the presence of a range of intermediate morphologies suggests that hybridisation and backcrossing has led to the formation of hybrid swarms. Molecular analyses of AFLP and chloroplast DNA sequences (*trn*L/F spacer) (Bleeker & Hurka 2001; Bleeker & Matthies 2005) and patterns of microsatellite variation of both parental species and putative hybrids (unpublished data E.H. McLean) have confirmed these morphological indications of introgressive hybridisation along the Elbe river. The availability of hybrids, and *Rorippa*'s position within the tribe Cardamineae (Al-Shehbaz *et al.* 2006), that belongs to the same phylogenetic lineage (I) as the genomic model species *Arabidopsis thaliana* (Beilstein *et al.* 2006) provide excellent opportunities for comparative genomics (Schranz *et al.* 2007). As such, *Rorippa* may function as a model system to unravel the genetic basis of traits that are associated with flooding tolerance.

The first aim of this research was to quantify the reactions of *R. amphibia* and *R. sylvestris* in coping with different flooding regimes. We measured growth, biomass allocation, dry/fresh weight ratios and leaf morphological traits under three different flooding regimes: well drained, waterlogged and fully submerged. In concordance with its stable wet to waterlogged habitat, we predicted that *R. amphibia* would grow well in both drained and waterlogged treatments, but that *R. sylvestris* would exhibit the most vigorous growth under well drained conditions and a growth reduction under waterlogged conditions. We expected submergence to reduce growth in both species and to affect leaf morphology and positioning (Voesenek *et al.* 2006). However, we hypothesised that *R. amphibia* shoots would grow more when submerged in an attempt to reach the surface, thus possibly depleting available carbohydrate resources (cf. *Rumex palustris*; Groeneveld & Voesenek 2003); such a strategy would be advantageous in a habitat where flooding is normally shallow and predictable. In contrast, we expected that *R. sylvestris* would arrest growth (thus storing carbohydrate reserves) and passively wait for better times, perhaps in a state of anaerobic dormancy (Laan & Blom 1990; Vartapetian & Jackson 1997). This would be advantageous in a habitat where flooding is deep and less predictable in duration. We also expected that *R. sylvestris* would generally allocate more biomass to roots, increasing its regeneration capacity after surviving adverse conditions (e.g. waterlogging and submergence).

The second aim of this research was to assess how the parental traits associated with the flooding regimes are expressed in first generation (F1) hybrids obtained from greenhouse crosses between wild-collected plants of both species. We asked whether their phenotypic expression is conducive for ecological divergence. More specifically, we tested whether F1 hybrids were intermediate with respect to the parental species (i.e., whether the expression of parental traits showed "genomic additivity"), or mostly resembled one of the parents ("genomic dominance"), or had trait values beyond those of either parent ("genomic overdominance"). In the case that genomic additivity prevails, physical isolation between F1's and their parents would be possible, but only if an intermediate habitat is available where the parental species are absent (Anderson 1948) or outcompeted by the hybrids. In the case that genomic dominance prevails, it is likely that the F1 hybrid's niche overlaps with the parent it resembles. This would make directional introgression toward this parent more likely than to the other (Anderson & Hubricht 1938). Finally, overdominance could lead to extreme hybrid phenotypes beyond that of either parent (Falconer 1960) and potentially to extreme

habitat preferences; in F1 hybrids this could be caused by complementary gene action or changed epistatic interactions (Rieseberg *et al.* 1996). Of course, in further hybrid generations, transgressive segregation can lead to even more extreme hybrid phenotypes by creating novel combinations of alleles (Rieseberg *et al.* 1999). Irrespective of the phenotypic expression of individual traits in the F1 hybrids relative to the parent, hybrids may exhibit a combination of traits not present in either of the parental species that allows them to grow and reproduce under different conditions than the parents (Anderson & Stebbins 1954; Arnold 2004).

Knowledge of how putatively adaptive traits (in this case associated with the different habitat preferences of *R. amphibia* and *R. sylvestris*) are expressed in F1 hybrids will contribute to answering broader evolutionary questions regarding the long-term consequences of hybridisation. Are hybrids merely present because of their constant formation in disturbed habitats (Schemske 2000), or do they have the potential to occupy a specific novel niche (Arnold 1997)? Do the ecological characteristics of first generation hybrids promote reproductive isolation, which might eventually lead to a separate hybrid lineage or even species (Rieseberg 1997; Buerkle *et al.* 2000)? Or is it more likely that it will set the stage for backcrossing (to one or both parental species), making introgression and gene exchange the most important consequence of hybridisation (Anderson & Hubricht 1938)?

Materials and methods

Plant material

During the growing seasons of 2002 and 2003, rhizomes of tetraploid *R. amphibia* (denoted A in Figures and Tables) and *R. sylvestris* (denoted S) were collected from several locations throughout Europe and grown in a greenhouse environment since. In the summer of 2004, reciprocal crosses were carried out between six independent pairs of *R. amphibia* and *R. sylvestris* of different origins (Table 1) to obtain reciprocal F1 hybrids (denoted AS and SA in Figures and Tables). In December 2005, of each of these 12 crosses five randomly picked seeds were germinated on sterile filter paper moistened with 2ml of a 3µM gibberellic acid solution. Seedlings were transferred to soil and placed in the same greenhouse compartment as the parental genotypes. In March 2006, we selected one genotype from each of the 12 F1 sibling groups (6 genotypes *R. amphibia* x *R. sylvestris*, 6 genotypes *R. sylvestris*

x *R. amphibia*). From these, and the 12 corresponding parental genotypes (6 genotypes *R. amphibia*, 6 genotypes *R. sylvestris*), we obtained uniform, similar sized rosettes by placing root fragments washed in a 0.5 g/L hypochlorite solution into petri dishes containing half strength Murashige and Skoog Basal medium with Gamborg's vitamins (Sigma) at pH 5.6 with 0.8% purified agar (Hispanagar). The roots were left to sprout for five full days in a growth cabinet (Sanyo MLR-350) at 16h light (36 µE), 23°C, and 8h dark, 15°C. Of each genotype 40 similar sized single sprouts were cut off and transferred to trays with net pots (55 mm diameter) containing sterilised sand (0.5-1.0 mm grain size, Filcom BV, Papendrecht, The Netherlands) drained with 0.5 g/L nutrient solution (Peat Lite special, Peters Professional). After three weeks in the greenhouse, 16 rosettes of each genotype were selected, based on uniformity in leaf number (4-5 leaves) and transferred to individual pots (100 mm diameter) containing the same sterilised sand with 5 grains per pot of controlled release fertiliser (Osmocote Plus 15+11+13+2MgO+Trace elements, Peters Professional). Four of the 16 rosettes were randomly assigned to each of three water treatments and an initial harvest group. The whole procedure was repeated two weeks later so that eight replicates were obtained per genotype.

Table 1. Sampling details of the six pairs of tetraploid genotypes of *R. amphibia* and *R. sylvestris* that were used in this study.

	R. amphibia			*R. sylvestris*		
Pair	River	Location[1], Country	Latitude Longitude[2]	River	Location[1], Country	Latitude Longitude[2]
1	Meuse	Herbricht, Belgium	N: 50°54'43" E: 05°42'26'	Elbe	Hitzacker, Germany	N: 53°09'16" E: 11°02'47"
2	Meuse	Swalmen, Netherlands	N: 51°14'36" E: 06°00'31"	Sure	Reisdorf, Luxembourg	N: 49°52'07" E: 06°16'04"
3	IJssel	Doesburg, Netherlands	N: 52°01'25" E: 06°08'42"	Rhine	Millingerwaard, Netherlands	N: 51°52'02" E: 05°59'18"
4	Zwarte Water	Zwartsluis, Netherlands	N: 52°37'30" E: 06°04'48"	Stour	Child Okeford, United Kingdom	N: 50°54'05" W: 02°15'01"
5	Elbe	Darchau, Germany	N: 53°14'01" E: 10°54'18"	Loire	Mesnil, France	N: 47°49'43" E: 02°15'38"
6	Elbe	Darchau, Germany	N: 53°14'01" E: 10°54'18"	Loire	Montjean, France	N: 47°23'36" W: 00°52'08"

[1] Settlements closest to the exact sampling location

[2] WGS84 coordinates

WATER TREATMENTS

Plants were subjected to three treatments mimicking conditions prevailing in the natural habitat: well drained (DRN), waterlogged (LOG) with a constantly inundated soil and an emerging shoot, and completely submerged (SUB). Treatments were applied in white plastic buckets (diameter: 30cm, height: 27cm, volume: 16L, Nipak BV, Papendrecht, The Netherlands) with overflow holes drilled at an appropriate height. Each bucket contained one plant, treatments lasted two weeks. The height and diameter of the buckets were such that leaves neither touched the wall nor reached the water surface (in the SUB-treatment) throughout the experiment. At the start of the experiment, all buckets were filled simultaneously with rain water. After one week, and after that daily, the water in all buckets was simultaneously flushed for three hours in order to control algal growth and nutrient accumulation. Throughout the experiment, temperature was regulated to stay between 20 and 23 °C during the 16h daylight (SON-T Agro 400W, Philips), and between 15 and 17 °C during the night.

RESPONSE VARIABLES

Response variables were assessed for each of the experimental plants at the start and end of the treatment period, unless specified otherwise. We estimated the length (mm) of the above ground roots (roots protruding from the soil surface) and counted the number of adventitious roots (roots developing from the leaf axils), and the number of leaves. From this, we calculated the number of leaves that formed during the experiment. Furthermore, for the longest leaf (LLF), we assessed the following leaf morphological traits (Figure 1): the length (LENLLF, mm), width (WIDLLF, mm) and petiole length (PETLLF, mm). From these, we calculated the blade width/length ratio (BLADE SHAPE ≡ WIDLLF/(LENLLF-PETLLF)) and the petiole length relative to the total length (PETLLF/LENLLF). We measured the angle between the horizontal plane and the base of the petiole of the longest leaf (degrees) and the youngest leaf (degrees), such that angles could

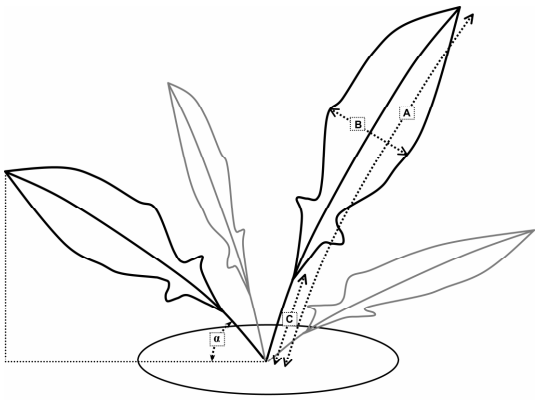


Figure 1. Schematic representation of a rosette of *Rorippa*. Measures that were taken on the longest leaf are indicated by dotted arrows: A: length; B: width; C: petiole length. A dotted line indicates the projection of the youngest leaf used to obtain a standardised measure of α: the leaf angle of the youngest leaf.

range from 0º (prostrate orientation) to 90º (upright orientation) (Figure 1). Then, after washing off all sand particles from roots and shoots and surface drying all material by softly pressing with dry tissue paper, we immediately assessed fresh weight of roots (FWR, g) and shoots (FWS, g). Finally, two random plants of each genotype were dried at 65ºC for 72 hours for assessment of dry weight (DW) of shoots (DWS, g) and roots (DWR, g). Similarly, DW had been assessed at the start of the experiment for the four plants of each genotype that had been assigned to the initial harvest group. From the DW measures, we calculated the relative growth of the shoot and root in the two-week period ($RG_{shoot} \equiv \ln DWS_{end} - \ln DWS_{start}$; $RG_{root} \equiv \ln DWR_{end} - \ln DWR_{start}$). Furthermore, we calculated the shoot weight fraction (SWF ≡ DWS/(DWS + DWR)) and the shoot dry matter content (SDMC ≡ DWS/FWS) for the plants at the end of the experiment. Arcsine square root or logarithmic transformations were applied where appropriate in order to approach normal distribution and variance homogeneity within groups.

Experimental design

The experiment had a split plot design with genomic class (A, S, AS, SA) and treatment (DRN, LOG, SUB) as the main fixed factors. Each table contained one replicate of each set (i.e., one crossed pair of *R. amphibia* and *R. sylvestris*, and their reciprocal F1 hybrid offspring) within each treatment. Table and set were treated as random factors.

Statistical analysis

All analyses were performed with SPSS v11.0 for Windows (SPSS Incorporated, Chicago, USA). We used ANOVA independent contrasts to compare trait expression of parents and both hybrid classes. Due to the skewed distribution of observations (i.e., many zeros), the number of adventitious roots and the length of the above ground roots were analysed in a separate non-parametric Kruskal-Wallis ANOVA procedure, using post-hoc Mann-Whitney U tests to test for pairwise differences among parents and hybrids.

Contrasting parental species and reciprocal hybrids

First, we contrasted the trait values of the parental species (A vs. S) for each treatment separately. Then, we tested for differences in the slope of the reaction norms of both parental species going from DRN to LOG and from LOG to SUB treatments. In the same manner, we contrasted the two reciprocal hybrid genomic classes (AS vs. SA).

Evaluation of expression of parental traits in F1 hybrids

If the parental species differed in their trait values and/or reaction norms, we proceeded to evaluate the hybrid trait values. We specifically tested hypotheses of dominance or partial dominance (i.e., hybrids resemble *R. amphibia* or *R. sylvestris*), additivity (i.e., hybrids are intermediate) and overdominance (i.e., hybrid trait values lie beyond the parental trait range). For this purpose, we defined "H" as the dominance coefficient for the hybrid's trait, with H=0 and H=1 representing the situation in which the hybrid is identical to parent *R. amphibia* and *R. sylvestris*, respectively, and H=1/2 representing the situation in which the hybrid is exactly intermediate. We then calculated this value for our experimental data (denoted $\hat{H}$) by rescaling the estimated mean trait values for the hybrids and their 95% confidence interval (CI) limits to the difference between the mean parental trait values. Finally, we evaluated whether H=0, H=0.5 and H=1 and were part of $\hat{H} \pm 95\%$ CI. If not, we rejected dominance and additivity, and further evaluated whether there was partial dominance of *R. amphibia* ($0 < \hat{H} \pm 95\%$ CI < 0.5) or *R. sylvestris* ($0.5 < \hat{H} \pm 95\%$ CI < 1), or overdominance ($\hat{H} \pm 95\%$CI < 0 or $1 < \hat{H} \pm 95\%$ CI).

Results

INITIAL MEASUREMENTS

Table 2 summarises all initial measurements and statistical evaluations (ANOVA independent contrasts). At the start of the experiment, the total DW of *R. amphibia* was around twice that of *R. sylvestris*. The longest leaf of *R. amphibia* had a much shorter relative petiole and a more elongated blade shape. *R. sylvestris* had more leaves. There were no significant differences in trait values among reciprocal hybrids (*R. amphibia* x *R. sylvestris* vs. *R. sylvestris* x *R. amphibia*, data not shown), except for the number of leaves, which was somewhat higher for the *R. sylvestris* x *R. amphibia* (*R. sylvestris* mother) hybrids (*R. amphibia* x *R. sylvestris*: 4.52, *R. sylvestris* x *R. amphibia*: 4.83, $F_{[1;567]}=11.1$, $p<0.005$). The F1 hybrids resembled *R. amphibia* (i.e., H=0 within $\hat{H} \pm 95\%$ CI) in total DW and *R. sylvestris* (i.e., H=1 within $\hat{H} \pm 95\%$ CI) in leaf number (Table 2). Hybrid blade shape of the longest leaf resembled that of *R. amphibia* (Table 2). The relative petiole length of the longest leaf was perfectly intermediate (i.e., H=0.5 within $\hat{H} \pm 95\%$ CI). For the length of the longest leaf and the angle of the youngest leaf, overdominance (H=0, H=0.5 and H=1 not within $\hat{H} \pm 95\%$ CI) was observed. Hybrids had a longer longest leaf and a more prostrate leaf orientation than either of the parental species (Table 2).

Table 2. Summary of whole plant and leaf morphological traits measurements (units) at the start of the experiment. Untransformed mean trait values for each genomic class (A: *R. amphibia*; AS: *R. amphibia x R. sylvestris*; SA: *R. sylvestris x R. amphibia*; S: *R. sylvestris*). F-value of ANOVA independent contrasts (A vs. S, bold indicates significance at the indicated level: *p<0.05; **p<0.005; ***p<0.0005). The square root of the Mean Squares of the error term ($\sqrt{MS_{error}}$) and the used degrees of freedom (df) are indicated in the first column. If A and S means differed significantly, dominance coefficients ($\hat{H}$) were calculated for the mean hybrid trait values (AS+SA) and the limits of the 95% confidence intervals (CI). These are evaluated with respect to the hypotheses of A dominance (H=0), additivity (H=0.5) and S dominance (H=1).

				Evaluation of A dominance (H=0), additivity (H=0.5), S dominance (H=1)		
	Genomic class	Mean trait values	Contrast (F) A vs. S $\hat{H}$ ± 95% CI	H=0 within CI?	H=0.5 within CI?	H=1 within CI?
Number of leaves	A[1]	4,11	**23.1*****	no	no	yes
(count)	AS[1]	4,52	1.25 ± 0.29		=> S dominance	
$\sqrt{MS_{error}}$: 0.796	SA[1]	4,83				
df: 1; 567	S[1]	4,56				
Total DW	A[2]	0,046	**62.3*****	yes	no	no
(g)	AS[2]	0,048	-0.18 ± 0.18		=> A dominance	
$\sqrt{MS_{error}}$: 0.014	SA[2]	0,051				
df: 1; 183	S[2]	0,023				
Relative DW shoot	A[2]	0,82	0.53			
(g/g)	AS[2]	0,8	n.a			
$\sqrt{MS_{error}}$: 0.044	SA[2]	0,8				
df: 1; 183	S[2]	0,81				
Length of longest	A[1]	61,5	**10.1****	no	no	no
leaf (mm)	AS[1]	70	-2.21 ± 0.44		=> Overdominance (A)	
$\sqrt{MS_{error}}$: 9.74	SA[1]	69,1				
df: 1; 567	S[1]	57,9				
Relative petiole length	A[1]	0,29	**486*****	no	yes	no
longest leaf (mm/mm)	AS[1]	0,39	0.52 ± 0.06		=> Additivity	
$\sqrt{MS_{error}}$: 0.078	SA[1]	0,41				
df: 1; 567	S[1]	0,5				
Blade shape longest	A[1]	0,58	**35.0*****	yes	no	no
leaf (mm/mm)	AS[1]	0,59	0.00 ± 0.23		=> A dominance	
$\sqrt{MS_{error}}$: 0.170	SA[1]	0,57				
df: 1; 567	S[1]	0,7				
Angle youngest leaf	A[1]	58	**6.97***	no	no	no
(deg)	AS[1]	55	-0.85 ± 0.53		=> Overdominance (A)	
$\sqrt{MS_{error}}$: 11.0	SA[1]	56				
df: 1; 567	S[1]	62				
Angle longest leaf	A[1]	54	0.03			
(deg)	AS[1]	47	n.a.			
$\sqrt{MS_{error}}$: 11.7	SA[1]	49				
df: 1; 567	S[1]	54				

[1] Based on n=144

[2] Based on n=48

FINAL MEASUREMENTS: CONTRASTING THE PARENTAL SPECIES

Whole plant traits

Figure 2 shows the untransformed mean trait values that were assessed at the end of the experiment and Table 3 summarises the statistical evaluations (ANOVA independent contrasts A vs. S). *R. amphibia* had formed fewer leaves than *R. sylvestris* in the well drained (DRN) and the waterlogged (LOG) treatments (Fig. 2A). However, the reduction going from DRN to LOG was much more extreme for *R. sylvestris*: *R. amphibia* formed the same number of leaves in the DRN and LOG treatments, whereas *R. sylvestris* formed fewer leaves in the LOG treatment. Leaf formation was reduced in both species going from LOG to SUB, but more so in *R. sylvestris*. In the DRN treatment, the relative growth of the shoot (Fig. 2B) was similar in *R. amphibia* and *R. sylvestris*, while the relative growth of the root (Fig. 2C) was higher in *R. sylvestris*. Going from DRN-LOG, relative growth of both shoot and root reacted stronger (i.e., had steeper slopes) in *R. sylvestris* than in *R. amphibia*. As a result, in the LOG treatment the *R. sylvestris* relative shoot growth had become significantly less than that of *R. amphibia*, and the relative growth of the roots had become similar. Submergence had a strong negative effect on the relative growth in both species (both in shoots and roots). Still, both species established significant shoot growth (Fig. 2B) over two weeks of submergence (mean relative growth shoot ± 95% CI (ln g/g, n=24), *R. amphibia*: 0.189 ± 0.119; *R. sylvestris*: 0.310 ± 0.172). In *R. amphibia*, this was associated with a significant biomass loss of the roots (mean relative growth roots ± 95% CI (ln g/g, n=24), *R. amphibia*: -0.263 ± 0.150), whereas in *R. sylvestris* the root biomass remained constant (mean relative growth roots ± 95% CI (ln g/g, n=24), *R. sylvestris*: 0.064 ± 0.164). In all treatments, the shoot weight fraction (Fig. 2D) was larger in *R. amphibia* than in *R. sylvestris*, while in both species the shoot weight fraction showed a similar increase going from DRN-LOG and from LOG-SUB. The shoot dry matter content (Fig. 2E) of *R. amphibia* was larger than that of *R. sylvestris* in the DRN treatment, there was no difference in the LOG treatment, and in the SUB treatment the situation was reversed due to a stronger negative reaction slope of *R. amphibia* going from LOG-SUB. Both species formed similar numbers of adventitious roots (*R. amphibia*: 0.23 roots/plant; *R. sylvestris*: 0.50 roots/plant; Mann Whitney Test: U=1115, n.s.) and had a similar above ground root length (*R. amphibia*: 11mm; *R. sylvestris*: 45mm; Mann Whitney Test: U=1051, n.s.) in the DRN treatment. However, the LOG treatment had a large effect on these traits in *R. amphibia* leading to significantly more

Table 3. Results and conclusions of ANOVA of whole plant and leaf morphological traits using independent contrasts between six independent genotypic pairs of *Rorippa amphibia* (A) and *R. sylvestris* (S). F tests were performed within each treatment (DRN = well drained; LOG = waterlogged; SUB = submerged) and for the species' plastic reactions (slopes) from DRN-LOG and LOG-SUB. F-values in bold are significant at the indicated level (*p<0.05, **p<0.005, ***p<0.0005). For each trait, the square root of the Mean Squares of the error term (√MSerror) and the degrees of freedom (df) are also indicated. If A and S means differed significantly, dominance coefficients ($\hat{H}$) were calculated for the mean hybrid trait values and reaction slopes (AS+SA) and the limits of the 95% confidence intervals (CI). These are evaluated with respect to the hypotheses of A dominance (H=0), additivity (H=0.5) and S dominance (H=1).

		F test parents contrasted	Conclusion	$\hat{H}$ ± 95% CI	H=0 within CI?	H=0.5 within CI?	H=1 within CI?	Conclusion hybrid phenotype
Number of leaves formed	DRN	**48.0*** **	A < S	0.56 ± 0.20	no	yes	no	Additivity
(count)	LOG	**16.0*** **	A < S	0.82 ± 0.35	no	yes	yes	Add/dom S
	SUB	0.95	No diff					
$\sqrt{MS_{error}}$: 1.046	DRN-LOG	**4.29***	A < S	0.20 ± 0.95	yes	yes	yes	No power
df: 1; 542	LOG-SUB	**4.58***	A < S	1.10 ± 0.92	no	yes	yes	Add/dom S
Relative growth shoot	DRN	3.74	No diff					
(ln, g/g)	LOG	**5.78***	A > S	0.97 ± 0.58	no	yes	yes	Add/dom S
	SUB	1.92	No diff					
$\sqrt{MS_{error}}$: 0.287	DRN-LOG	**9.45****	A < S	0.45 ± 0.64	yes	yes	yes	No power
df: 1; 254	LOG-SUB	**7.20***	A > S	0.79 ± 0.74	no	yes	yes	
Relative growth root	DRN	**8.49****	A < S	-0.22 ± 0.48	yes	no	no	Dom A
(ln, g/g)	LOG	0.79	No diff					
	SUB	**8.51****	A < S	0.25 ± 0.48	yes	yes	no	Add/dom A
$\sqrt{MS_{error}}$: 0.372	DRN-LOG	**7.25***	A < S	0.46 ± 0.73	yes	yes	yes	No power
df: 1; 254	LOG-SUB	**7.27***	A > S	0.82 ± 0.73	no	yes	yes	Add/dom S
Shoot Weight Fraction	DRN	**11.7****	A > S	0.71 ± 0.41	no	yes	yes	Add/dom S
(arcsin√p, g/g)	LOG	**5.03***	A > S	0.62 ± 0.62	yes	yes	yes	No power
	SUB	**5.07***	A > S	0.67 ± 0.62	no	yes	yes	Add/dom S
$\sqrt{MS_{error}}$: 0.0228	DRN-LOG	0.69	No diff					
df: 1; 254	LOG-SUB	<0.01	No diff					
Shoot Dry Matter Content	DRN	**7.43****	A > S	-0.75 ± 0.51	no	no	no	Overdom (A)
(10log, g/g)	LOG	3.33	No diff					
	SUB	**14.2*****	A < S	0.88 ± 0.37	no	no	yes	Dom S
$\sqrt{MS_{error}}$: 0.0639	DRN-LOG	0.41	No diff					
df: 1; 254	LOG-SUB	**15.7*****	A > S	0.50 ± 0.50	no	yes	yes	Additivity

Table 3, continued

		F test parents contrasted	Conclusion	Ĥ ± 95% CI	H=0 within CI?	H=0.5 within CI?	H=1 within CI?	Conclusion hybrid phenotype
Length of longest leaf	DRN	**4.60***	A > S	0.89 ± 0.65	no	yes	yes	Add/dom S
(10log, mm)	LOG	**80.1*****	A > S	0.22 ± 0.16	no	no	no	Partial dom A
	SUB	**78.4*****	A > S	0.20 ± 0.16	no	no	no	Partial dom A
$\sqrt{MS_{error}}$: 0.0713	DRN-LOG	**23.1*****	A > S	0.01 ± 0.41	yes	no	no	Dom A
df: 1; 542	LOG-SUB	<0.01	No diff					
Petiole/length ratio longest	DRN	**81.3*****	A < S	0.54 ± 0.15	no	yes	no	Additivity
leaf (arcsin√p, mm/mm)	LOG	**59.2*****	A < S	0.49 ± 0.18	no	yes	no	Additivity
	SUB	**170*****	A < S	0.38 ± 0.11	no	no	no	Partial dom A
$\sqrt{MS_{error}}$: 0.0751	DRN-LOG	0.88	No diff					
df: 1; 542	LOG-SUB	**14.3*****	A < S	0.22 ± 0.52	yes	yes	no	Add/dom A
Width/length ratio blade	DRN	**31.1*****	A < S	0.70 ± 0.25	no	yes	no	Additivity
longest leaf	LOG	**32.5*****	A < S	0.52 ± 0.24	no	yes	no	Additivity
(10log, mm/mm)	SUB	**142.4*****	A < S	0.47 ± 0.12	no	yes	no	Additivity
$\sqrt{MS_{error}}$: 0.0792	DRN-LOG	<0.01	No diff					
df: 1; 542	LOG-SUB	**19.4*****	A < S	0.43 ± 0.45	yes	yes	no	Add/dom A
Angle longest leaf	DRN	**24.6*****	A > S	0.91 ± 0.28	no	no	yes	Dom S
(arcsin√p, deg)	LOG	0.85	No diff					
	SUB	**6.53***	A > S	0.49 ± 0.54	yes	yes	yes	No power
$\sqrt{MS_{error}}$: 0.172	DRN-LOG	**8.13***	A < S	0.75 ± 0.69	no	yes	yes	Add/dom S
df: 1; 542	LOG-SUB	1.33	No diff					
Angle youngest leaf	DRN	3.72	No diff					
(arcsin√p, deg)	LOG	2.38	No diff					
	SUB	**28.6*****	A < S	0.40 ± 0.26	no	yes	no	Additivity
$\sqrt{MS_{error}}$: 0.113	DRN-LOG	**6.02***	A < S	0.69 ± 0.80	yes	yes	yes	No power
df: 1; 542	LOG-SUB	**7.24***	A < S	0.82 ± 0.73	no	yes	yes	Add/dom S

adventitious roots in *R. amphibia* (*R. amphibia*: 0.85 roots/plant; *R. sylvestris*: 0.29 roots/plant; Mann Whitney Test: U = 822, p<0.005) as well as a significantly longer above ground root length (*R. amphibia*: 184mm, *R. sylvestris*: 31mm; Mann Whitney Test: U = 585, p<0.0005). In contrast, in the SUB treatment, *R. amphibia* formed significantly fewer (i.e., hardly any) adventitious roots than *R. sylvestris* (*R. amphibia*: 0.02 roots/plant; *R. sylvestris*: 0.29 roots/plant; Mann Whitney Test: U=1030, p<0.05), and had a shorter above ground root length (*R. amphibia*: 8.3mm; *R. sylvestris*: 45mm Mann Whitney Test: U=959, p<0.05).

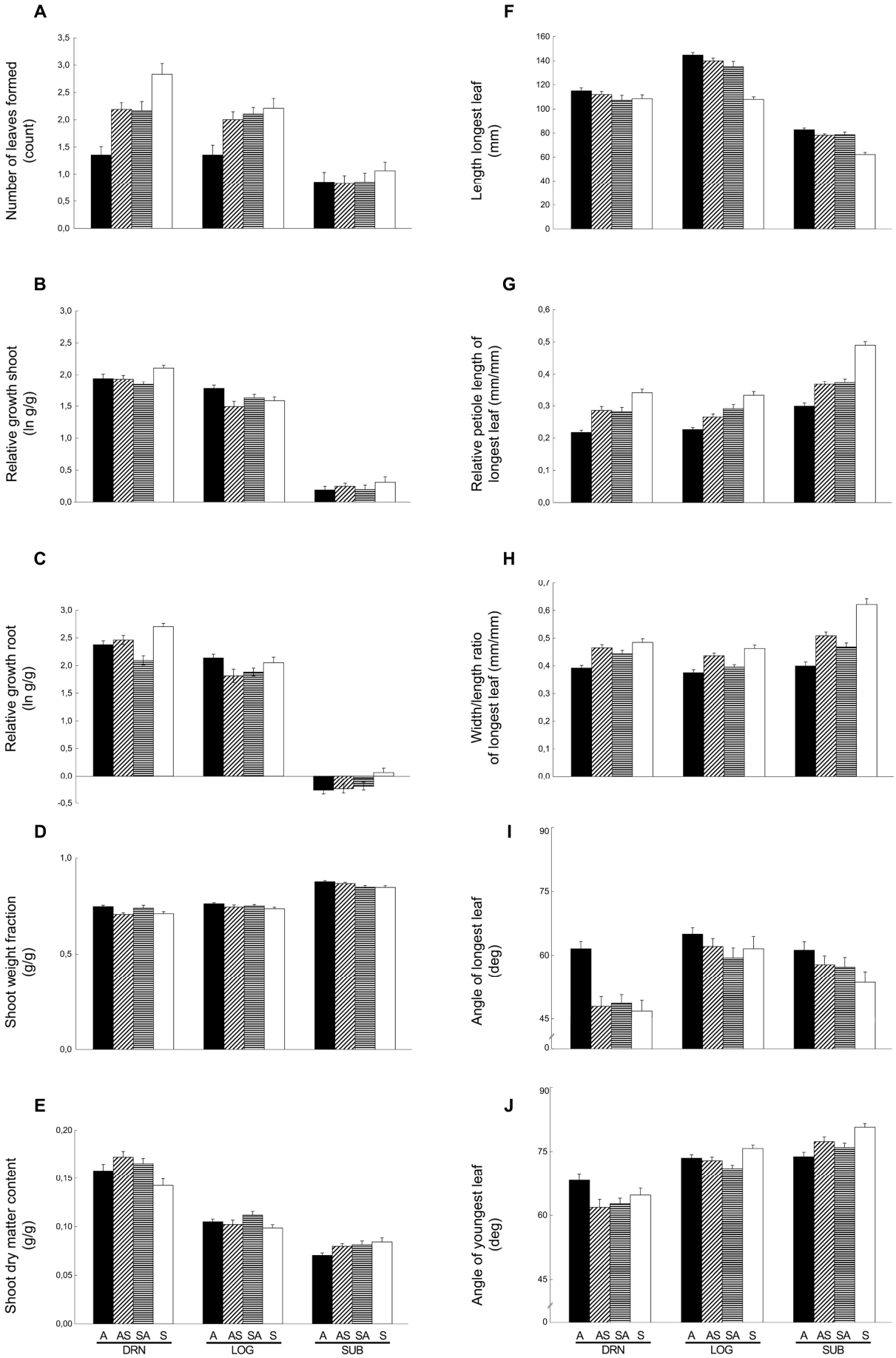
Leaf morphological traits

In all treatments, the longest leaf of *R. amphibia* was longer than that of *R. sylvestris* (Fig. 2F). Upon waterlogging, *R. amphibia* length of longest leaf increased strongly, but remained the same for *R. sylvestris*. Both species had a similar negative reaction going from LOG to SUB, i.e., leaves became smaller upon submergence. Irrespective of treatment, relative petiole length of the longest leaf (Fig. 2G) was longer in *R. sylvestris*. Waterlogging had no effect on this trait in either species. Going from LOG to SUB, relative petiole length increased in both species, but the slope of the reaction was much steeper in *R. sylvestris*. A similar pattern was observed for the blade shape of the longest leaf (Fig. 2H). Irrespective of treatment, *R. sylvestris* blade shape was rounder (i.e., larger width/length ratio). In *R. amphibia* blade shape hardly differed among treatments, whereas *R. sylvestris* leaves became rounder in the SUB treatment. The angle of the longest leaf (Fig. 2I) of *R. amphibia* was larger (i.e., orientated more upward) in the DRN treatment. Going from DRN to LOG, leaf orientation of *R. sylvestris* increased to become similar to that of the *R. amphibia*. In both species the leaf angle of the longest leaf decreased going from LOG to SUB, such that the leaf angle of *R. amphibia* in the SUB treatment became larger than that of *R. sylvestris*. The leaf angle of the youngest leaf (Fig. 2J) was similar in both species in the DRN and LOG treatments. Going from DRN-LOG, leaf angle of the youngest leaf increased in both species, but the reaction slope was steeper in *R. sylvestris*. Going from LOG to SUB, *R. sylvestris* leaf angle increased further leading to an almost vertical orientation (i.e., more than 80 degrees) in the SUB treatment, whereas in *R. amphibia* the leaf angle remained constant.

FINAL MEASUREMENTS: CONTRASTING THE RECIPROCAL HYBRIDS

For all response variables the reciprocal hybrids were similar in their trait values and responses to both flooding treatments (data not shown). In other words, species specific maternal effects and/or cytoplasmic factors did not affect the hybrid phenotypic responses to the treatments. Therefore, the two reciprocal hybrid classes were pooled (referred to as "hybrids") in subsequent analyses.

Figure 2. Mean trait values (untransformed) of whole plant traits (A-E) and leaf morphological traits (F-J) with standard errors (n=48) after each treatment for the genomic classes: A, *Rorippa amphibia* (black); S, *R. sylvestris* (white); AS, F1 hybrid A x S (diagonal stripes); SA, F1 hybrid S x A (horizontal stripes). Treatments were DRN (well drained), LOG (waterlogged), and SUB (submerged). A. Number of leaves formed; B. Relative growth shoot; C. Relative growth root; D. Shoot weight fraction; E. Shoot dry matter content; F. Length longest leaf; G. Petiole length relative to total length longest leaf; H. Width/length ratio (blade shape) of longest leaf; I. Angle of longest leaf; J. Angle of youngest leaf.


A
Number of leaves formed (count)
B
Relative growth shoot (ln g/g)
C
Relative growth root (ln g/g)
D
Shoot weight fraction (g/g)
E
Shoot dry matter content (g/g)
F
Length longest leaf (mm)
G
Relative petiole length of longest leaf (mm/mm)
H
Width/length ratio of longest leaf (mm/mm)
I
Angle of longest leaf (deg)
J
Angle of youngest leaf (deg)
A AS SA S
DRN
LOG
SUB

FINAL MEASUREMENTS: EVALUATION OF EXPRESSION OF PARENTAL TRAITS IN F1 HYBRIDS

Figure 2 shows the untransformed mean trait values that were assessed at the end of the experiment and Table 3 summarises the evaluation of hypotheses regarding the expression of parental traits (H=0 ~ *R. amphibia*, H=1 ~ *R. sylvestris*) in the hybrids.

Hybrids resembled *R. amphibia* (i.e., H=0 within $\hat{H} \pm 95\%$ CI) in their lower relative root growth in the DRN treatment as compared to *R. sylvestris* (Fig. 2C). In the SUB treatment, hybrids (similar to both parental species) established significant shoot growth (mean relative growth shoot ± 95% CI (ln g/g, n=48), hybrids: 0.220 ± 0.086). Similar to *R. amphibia*, this shoot growth was associated with a significant reduction in root biomass (mean relative growth roots ± 95% CI (ln g/g, n=48), hybrids: -0.216 ± 0.107). Hybrids also showed the typical *R. amphibia* waterlogging response to form more adventitious roots (*R. amphibia*: 0.85; *R. sylvestris*: 0.29; hybrids: 0.68, Mann Whitney Test *R. amphibia* vs. hybrids: U=2101, n.s.; *R. sylvestris* vs. hybrids: U=1844, $p<0.05$) and to increase above ground root length (*R. amphibia*: 184mm; *R. sylvestris*: 31mm; hybrids: 141mm, Mann Whitney Test *R. amphibia* vs. hybrids: U=1979, n.s.; *R. sylvestris* vs. hybrids: U=1466, $p<0.0005$). The increasing length of the longest leaf of the hybrids going from DRN to LOG resembled the waterlogging response of *R. amphibia* (Fig. 2F). Hybrid leaf lengths resembled those of *R. amphibia* in the LOG and the SUB treatment (partial dominance *R. amphibia*). Finally, relative petiole length of hybrids was more similar to *R. amphibia* in the SUB treatment (partial dominance *R. amphibia*), while it was intermediate to the parental species (i.e., 0.5 within $\hat{H} \pm 95\%$ CI) for the other treatments (Fig. 2G).

Hybrids only resembled *R. sylvestris* (i.e., H=1 within $\hat{H} \pm 95\%$ CI) in their larger shoot dry matter content in the SUB treatment (Fig. 2E) and their more prostrate longest leaf orientation in the DRN treatment (Fig. 2I). In all other cases where hybrids resembled *R. sylvestris*, there was enough power to reject *R. amphibia* dominance (H=0 not within $\hat{H} \pm 95\%$ CI), but not to distinguish between *R. sylvestris* dominance and additivity. This was the case for the smaller relative shoot growth in the LOG-treatment and for the overall smaller shoot weight fraction (as compared to *R. amphibia*). Hybrids were also more similar to *R. sylvestris* in that the reaction slopes were steeper for the angle of the longest leaf from DRN to LOG (Fig. 2I) and for the angle of the youngest leaf from LOG to SUB (Fig. 2J). The higher number of leaves that hybrids formed in the LOG treatment and the negative reaction going from LOG-SUB (Fig. 2A) also indicated a tendency towards *R. sylvestris* dominance.

In the DRN treatment, hybrids had a larger shoot dry matter content than either parent (Fig. 2E), which was the only case in which overdominance (i.e., $\hat{H} \pm 95\%$ CI < 0) was observed. In all other cases, hybrids were intermediate in their trait values or reaction slopes, or power was insufficient to distinguish among any of the hypotheses (i.e., H=0, H=0.5, H=1 all within $\hat{H} \pm 95\%$ CI).

Discussion

The first aim of this paper was to compare *R. amphibia* (denoted A in Figures and Tables) and *R. sylvestris* (denoted S in Figures and Tables) in their responses to different flooding regimes. The former species occurs in more stable, constantly wet to waterlogged habitats, and the latter in habitats with a more unpredictable regime of intermittent (often prolonged and deep) flooding and drought episodes. We set out with a specific set of expectations of species specific responses to three treatments, chosen to be representative of the naturally prevailing water regimes (except drought). Our data indeed showed pronounced differences between the species.

According to expectations, and in contrast with *R. sylvestris*, *R. amphibia* growth hardly differed between the well drained and waterlogging conditions. Leaf formation, morphology and positioning were not much affected by waterlogging either. Apparently, these treatments do not constitute a great difference with respect to these traits for a species like *R. amphibia* that is adapted to surviving in a habitat with ample water available. Still, there were some unexpected differences. Leaf size of the longest leaf of *R. amphibia* increased significantly upon waterlogging, an effect not seen in *R. sylvestris*. Future studies should provide insight into the physiological basis of this leaf size difference, and its potential adaptive significance.

Another *R. amphibia*-specific response to waterlogging is the development of adventitious and above ground roots in the water surface layer, a typical response for plants of waterlogged environments (Armstrong *et al.* 1994; Vartapetian & Jackson 1997). In *Rumex* ssp. similar species differentiation was found in the ability to form adventitious roots (Visser *et al.* 1996). Our results support the hypothesis that *R. amphibia* is better able to cope with waterlogging than *R. sylvestris* and provide insight in the traits associated with *R. amphibia*'s specialisation to waterlogged habitats.

According to expectations, *R. sylvestris* showed the most vigorous growth under well drained conditions and indeed waterlogging reduced *R. sylvestris* growth to a level similar to *R. amphibia* growth. Our data showed that *R. sylvestris* allocates more biomass to the roots. This corresponds to the hypothesis that under favourable conditions plants build up carbohydrate reserves that may be stored in the roots (Mooney 1972), thereby increasing their regeneration capacity after adverse conditions (e.g. waterlogging or submergence). Drought tolerance may play an important additional role in explaining the zonation in the occurrence of species along an elevation gradient (cf. Lenssen & De Kroon 2005).
The effect of submergence was comparable between the parental species in that both species showed reduced growth, an increase in the shoot weight fraction and a decrease in the shoot dry matter content. Contrary to our expectations that *R. sylvestris* would arrest growth when submerged, both *R. amphibia* and *R. sylvestris* established significant growth over two weeks under submerged conditions. Growth was restricted to the shoot, and in *R. amphibia* shoot growth was associated with a loss of root biomass. This suggests that submerged *R. amphibia* deplete their carbohydrate reserves in the root in an attempt to reach the surface. Mobilisation of starch reserves upon submergence has been previously reported in rice (Raskin & Kende 1984) and in *Rumex palustris* (Groeneveld & Voesenek 2003). This would be advantageous if flooding is shallow so that restoring air contact is indeed possible. In contrast to *R. amphibia*, the shoot growth in *R. sylvestris* was not accompanied by a reduction of root biomass. This suggests that *R. sylvestris* can survive (and even continue to grow) under submerged conditions without depleting its reserves. It appears that *R. sylvestris* may be capable of underwater photosynthesis (reviewed in Mommer & Visser 2005) and does not passively wait for better times in a state of anaerobic dormancy (Laan & Blom 1990; Vartapetian & Jackson 1997), at least not under the (light) conditions in our experiment.

Upon submergence *R. amphibia* did not show any striking alterations in leaf morphology and orientation, apart from a decrease in general leaf size (which likely reflects reduced growth). In contrast, the leaf petiole of *R. sylvestris* elongated and the orientation of the youngest leaf became almost vertical, suggesting a hyponastic response (Voesenek *et al.* 2006). Such a response is known in *Rumex palustris*, another flood-tolerant terrestrial species that can perform underwater photosynthesis (Laan & Blom 1990; Mommer *et al.* 2005) and thus prevent depletion of stored reserves in the roots (Groeneveld & Voesenek 2003).

More detailed studies are needed to confirm the different strategies of the two species under submergence, and many questions present themselves. Is *R. amphibia* indeed depleting its carbohydrate resources if flooding is prolonged and too deep to reach the surface? Does this depletion have a negative effect upon survival and recovery after prolonged submergence? Does *R. sylvestris* indeed not deplete its carbohydrate resources, and if so, what energy source is used for growth? What role does underwater photosynthesis (Mommer & Visser 2005) play in *Rorippa*? Does *R. sylvestris* indeed have a better survival or recovery than *R. amphibia* after prolonged submergence?

Our second aim was to assess how parental traits associated with flooding are expressed in first generation (F1) hybrids. For each trait, we explicitly tested whether the hybrids' average deviated from the hypothesis of intermediacy. If so, we subsequently tested for dominance and overdominance. For a number of traits, we rejected the intermediacy hypothesis. From a genetic perspective, this is a surprising result, particularly for growth related traits for which it seems unlikely that they are determined by just a few genes. This may indicate that the parental species have fixed genetic differences for a few genes with a major effect on growth and non-additive gene action, or that many individual genetic effects are channelled to a common metabolic pathway that results in non-additivity of the phenotypic expression. Current research is comparing expression profiles of the parental species with those of F1 and further hybrid (backcross) generations. From an ecological perspective, non-additive expression of traits may have consequences for the habitat preference of hybrids. If hybrids resemble one of the parents (dominance), hybrid habitat may overlap with that of the parent it resembles (Anderson 1948). Particularly the typical *R. amphibia* waterlogging responses were dominantly expressed in hybrids. Like in *R. amphibia*, waterlogging did not have an effect on growth and triggered the formation of adventitious roots, above ground roots and a longer longest leaf. When submerged, hybrids also showed the same root biomass reduction, indicating that (like *R. amphibia*) hybrids are perhaps less efficient in underwater photosynthesis compared to *R. sylvestris*. If so, the hybrid habitat may thus mostly overlap with that of *R. amphibia* in locations where the occurrence of the parental species is mainly determined by flooding. In such a setting, introgression and (further) backcrossing would be more likely to happen in the direction of *R. amphibia* (Anderson & Hubricht 1938).

In summary, our results supported our hypothesis that *R. amphibia* is better able to cope with waterlogging than *R. sylvestris* and provide insight in the traits that underlie *R. amphibia*'s

specialisation to waterlogged habitats. Additionally, we have shown that growth under submerged conditions was causing root biomass loss in *R. amphibia*, whereas *R. sylvestris* could prevent such loss. Furthermore, we have shown that *R. amphibia* leaf morphology remained constant, while *R. sylvestris* leaf morphology changed markedly upon submergence. Altogether, our results indicate that the two species are clearly different in their way of coping with flooding. We are currently further unravelling the mechanisms that underlie these differences. We found that hybrids combined a complex suite of traits from both parents, sometimes determined by additive, sometimes by dominant and rarely by overdominant expression of the parental phenotypes. This means that *Rorippa x anceps* F1 hybrids possess a unique phenotype, consisting of a unique combination of traits of both parental species. This specific hybrid phenotype may facilitate establishment of hybrids in free meandering rivers, particularly in the floodplains of rivers like (amongst others) the Elbe and Wisla. Greenhouse crosses have corroborated field observations that hybrids are fertile and backcross readily to both parental species (Chapter 4), making introgressive hybridisation (Anderson & Hubricht 1938) the most likely evolutionary consequence of hybridisation in *Rorippa*. Neutral microsatellite markers have revealed an interesting mixture of disomic and tetrasomic inheritance in F1 hybrids (Chapter 2), which raises questions as to what may be the potential of transgressive segregation in further hybrid generations (backcrosses). We are currently investigating these further generations both under natural and greenhouse conditions, at the phenotypic, genetic and transcriptional level.

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References

Al-Shehbaz, I.A., Beilstein, M.A. & Kellogg, E.A. (2006) Systematics and phylogeny of the Brassicaceae (Cruciferae): an overview. *Plant Systematics and Evolution*, **259**, 89-120.

Anderson, E. (1948) Hybridization of the habitat. *Evolution*, **2**, 1-9.

Anderson, E. (1949) *Introgressive hybridization*. John Wiley and Sons, Inc.; Chapman & Hall, Ltd, New York, London.

Anderson, E. & Hubricht, L. (1938) Hybridization in *Tradescantia*. III The evidence for introgressive hybridization. *American Journal of Botany*, **25**, 396-402.

Anderson, E. & Stebbins, G.L. (1954) Hybridization as an evolutionary stimulus. *Evolution*, **8**, 378-388.

Armstrong, W., Brändle, R. & Jackson, M.B. (1994) Mechanisms of flood tolerance in plants. *Acta Botanica Neerlandica*, **43**, 307-358.

Arnold, M.L. (1992) Natural hybridization as an evolutionary Process. *Annual Review of Ecology and Systematics*, **23**, 237-261.

Arnold, M.L. (1997) *Natural hybridization and evolution*. Oxford University Press, Oxford, UK.

Arnold, M.L. (2004) Transfer and origin of adaptations through natural hybridization: Were Anderson and Stebbins right? *Plant Cell*, **16**, 562-570.

Barton, N.H. & Hewitt, G.M. (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16**, 113-148.

Beilstein, M.A., Al-Shehbaz, I.A. & Kellogg, E.A. (2006) Brassicaceae phylogeny and trichome evolution. *American Journal of Botany*, **93**, 607-619

Bleeker, W. & Hurka, H. (2001) Introgressive hybridization in *Rorippa* (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. *Molecular Ecology*, **10**, 2013-2022.

Bleeker, W. & Matthies, A. (2005) Hybrid zones between invasive *Rorippa austriaca* and native *R-sylvestris* (Brassicaceae) in Germany: ploidy levels and patterns of fitness in the field. *Heredity*, **94**, 664-670.

Blom, C. & Voesenek, L. (1996) Flooding: The survival strategies of plants. *Trends in Ecology & Evolution*, **11**, 290-295.

Blom, C.W.P.M. (1999) Adaptations to flooding stress: From plant community to molecule. *Plant Biology*, **1**, 261-273.

Buerkle, C.A., Morris, R.J., Asmussen, M.A. & Rieseberg, L.H. (2000) The likelihood of homoploid hybrid speciation. *Heredity*, **84**, 441-451.

Coyne, J.A. & Orr, H.A. (1998) The evolutionary genetics of speciation. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **353**, 287-305.

Dowling, T.E. & Secor, C.L. (1997) The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics*, **28**, 593-619.

Falconer, D.S. (1960) *Introduction to quantitative genetics*. The Ronald Press Company, New York.

Groeneveld, H.W. & Voesenek, L. (2003) Submergence-induced petiole elongation in *Rumex palustris* is controlled by developmental stage and storage compounds. *Plant and Soil*, **253**, 115-123.

Gross, B.L. & Rieseberg, L.H. (2005) The ecological genetics of homoploid hybrid speciation. *Journal of Heredity*, **96**, 241-252.

Johnston, J.A., Donovan, L.A. & Arnold, M.L. (2004) Novel phenotypes among early generation hybrids of two Louisiana *Iris* species: flooding experiments. *Journal of Ecology*, **92**, 967-976.

Jonsell, B. (1968) Studies in the North-West European species of *Rorippa* s. str. *Symbolae Botanicae Upsalienses*, **19**, 1-221.

Laan, P. & Blom, C. (1990) Growth and Survival Responses of Rumex Species to Flooded and Submerged Conditions - the Importance of Shoot Elongation, Underwater Photosynthesis and Reserve Carbohydrates. *Journal of Experimental Botany*, **41**, 775-783.

Lenssen, J.P.M. & De Kroon, H. (2005) Abiotic constraints at the upper boundaries of two *Rumex* species on a freshwater flooding gradient. *Journal of Ecology*, **93**, 138-147.

Mommer, L., Pons, T.L., Wolters-Arts, M., Venema, J.H. & Visser, E.J.W. (2005) Submergence-induced morphological, anatomical, and biochemical responses in a terrestrial species affect gas diffusion resistance and photosynthetic performance. *Plant Physiology*, **139**, 497-508.

Mommer, L. & Visser, E.J.W. (2005) Underwater photosynthesis in flooded terrestrial plants: A matter of leaf plasticity. *Annals of Botany*, **96**, 581-589.

Mooney, H.A. (1972) The carbon balance of plants. *Annual Review of Ecology and Systematics*, **3**, 315-346.

Otto, S.P. & Whitton, J. (2000) Polyploid incidence and evolution. *Annual Review of Genetics*, **34**, 401-437.

Ramsey, J. & Schemske, D.W. (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics*, **29**, 467-501.

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

Rand, D.M. & Harrison, R.G. (1989) Ecological genetics of a mosaic hybrid zone: mitochondrial, nuclear, and reproductive differentiation of crickets by soil type. *Evolution*, **43**, 432-449.

Raskin, I. & Kende, H. (1984) Effect of submergence on translocation, starch content and amylolytic activity in deep-water rice. *Planta*, **162**, 556-559.

Rieseberg, L.H. (1997) Hybrid origins of plant species. *Annual Review of Ecology and Systematics*, **28**, 359-389.

Rieseberg, L.H., Archer, M.A. & Wayne, R.K. (1999) Transgressive segregation, adaptation and speciation. *Heredity*, **83**, 363-372.

Rieseberg, L.H., Baird, S.J.E. & Gardner, K.A. (2000) Hybridization, introgression, and linkage evolution. *Plant Molecular Biology*, **42**, 205-224.

Rieseberg, L.H., Sinervo, B., Linder, C.R., Ungerer, M.C. & Arias, D.M. (1996) Role of gene interactions in hybrid speciation: Evidence from ancient and experimental hybrids. *Science*, **272**, 741-745.

Rieseberg, L.H. & Wendel, J.F. (1993) Introgression and its consequences in plants. *Hybrid zones and the evolutionary process*. (eds R.G. Harrison). Oxford University Press, New York.

Rosenthal, D.M., Schwarzbach, A.E., Donovan, L.A., Raymond, O. & Rieseberg, L.H. (2002) Phenotypic differentiation between three ancient hybrid taxa and their parental species. *International Journal of Plant Sciences*, **163**, 387-398.

Schemske, D.W. (2000) Understanding the origin of species. *Evolution*, **54**, 1069-1073.

Schranz, M.E., Song, B.H., Windsor, A.J. & Mitchell-Olds T. Comparative genomics in the Brassicaceae: a family-wide perspective. *Current Opinion in Plant Biology*, **10**, 168-175.

Schwarzbach, A.E., Donovan, L.A. & Rieseberg, L.H. (2001) Transgressive character expression in a hybrid sunflower species. *American Journal of Botany*, **88**, 270-277.

Seehausen, O. (2004) Hybridization and adaptive radiation. *Trends in Ecology & Evolution*, **19**, 198-207.

Soltis, D.E. & Soltis, P.S. (1999) Polyploidy: recurrent formation and genome evolution. *Trends in Ecology & Evolution*, **14**, 348-352.

Soltis, D.E., Soltis, P.S. & Tate, J.A. (2004) Advances in the study of polyploidy since Plant Speciation. *New Phytologist*, **161**, 173-191.

Stebbins, G.L. (1947) Types of polyploids: their classification and significance. *Advances in Genetics*, **1**, 403-429.

Stebbins, G.L. (1950) *Variation and evolution in plants*. Columbia University Press, New York & London.

Vartapetian, B.B. & Jackson, M.B. (1997) Plant adaptations to anaerobic stress. *Annals of Botany*, **79**, 3-20.

Visser, E.J.W., Blom, C. & Voesenek, L. (1996) Flooding-induced adventitious rooting in *Rumex*: Morphology and development in an ecological perspective. *Acta Botanica Neerlandica*, **45**, 17-28.

Voesenek, L., Colmer, T.D., Pierik, R., Millenaar, F.F. & Peeters, A.J.M. (2006) How plants cope with complete submergence. *New Phytologist*, **170**, 213-226.

Welch, M.E. & Rieseberg, L.H. (2002) Habitat divergence between a homoploid hybrid sunflower species, *Helianthus paradoxus* (Asteraceae), and its progenitors. *American Journal of Botany*, **89**, 472-478.

Winge, Ø. (1917). The chromosomes: their number and general importance. *Comptes rendus des travaux du laboratoire Carlsberg*, **13**, 131-275.

Yatabe, Y., Kane, N.C., Scotti-Saintagne, C. & Rieseberg, L.H. Rampant gene exchange across a strong reproductive barrier between the annual sunflowers, *Helianthus annuus* and *H. petiolaris*. *Genetics*, in press.

CHAPTER 6

Development of highly conserved primers for 12 new polymorphic microsatellite loci for the genus Yellow-cress (*Rorippa* Scop., Brassicaceae)

Marc Stift, Peter Kuperus and Peter H. van Tienderen

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Abstract

We isolated 12 highly conserved polymorphic microsatellite loci for the Yellow-cress species *Rorippa amphibia* and *R. sylvestris*. We used a partial genomic library enriched for several repeat motifs. Obtained sequences containing repetitive elements were blasted and aligned with the *A. thaliana* sequence. We evaluated the cross-species compatibility of primers designed from sequences either aligning strongly or weakly with *A. thaliana*. The former proved much more efficient in obtaining primers that worked in both species. The developed conserved primers for microsatellite loci provide excellent markers for studying segregation, gene flow and hybridisation in the genus *Rorippa*.

Introduction

The self-incompatible, polyploid Brassicaceae species *Rorippa amphibia* (diploid and tetraploid) and *R. sylvestris* (tetraploid to hexaploid) occur throughout Europe in patchy and line shaped riverside habitats. When sympatric, hybridisation sometimes leads to hybrid swarms (Jonsell 1968, Bleeker & Hurka 2001). Both *Rorippa* species are highly clonal. To get insight in patterns of inheritance, gene flow and hybridisation we developed microsatellite markers that are compatible across species.

Materials & methods

After freezing in liquid nitrogen, fresh leaf material (100mg) of four *R. amphibia* and four *R. sylvestris* plants of different origins was grinded to powder. We used a modified CTAB procedure to isolate total genomic DNA (Doyle & Doyle 1990) and pooled the DNA for each species separately. For the isolation of the microsatellite loci we used a modified FIASCO protocol (Fast Isolation by AFLP of Sequences Containing repeats) (Zane et al. 2002). Genomic DNA was digested with restriction enzymes *Eco RI*, *Mse I*, *Pst I* and *Taq I*, followed by adaptor ligation, PCR amplification and cleaning. Directly after denaturisation (94°C, 3min), the DNA fragments derived from the *R. amphibia* pooled DNA were hybridised with four different mixtures of biotin labelled probes: (i) $(CA)_{20}$, $(CCA)_{15}$ and $(CAA)_{15}$; (ii) $(CT)_{20}$ and $(CTT)_{15}$, (iii) $(GACA)_{11}$ and $(GATA)_{11}$, and (iv) $(GAC)_{15}$ and $(CAT)_{15}$. The DNA fragments derived from the *R. sylvestris* pooled DNA were hybridised with two separate biotin labelled probes: $(CT)_{20}$ and $(CA)_{20}$. The resulting hybridisation complexes were lifted out with streptavidin-coated magnetic spheres (Promega) following manufacturer's protocol, re-amplified and cloned using the pGEM T Easy Vector System II (Promega) following manufacturer's protocol. We screened the colonies for the presence of a repeat-insert using PCR-based isolation of microsatellite arrays (PIMA) (Lunt et al. 1999). This revealed 56 positives out of 288 screened colonies for *R. amphibia*, and 110 positives out of 288 for *R. sylvestris*. Of those, we recovered plasmids of 26 and 110 positive colonies using Alkaline Lysis (Sambrook et al. 1989) and sequenced them with the BigDye Terminator v1.1 kit (Applied Biosystems). All sequences were blasted and aligned with *Arabidopsis thaliana*. Of the 26 *R. amphibia* derived sequences, 21 contained microsatellites

of which 13 had a strong alignment with *A. thaliana*. Of the 110 *R. sylvestris* derived sequences, 107 contained microsatellites of which 41 had a strong alignment with *A. thaliana*.

We selected 54 sequences (9 from *R. amphibia*, 45 from *R. sylvestris*) with suitable flanking regions for primer design with Primer3 software (Rozen & Skaletsky 2000). Of those, 35 (8 from *R. amphibia*, 27 from *R. sylvestris*) had a strong alignment with *A. thaliana* and we designed primers for the regions that were most conserved between *Rorippa* and *Arabidopsis*. We tested PCR amplification of these primer pairs (unlabelled) on 20 plants of each species. The 20µl PCRs contained 1 x PCR buffer (HT Biotechnology: 100mM Tris-HCl, pH 9.0, 15mM MgCl2, 500mM KCl, 1% Triton X-100, 0.1% (w/v) stabiliser), 0.1mM of each dNTP, 0.5mg/ml Bovine Serum Albumin, 0.15µM of each primer, 0.04units/µl Taq DNA polymerase (HT Biotechnology) and approximately 20ng DNA. PCR reactions were carried out in a PTC 100 thermocycler (MJ Research): 2 min at 94 °C, 32 cycles of 30s at 94 °C, 30s at primer specific annealing temperature T_a (Table 1) and 30s at 72 °C, and a final extension of 3 min at 72 °C. From the 35 primer pairs based on the sequences with strong alignment with *A. thaliana*, 30 amplified a clean fragment for both species, four for *R. sylvestris* only and one did not amplify at all. From the 19 primer pairs based on sequences with no *A. thaliana* match, two amplified a clean fragment for both species, three for *R. amphibia* only, whereas 14 did not amplify at all.

We selected 12 loci that showed variability across species (SEA 200, Elchrom Scientific) and that aligned to different *A. thaliana* chromosomes (Table 1). Using labelled forward primers (IRD700) we analysed a sample of 15 diploid *R. amphibia* plants from the river Loire (N47°22'26", W00°59'15"). We additionally tested the loci across species on 9 plants of each species with different geographic origins. PCR conditions were as described above. One µl of the PCR product was diluted with 4µl water and 5µl loading dye (20mM EDTA, 0.08% Bromophenolblue in de-ionised formamide) and analysed on a 6.5 % KB+ polyacrylamide gel (Licor 4200). We genotyped the samples by hand using the 50-350bp sizing standard (Licor).

Table 1. Loci characteristics: name and Genbank accession code; repeat motif; Genbank accession code of *A. thaliana* homologue (corresponding chromosome number, - if unknown); primer sequences (lower cases indicate nucleotides different from *A. thaliana*); annealing temperature (T_a). Based on diploid *R. amphibia* population (n=14): observed heterozygosity; expected heterozygosity; p-value of HWE test (values in bold fall within the Bonferroni corrected significance threshold of 0.00625). Based on Europe-wide sample of *R. amphibia* (n=9) and *R. sylvestris* (n=9): number of observed alleles (N_a) and allele size range (bp).

Locus name, genbank accession nr	Repeat motif	*A. thaliana* homologue (chromosome number)	Primer sequences (5'–3')	T_a (°C)	Based on diploid *R. amphibia* population (n=14)			Based on samples from different geographic origins	
					H_0	H_e	P value HWE test	*R. amphibia* (n = 9) N_a, allele size range (bp)	*R. sylvestris* (n = 9) N_a, allele size range (bp)
Rs 101	$(CT)_{11}$	AL163527	aGcTACTTTGTGTGGAGTG	55	0.643	0.659	0.87	8, 140-160	12, 138-170
DQ294637		(3)	tTTATTACACAAGTGtCACATG						
Rs 60	$(CA)_7$	AL161588.2	CCAATcTTAACTGCACACACA	55	n.a., monomorphic			3, 100-113	5, 100-113
DQ294638		(4)	GcAGTATATTTGTTTTgCACACT						
Rs 62	$(TG)_7$	AB026636.1	TCAAGCAAATCTTGGTaTTgG	55	0.071	0.386	**0.0048**	3, 161-172	4, 161-171
DQ294639		(3)	CCtTTCAAACCTCCTTCATTC						
Rs 89	$(TC)_{13}$	AL163652.1	ATTACAACAAATCTTCCACCAC	58	0.786	0.897	0.32	3, 171-177	7, 165-177
DQ294641		(5)	GCTTGATCTCACATTACATTATTTC						
Rs 46	$(TC)_{10}$ TGTG	AL163818.1	GTGTgCTTCGATGTTGGAG	58	n.a., monomorphic			4, 144-157	7, 144-155
DQ294640		(3)	GGAGACACAACAGGAACATAAAC						
Rs 44	$(CT)_{22}$ TTATAT	AF296834.1	AGCCgGgTATAGTATGTAAGTG	58	0.500	0.519	0.39	4, 151-176	18, 140-183
DQ294642	(CT)4	(-)	AGTTGCCTCATAGTTCAGGTC						
Ra 12	$(CAG)_2$ $(CAA)_5$	U90439.3	GTtCTCGtGTTTCCATGTTG	60	0.714	0.746	0.95	8, 110-156	4, 130-143
DQ294643		(2)	aATTGGGCTtGAGTTTGTTC						
Rs 30	$(AG)_{12}$	AC006341.2	cTCATATGGGTCAAGGTCTTC	60	0.273	0.801	**0.0019**	5, 126-134	8, 126-152
DQ294644		(1)	GAAGTTCTcTTCgGGtTCAG						
Ra 01	$(CTT)_7$	AL161553.2	CACACAAAGCACAAAAtGAGAG	62	n.a., monomorphic			3, 192-201	5, 185-201
DQ294646		(4)	GCTACAGTCGGTGAAGAGGAG						
Ra 13	$(CAAA)_2$ $(CAA)_8$	NM_128075.2	ATGCCTTTAGAGTTCGACCAG	62	0.643	0.799	0.75	4, 135-147	5, 132-153
DQ294647		(-)	GTTGTTAGGAGCACCAATGAG						
Rs 64	$(GAT)_5$	AB019229.1	GGTCTCGATGTAGCCCTTG	62	n.a., monomorphic			4, 135-144	6, 135-147
DQ294648		(3)	TGACCACCGCGTAATAGATG						
Rs 10	$(AC)_{10}$	AB025633.2	GCTCCATACGTCACATTCAC	62	0.071	0.140	1.00	2, 186-192	3, 186-194
DQ294649		(5)	GCACATTGATCCCATCTTTC						

Results & discussion

In the *R. amphibia* sample, two plants had identical multilocus genotypes, indicating clonality. We calculated heterozygosities and tested for HWE for the 8 variable loci (Table 1). Of these, 6 were in HWE. For 2 loci, null-alleles (RS30) and low levels of variation (RS62) are probably responsible for deviation from HWE. We found no significant linkage disequilibrium between any pair of loci. In the species comparison, all 12 loci were variable. Across species, allele size ranges overlapped, while the number of alleles varied (Table 1).

Our method to first check alignment with *A. thaliana*, and subsequently develop primers only for the most conserved parts of the flanking regions proved efficient to obtain cross-compatible markers. Out of 35 primer pairs, 86% (30) amplified in both study species and are likely to amplify for other species as well. By contrast, for sequences with a weaker alignment with *A. thaliana*, only 11% (2) amplified in both species. Moreover, a remarkably high proportion (74%) did not amplify at all.

The 12 characterised loci (Genbank accession codes DQ294637-DQ294649) will be particularly useful for studying segregation, gene flow and hybridisation in *Rorippa*.

References

Bleeker W, Hurka H (2001) Introgressive hybridization in *Rorippa* (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. *Molecular Ecology* **10**, 2013-2022.

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* **12**, 13-15.

Jonsell B (1968) Studies in the north-west European species of *Rorippa* s. str. *Symbolae Botanicae Upsaliensis* **19**, 1-221.

Lunt DH, Hutchinson WF, Carvalho GR (1999) An efficient method for PCR-based isolation of microsatellite arrays (PIMA). *Molecular Ecology* **8**, 891-894.

Rozen S, Skaletsky HJ (2000) PRIMER3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, NJ.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology* **11**, 1-16.

CHAPTER 7

General discussion

The general discussion is centred around some recurrent themes in this thesis. The first theme is species divergence between *R. amphibia* and *R. sylvestris*. I will discuss some morphological and ecological differences between the species, the degree of reproductive isolation between them and the conditions under which hybridisation occurs. Moreover, chromosomal divergence between the species will be addressed by studying chromosome pairing preference inferred from marker inheritance in artificial F1 hybrids. The second theme is the potential evolutionary role of hybridisation. I will discuss the conditions under which hybridisation between *R. amphibia* and *R. sylvestris* is likely and the possible consequences in terms of hybrid breakdown, hybrid speciation and introgressive hybridisation. The third theme is polyploidy. In particular, the most likely origin of tetraploid *R. amphibia* and *R. sylvestris*, tetraploid genetic diversity and potential sources of this diversity will be discussed. Finally, I will give some directions for future research.

Species differences and hybridisation

Morphology and ecology

R. amphibia and *R. sylvestris* are listed as separate species in all current European floras (e.g., van der Meijden 1996), and I followed this throughout this thesis. Indeed, the two species can be easily distinguished based on leaf morphology and reproductive characters (Jonsell 1968). At the same time though, there have been many reports of hybrid individuals that typically possess a mixture of the parental morphological traits (e.g., Jonsell 1968; Bleeker & Hurka 2001), and the hybrid *R. x anceps* is include, or at least mentioned, in most floras (e.g., van der Meijden 1996). At the tetraploid level there are hardly any barriers to hybridisation between *R. amphibia* and *R. sylvestris* and to backcrossing to either parent (Chapter 4). Hybrids are also self-incompatible so backcrossing with the parents is the most likely option for a newly formed hybrid while still rare. Nevertheless, my thesis provides

novel information that justifies a treatment of *R. amphibia* and *R. sylvestris* as separate entities while at the same time there is ample evidence that hybridisation occurs and has an impact on the species. The species differ in their responses to different flooding treatments (Chapter 5) which appears to be linked to differences in their habitat: *R. amphibia* typically grows in constantly wet or waterlogged habitats; *R. sylvestris* typically grows in ephemeral sites that can be flooded for longer or shorter periods throughout the year (Blom 1999; Chapter 1).

Hybridisation

In most situations the species are spatially well separated, so that their identity is maintained. Only in the mosaic of habitats in the floodplains of more or less free meandering rivers the species grow sufficiently close together to allow pollen transfer between them, resulting in hybridisation. This supports the notion that hybridisation is associated with or even promoted by disturbance (see Chapter 1). The disturbance in this system has a natural rather than an anthropogenic origin. In fact, human interventions like canalisation of rivers will likely have the opposite effect, as they will result in a reduction of river dynamics and so create fewer opportunities for hybridisation.

Distribution of hybrids

Although *R. x anceps* hybrids are certainly not exceptional in locations where *R. amphibia* and *R. sylvestris* grow closely together, they are not found throughout the whole distribution range of both species. Hybrid swarms have only been observed in regions where both species are tetraploid, e.g., along the rivers Elbe (Bleeker & Hurka 2001), or the Wisla and the Dnepr (personal observations). But even the sympatric occurrence of tetraploids of both species does not always result in the formation of hybrid swarms. Although the river Danube appeared to be comparable to the Elbe and Wisla (i.e., with respect to floodplain dynamics, area and species abundances) we observed no morphological indications of hybridisation with a comparable sampling effort for the different systems. We did not find any postzygotic isolation between the species in our greenhouse crossing experiments (Chapter 4). This suggests that prezygotic isolating mechanisms must operate in the populations along the river Danube. These mechanisms could have an ecological rather than a genetic basis (e.g., related to pollinator visitation, flowering phenology or fine-scale habitat preferences).

Chromosomal divergence?

Flow cytometric measurements show that *R. amphibia* has a larger genome than *R. sylvestris* (Chapter 1, Figure 1.1). Given the divergence between the species in genome size it becomes interesting to see whether this chromosomal divergence affects the pairing of chromosomes in meiosis. From genotyping the offspring of tetraploid artificial F1 hybrids, I concluded that the homologous chromosomes (i.e., the chromosomes derived from the same parental species) pair preferentially during meiosis, but not exclusively so (Chapter 2). Thus, the tetraploid hybrids do not behave as typical allotetraploids with fixed heterozygosities. This suggests that the (homeologous) chromosomes of *R. amphibia* and *R. sylvestris* have sufficiently diverged to reduce the frequency of cross-species bivalent formation. However, the regulation of chromosome pairing in meiosis is still poorly understood and sequence homology may only be indirectly responsible for initial pairing, a process that is mainly epigenetically regulated (reviewed in Sybenga 1999).

Consequences of hybridisation

Hybrid breakdown?

Are hybrids a dead end in *Rorippa*? First generation hybrids are equally successful as their parents in terms of seeds production (Chapter 4), which indicates that they are certainly not sterile dead ends. In later generations, hybrid breakdown may be expected if tetrasomic segregation leads to the breaking up of co-adapted gene complexes (Dobzhansky & Pavlovsky 1958). *Rorippa* hybrids do not behave as strict allopolyploids with fixed heterozygosity (see above and Chapter 2). This means that later generation hybrids may possess a ratio of *R. amphibia* to *R. sylvestris* genes ranging from 0:4 to 4:0, including all intermediates. Such a pattern of inheritance that is intermediate between disomic and tetrasomic can have different consequences: it may indeed lead to hybrid breakdown if these gene ratios and their combinations lead to a reduction of fitness. On the other hand it may also result in transgressive phenotypes (Rieseberg *et al.* 1999), i.e., phenotypes outside the range covered by the parental species. Some combinations could be particularly bad, but some may combine the best characteristics of the two parents, with new epistatic interactions

between genes derived from the two parental species adding to the range of phenotypes produced.

Hybrid speciation?

Do *Rorippa x anceps* hybrids have the potential to form a new lineage? Extensive backcrossing makes introgression (Anderson & Hubricht 1938) rather than hybrid speciation (Rieseberg 1997; Buerkle et al. 2000) the most likely evolutionary consequence of hybridisation in *Rorippa*. In natural populations, putative (i.e., morphologically intermediate) hybrids have always been found together with forms closely resembling the parental species. There appeared to be no reproductive isolation between F1 hybrids and their parents in terms of a significantly reduced seed production. Backcrosses to the parental species appeared as successful as hybrid x hybrid crosses (Chapter 4). This agrees with morphological and molecular indications of introgression on a local scale, i.e., the river Elbe (Bleeker & Hurka 2001). However, more work is needed to establish whether further recombination and segregation of genes lead to novel phenotypes with the potential to occupy new (isolated) habitats in which the parental species are not successful. In *Helianthus*, such transgressive hybrid phenotypes gave rise to three hybrid species - *H. anomalus*, *H. deserticola* and *H. paradoxus* - all occurring in extreme habitats as compared to the parental species *H. annuus* and *H. petiolaris* (Rieseberg *et al.* 2003).

Introgressive hybridisation

Introgression is not necessarily symmetric. Unilateral introgression can be the result of genetic incompatibilities (e.g., Keim *et al.* 1989). Backcrosses of *R. x anceps* F1 hybrids to either species were equally successful in terms of seed number (Chapter 4), indicating that there are no genetic incompatibilities that would promote unilateral introgression. Bilateral introgression is expected if pollen transfer is equally likely in both directions, which is in agreement with the evidence for introgression between *R. amphibia* and *R. sylvestris* in some Elbe populations (Bleeker & Hurka 2001). However, pollen transfer may not always be equally likely in both directions. Hybrid flower morphology and pollinator attraction, phenology or local adaptation could affect the direction of pollen transfer in natural populations. Pollinator visitation patterns in *Iris fulva x I. hexagona* hybrid zones promoted backcrossing of F1 hybrids to the locally dominant parental species (Emms & Arnold 2000). If hybrids are locally

adapted to a 'hybrid habitat' that overlaps with that of only one of the parents, pollen transfer to that parental species is more likely to occur (Anderson & Hubricht 1938). In Chapter 5 we concluded that the hybrid habitat could overlap with that of *R. amphibia* in locations where the occurrence of the parental species is mainly determined by flooding. This conclusion was based on their reactions to flooding and waterlogging in a greenhouse experiment, so clearly more work is needed to determine what happens in the field. A working hypothesis would be that (further) backcrossing towards *R. amphibia* is more likely than towards *R. sylvestris*. More work is also needed to test whether introgression of adaptive traits is possible. Introgression of adaptive traits was recently detected using transplantation experiments under highly selective field conditions in the *I. fulva x I. hexagona* system (Martin *et al.* 2006). Martinsen *et al.* (2001) suggested that hybrids may act as selective filters favouring introgression of some genes over others in a *Populus* hybrid zone.

Distribution and origin of polyploids

Polyploidisation clearly plays an important role in the genus *Rorippa*. In *R. sylvestris*, diploids are completely absent, and all forms are tetraploid or hexaploid. We have tried to locate a putative diploid ancestor, *R. sylvestris* subsp. *kerneri* that had been described in Flora Europaea (Jalas & Suominen 1994). Based on sampling details of herbarium specimens from the Budapest Herbarium, we managed to locate and sample eight putative individuals of *R. sylvestris* subsp. *kerneri.* However, these turned out to be tetraploid (based on flow cytometry). In *R. amphibia*, diploid and tetraploid forms are found. The cytotypes are separated geographically (Chapter 1, Figure 1.2), with contact zones in northern France, Germany and the Thames Valley in England. Both diploids and tetraploids are self-incompatible and morphologically very similar (Jonsell 1968). Tetraploids of both species presumably have an autotetraploid origin based on tetrasomic inheritance of microsatellite markers (Chapter 2). The more northern distribution of *R. amphibia* tetraploids fits the common pattern that polyploids are found more frequently at higher latitudes than diploids (Vandel 1940; Stebbins 1984; 1985; Little *et al.* 1997; Abbott & Brochmann 2003; Brochmann *et al.* 2004). It has been suggested that polyploids are better capable

of dealing with harsh or novel environments than diploids (Hagerup 1931; Manton 1950; Stebbins 1950; Johnson & Packer 1965). Stebbins (1984; 1985) hypothesised that a greater genetic variation may underlie these patterns.

Genetic variation in tetraploids

Neutral theory predicts that tetraploids can have a larger genetic diversity than diploids if equilibrium between mutation and drift has been reached due to a doubling of the effective population size (Moody *et al.* 1993). Only a few studies have tested these predictions, possibly because not many diploid-tetraploid pairs allow for a fair comparison due to confounding differences in breeding system or life history. Diploid and tetraploid *R. amphibia* share the same breeding system and are both perennial. We showed that tetraploid *R. amphibia* harboured more neutral genetic diversity than diploids, consistent with the predictions of neutral theory (Chapter 3). This adds to a growing number of examples (Soltis & Soltis 1989; Brown & Young 2000; Hardy *et al.* 2001) that support the predictions from neutral theory, and fit Stebbins' hypothesis of a greater genetic variability underlying the success of polyploids.

The neutral predictions apply to a situation in which mutation-drift equilibrium has been reached. This may be the case if the origin of *R. amphibia* tetraploids is not recent, since it takes considerable time (on an evolutionary scale) for mutation-drift equilibrium to establish, especially if newly formed polyploids have undergone a formation bottleneck (Stebbins 1950). However, the observed pattern of genetic diversity does not exclude a scenario of recent tetraploid origin. Ongoing gene flow from diploids may provide a source of tetraploid genetic diversity that works much faster than mutation. Diploids could contribute to tetraploid genetic diversity through multiple formation of tetraploids (Soltis & Soltis 1993) or through interbreeding of diploids and tetraploids (Ramsey & Schemske 1998; Petit *et al.* 1999). Both multiple formation of tetraploids and interbreeding are more likely if unreduced gametes are produced by diploids (Bretagnolle & Thompson 1995). Unreduced gametes from diploids can give rise to new tetraploid lineages (whether or not through a triploid intermediate generation) and can produce fertile tetraploid offspring when fused with normal reduced gametes from tetraploids (reviewed in Bretagnolle & Thompson 1995). In Chapter 4 we showed that unreduced gametes can account for gene flow

between diploids and tetraploids of *R. amphibia*. Although there is a strong postzygotic reproductive barrier between the cytotypes, interploidal crosses sometimes yield triploid and tetraploid offspring.

There are no reproductive barriers to hybridisation with tetraploids of *R. sylvestris* (Chapter 4). Therefore, introgression may provide an additional source of genetic variation for (newly formed) autotetraploid *R. amphibia*. A preliminary analysis showed that the tetraploid-specific class of alleles in *R. amphibia* could not be accounted for by introgression from the *R. sylvestris* populations that were screened (Chapter 3). However, the sample sizes were too small to draw definitive conclusions. More work is needed to test whether tetraploid-specific alleles originate from hybridisation with *R. sylvestris*, or perhaps from other *Rorippa* species (*R. palustris* or *R. austriaca*). Both gene flow from conspecific diploids and heterospecific tetraploids may also help to overcome the minority cytotype disadvantage in the initial phases of tetraploid establishment (Levin 1975).

Directions for future research in *Rorippa*

Tetraploid inheritance

We found that hybridisation between autotetraploids may result in polyploid forms intermediate between auto- and allopolyploids, i.e., showing inheritance that is intermediate between disomic and tetrasomic (Chapter 2). This has important implications, both for the process of generating variation for selection to act on in nature, as well as for linkage mapping and population genetic studies of tetraploids. Approaches have been developed to account for the complexities of tetrasomic inheritance in population genetic analyses (Moody *et al.* 1993; Ronfort *et al.* 1998; Luo, Zhang, Zhang *et al.* 2006) and linkage mapping (e.g., Luo *et al.* 2004; Luo, Zhang, Leach *et al.* 2006), but generally do not apply to an inheritance pattern that is intermediate between disomic and tetrasomic, for example after hybridization of closely related autotetraploids (Chapter 2). Stebbins (1950) and Sybenga (1996) postulated that a condition of inheritance intermediate between disomic and tetrasomic would be unstable. They hypothesised that homeologous paring would either lead to quick homogenisation of the initially homeologous chromosomes through intergenomic recombination, or – if intergenomic recombination is associated

with a fitness disadvantage – to the evolution of pairing regulators that prevent homeologous pairing (Sybenga 1996). The offspring of crosses involving *Rorippa x anceps* F1 hybrids provide unique material to test this hypothesis, and to eventually infer how many generations of intermediate pairing preferences may precede complete homogenisation or diploidisation.

Genetic basis of flooding adaptation

The interfertility of *R. amphibia* and *R. sylvestris*, and the fact that backcrosses can be produced easily (Chapter 4), provides excellent opportunities for mapping of traits associated with flooding tolerance (Chapter 5). Hybrids can be used to increase segregation variance in mapping populations (Lexer *et al.* 2003). *Rorippa*'s position within the tribe Cardamineae (Al-Shehbaz et al. 2006), that belongs to the same phylogenetic lineage (I) as the genomic model species *Arabidopsis thaliana* (Beilstein et al. 2006) provides additional advantages for comparative genomics (Schranz *et al.* 2007). This means that we can address questions such as "What are the genes underlying the species specific flooding responses? Is there variation for these genes in wild populations? Are these genes expressed constitutively? If not, how does a plant 'know' when to express them? Is there a fitness cost to being flooding tolerant?"

The significance of hybridisation and introgression

More work is also needed to test to what extent introgression may contribute to the evolution of *R. amphibia* and *R. sylvestris*. What is the fate of the hybrid swarms that exist to date? How much introgression occurs in hybrid swarms? Is introgression symmetrical or not? Does introgression lead to the transfer of adaptations?

What is the role of transgressive segregation in further hybrid and backcross generations (Rieseberg *et al.* 1999)? Is there any evidence of past introgression in areas where hybridisation does not seem to occur now, despite sympatric occurrence of tetraploids of both species? What factors prevent hybridisation in these areas?

The origin of polyploids

The autotetraploid origin of tetraploid of *R. amphibia* and *R. sylvestris* (Chapter 2), the higher genetic variation in tetraploid *R. amphibia* (Chapter 3) and the potential role of diploid conspecifics and tetraploid *R. sylvestris* as a source of genetic variation (Chapter 5) give rise to a wealth of new questions in this area. How frequent is gene

flow from diploids to tetraploids? Do tetraploids of *R. amphibia* harbour more genetic variation in areas where hybridisation with tetraploid *R. sylvestris* occurs? Do tetraploid *R. amphibia* have multiple origins and do they continue to form? Is there genetic and ecological differentiation between diploids and tetraploids?

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It is clear from this work and the earlier work of Bengt Jonsell and Walter Bleeker (and their respective co-workers) that hybridisation between the closely related autotetraploid *R. amphibia* and *R. sylvestris* occurs, and results in introgression. The combination of the presence of autopolyploidy in related species and successful hybridisation between the two appears to be no coincidence. Of course, (auto)polyploid species are expected to hybridise more easily than diploid species with a comparable level of divergence. But the intriguing question is whether autopolyploidy merely facilitates hybridisation between established lineages, or – vice versa - whether hybridisation also contributes to the success of the autopolyploid parental lineages.

Hybridisation may be an important mechanism by which autopolyploid lineages can pick-up genetic variation. This may aid newly formed autopolyploid lineages to overcome a formation bottleneck. Picking-up self-incompatibility haplotypes from other species is one way by which this could happen, promoted by negative frequency dependent selection favouring rare S-haplotypes. More in general, the bilateral exchange of genes that evolved independently in separate lineages could benefit the new bearers, and further contribute to the success of (auto)polyploids.

References

Abbott, R.J. & Brochmann, C. (2003) History and evolution of the arctic flora: in the footsteps of Eric Hultén. *Molecular Ecology*, **12**, 299-313.

Bleeker, W. & Hurka, H. (2001) Introgressive hybridization in *Rorippa* (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. *Molecular Ecology*, **10**, 2013-2022.

Blom, C.W.P.M. (1999) Adaptations to flooding stress: From plant community to molecule. *Plant Biology*, **1**, 261-273.

Bretagnolle, F. & Thompson, J.D. (1995) Tansley Review No-78 - Gametes with the somatic chromosome-number - mechanisms of their formation and role in the evolution of autopolyploid plants. *New Phytologist*, **129**, 1-22.

Brochmann, C., Brysting, A.K., Alsos, I.G., Borgen, L., Grundt, H.H., Scheen, A.C. & Elven, R. (2004) Polyploidy in arctic plants. *Biological Journal of the Linnean Society*, **82**, 521-536.

Brown, A.H.D. & Young, A.G. (2000) Genetic diversity in tetraploid populations of the endangered daisy Rutidosis leptorrhynchoides and implications for its conservation. *Heredity*, **85**, 122-129.

Dobzhansky, T. & Pavlovsky, O. (1958) Interracial hybridization and breakdown of coadapted gene complexes in *Drosophila paulistorum* and *Drosophila willistoni*. *Proceedings of the National Academy of Sciences of the United States of America*, **44**, 622-629.

Emms, S.K. & Arnold, M.L. (2000) Site-to-site differences in pollinator visitation patterns in a Louisiana Iris hybrid zone. *Oikos*, **91**, 568-578.

Hagerup, O. (1931) Über Polyploidie in Beziehung zu Klima, Ökologie und Phylogenie. *Hereditas*, **16**, 19-40.

Hardy, O.J., De Loose, M., Vekemans, X. & Meerts, P. (2001) Allozyme segregation and inter-cytotype reproductive barriers in the polyploid complex *Centaurea jacea*. *Heredity*, **87**, 136-145.

Jalas, J. & Suominen, J. (1994) *Atlas Flora Europaeae, Volume 12*., Helsinki.

Johnson, A.W. & Packer, J.G. (1965) Polyploidy and environment in Arctic Alaska. *Science*, **148**, 237-239.

Jonsell, B. (1968) Studies in the North-West European species of *Rorippa* s. str. *Symbolae Botanicae Upsalienses*, **19**, 1-221.

Keim, P., Paige, K.N., Whitham, T.G. & Lark, K.G. (1989) Genetic analysis of an interspecific hybrid swarm of *Populus* - occurrence of unidirectional introgression. *Genetics*, **123**, 557-565.

Levin, D.A. (1975) Minority cytotype exclusion in local plant populations. *Taxon*, **24**, 35-43.

Lexer, C., Randell, R.A. & Rieseberg, L.H. (2003) Experimental hybridization as a tool for studying selection in the wild. *Ecology*, **84**, 1688-1699.

Little, T.J., Demelo, R., Taylor, D.J. & Hebert, P.D.N. (1997) Genetic characterization of an arctic zooplankter: insights into geographic polyploidy. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **264**, 1363-1370.

Luo, Z.W., Zhang, R.M. & Kearsey, M.J. (2004) Theoretical basis for genetic linkage analysis in autotetraploid species. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 7040-7045.

Luo, Z.W., Zhang, Z., Leach, L., Zhang, R.M., Bradshaw, J.E. & Kearsey, M.J. (2006) Constructing genetic linkage maps under a tetrasomic model. *Genetics*, **172**, 2635-2645.

Luo, Z.W., Zhang, Z., Zhang, R.M., Pandey, M., Gailing, O., Hattemer, H.H. & Finkeldey, R. (2006) Modeling population genetic data in autotetraploid species. *Genetics*, **172**, 639-646.

Manton (1950) *Problems of cytology and evolution in the Pteridophyta*. Cambridge University Press, Cambridge, United Kingdom.

Martin, N.H., Bouck, A.C. & Arnold, M.L. (2006) Detecting adaptive trait introgression between *Iris fulva* and *I. brevicaulis* in highly selective field conditions. *Genetics*, **172**, 2481-2489.

Martinsen, G.D., Whitham, T.G., Turek, R.J. & Keim, P. (2001) Hybrid populations selectively filter gene introgression between species. *Evolution*, **55**, 1325-1335.

Moody, M.E., Mueller, L.D. & Soltis, D.E. (1993) Genetic variation and random drift in autotetraploid populations. *Genetics*, **134**, 649-657.

Petit, C., Bretagnolle, F. & Felber, F. (1999) Evolutionary consequences of diploid-polyploid hybrid zones in wild species. *Trends in Ecology & Evolution*, **14**, 306-311.

Ramsey, J. & Schemske, D.W. (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics*, **29**, 467-501.

Rieseberg, L.H., Archer, M.A. & Wayne, R.K. (1999) Transgressive segregation, adaptation and speciation. *Heredity*, **83**, 363-372.

Rieseberg, L.H., Raymond, O., Rosenthal, D.M., Lai, Z., Livingstone, K., Nakazato, T., Durphy, J.L., Schwarzbach, A.E., Donovan, L.A. & Lexer, C. (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. *Science*, **301**, 1211-1216.

Ronfort, J.L., Jenczewski, E., Bataillon, T. & Rousset, F. (1998) Analysis of population structure in autotetraploid species. *Genetics*, **150**, 921-930.

Schranz, M.E., Song, B.H., Windsor, A.J. & Mitchell-Olds, T. (2007) Comparative genomics in the Brassicaceae: a family wide perspective. *Current Opinion in Plant Biology*, **10**, 168-175.

Soltis, D.E. & Soltis, P.S. (1989) Genetic Consequences of Autopolyploidy in Tolmiea (Saxifragaceae). *Evolution*, **43**, 586-594.

Soltis, D.E. & Soltis, P.S. (1993) Molecular data and the dynamic nature of polyploidy. *Critical Reviews in Plant Sciences*, **12**, 243-273.

Stebbins, G.L. (1950) *Variation and evolution in plants*. Columbia University Press, New York & London.

Stebbins, G.L. (1984) Polyploidy and the distribution of the arctic-alpine flora: new evidence and a new approach. *Botanica Helvetica*, **94**, 1-13.

Stebbins, G.L. (1985) Polyploidy, hybridization, and the invasion of new habitats. *Annals of the Missouri Botanical Garden*, **72**, 824-832.

Sybenga, J. (1996) Chromosome pairing affinity and quadrivalent formation in polyploids: Do segmental allopolyploids exist? *Genome*, **39**, 1176-1184.

Sybenga, J. (1999) What makes homologous chromosomes find each other in meiosis? A review and an hypothesis. *Chromosoma*, **108**, 209-219.

van der Meijden, R. (1996) *Heukel's Flora van Nederland 22ste druk*. Wolters-Noordhoff, Groningen, The Netherlands.

Vandel, A. (1940) La parthenogenese geographique. IV. Polyploidie et distribution geographique. *Bulletin Biologique de la France et de la Belgique*, **74**, 94-100.

Summary

The evolutionary significance of hybridisation has been the subject of a long debate. Many argue that hybrids are exceptional, generally unfit and thus merely an evolutionary dead end. However, also unfit hybrids may play a role and may contribute to speciation of the parental lineages through a process called reinforcement: The phenomenon that already diverged lineages become more and more reproductively isolated, because the production of hybrids is detrimental for both. Others argue that – even if the mean hybrid fitness is low – some hybrid genotypes may be as fit or even fitter than that of the parents. Such hybrids could backcross to the parental species and cause introgressive hybridisation, exchange of genes between diverged lineages (e.g., species). Alternatively, if hybrids do not backcross to the parental species, they could eventually form new true breeding lineages (hybrid speciation).

A common mode of hybrid speciation is through a process called allopolyploidisation, in which hybridisation between species is coupled to genome doubling. Allopolyploids are usually characterised by having a pattern of disomic inheritance: they produce gametes that contain two sets of chromosomes, one set from each parental species. Consequently, the hybrids are always heterozygous at loci for which the parents had different alleles, which is referred to as 'fixed heterozygosity'.

Polyploids can also have their origin from a cross within a single species, giving rise to a type of polyploids that is commonly referred to as autopolyploids. These are often characterised by polysomic inheritance: they produce gametes with all possible combinations of chromosomes and hence allelic combinations. Allo- and autopolyploids form the extremes of a range, depending on the level of divergence between the lineages involved, with so-called segmental allopolyploids in between. In the classical sense, the latter are characterised by showing disomic inheritance for some loci, and polysomic inheritance for others. Inheritance could also be intermediate, although such a condition may be unstable due to recombination.

Up to 70% of all angiosperms have a history of polyploidy and many extant diploids are probably ancient polyploids. The evolutionary success of polyploids is mainly attributed to the hybrid origin and increased genetic diversity of allopolyploids. Although autopolyploids may not be as frequent as allopolyploids, they are certainly not uncommon. Moreover, while

autopolyploids do not have fixed heterozygosity, they can theoretically have an increased genetic variance compared to diploids (e.g., a tetraploid harbours twice as many independent alleles per locus as a diploid). The question arises if and under what conditions autopolyploidy may be an evolutionary driving force.

Rorippa amphibia occurs in diploid and tetraploid forms and *R. sylvestris* in tetraploid and hexaploid forms. The species are clearly different in morphology and habitat preference, but are completely interfertile at the tetraploid level. Under natural conditions, hybrid swarms have been documented in several river systems. The presence of hybrids, polyploids, and the close relatedness to the model species *Arabidopsis thaliana*, make *Rorippa* a unique system to address questions related to hybridisation and polyploidisation. In chapter 6, the sequence similarity between *Rorippa* and *Arabidopsis* has been used to design a set of conserved primers for microsatellite loci, facilitating cross-species amplification. These primers were used in the work presented in Chapter 2 and 3.

The first focal point of this thesis concerns the origin of tetraploids. To establish whether tetraploid *R. amphibia* and *R. sylvestris* had an allo- or autopolyploid origin, I studied the segregation of microsatellite alleles in the offspring of tetraploid *R. amphibia* and *R. sylvestris* (Chapter 2). Traditional chi-square based methods only allow to test whether disomic or tetrasomic models of inheritance can be rejected. I developed a new likelihood-based approach that also accommodates intermediate inheritance. Inheritance appeared to be tetrasomic for both *R. amphibia* and *R. sylvestris*, so that tetraploids in this system are most likely of autotetraploid origin. I further tested whether intermediate inheritance models may apply to first generation hybrids. This may be expected since *R. amphibia* and *R. sylvestris* are similar enough to allow successful hybridisation, yet divergent enough to earn their species status. Indeed, inheritance models intermediate between disomic and tetrasomic fitted the observed segregation patterns better than fully disomic or fully tetrasomic models. The existence of inheritance intermediate to disomic and tetrasomic has important implications for linkage mapping and population genetics and hence breeding programs of tetraploids. Methods that have been developed for either disomic or tetrasomic tetraploids may not be generally applicable, particularly in systems where hybridisation is common.

The second focus of this thesis was the success of polyploids. In *R. sylvestris* diploids have probably gone extinct, and in *R. amphibia* tetraploids are more widespread than diploids.

Neutral theory predicts that (auto)tetraploids may harbour more genetic variation than diploids if an equilibrium between mutation and drift has been reached. However, autotetraploids may have undergone a strong formation bottleneck, so that it may take considerable evolutionary time before to reach such an equilibrium. The presence of diploids and tetraploids with the same breeding system (both self incompatible) and a very similar morphology (and probably ecology) provides an excellent opportunity to test whether an increased genetic diversity may indeed underlie the success of polyploids in this system. We therefore compared neutral (microsatellite) variation between diploid and tetraploid *R. amphibia* (Chapter 3) and showed that tetraploid *R. amphibia* harboured more genetic diversity than diploid counterparts, in agreement with neutral theory. Tetraploids may have attained this variation through a combination of mutation, recurrent formation, ongoing gene exchange with diploids or introgression of genes from related, tetraploid species. I investigated the possibility of the latter two processes (Chapter 4). Tetraploid *R. amphibia* were crossed with conspecific diploids, tetraploid *R. sylvestris* and F1 hybrids. The results indicated that there is a strong crossing barrier between tetraploid and diploid *R. amphibia*. However, gene exchange with diploids is not impossible, due to the production of unreduced gametes by diploids. This may be particularly important if tetraploids lack compatible partners, for example in the initial phases of their establishment. The results further indicated that there is hardly any crossing barrier between tetraploids of *R. amphibia* and *R. sylvestris*, and that F1 hybrids can readily backcross to both parental species. Thus, introgression of genes from tetraploid *R. sylvestris* is a potential second source of variation in tetraploid *R. amphibia*.

This brings us to the third focus of this thesis: the role of ecological divergence. The study species differ in their habitat preference. *R. amphibia* grows in sites with stable water tables, often as emergent plants or in dense reed vegetation. *R. sylvestris* grows in more open sites that can be flooded for longer periods, but can also dry out completely in summer. An experimental approach was takes to identify potential species differences in responses associated with flooding tolerance (Chapter 5). Three water treatments were designed to mimick natural conditions: well-drained soils, waterlogged soils, and fully submerged plants. Waterlogging significantly reduced growth in *R. sylvestris* compared to well-drained conditions, whereas it had hardly any effect on the growth of *R. amphibia*. Both species showed some shoot growth in the course of two weeks of submergence, but only in *R. sylvestris* this did not occur at the expense of reduction in root biomass. This suggests that

R. sylvestris has a better capability of underwater photosynthesis, thus preventing depletion of (stored) carbohydrates in the roots. The experiment also demonstrated that hybrids mostly resemble *R. amphibia* in their response to waterlogging and complete submergence. This suggests that the hybrid habitat overlaps with that of *R. amphibia* in locations where the occurrence of the parental species is mainly determined by flooding. This partial overlap may have significant consequences for the likelihood and direction of introgression.

Conclusions

Tetraploid *R. amphibia* and *R. sylvestris* have tetrasomic inheritance and are thus of autotetraploid origin. First generation hybrids between these species do neither behave as strict auto-, nor as strict allopolyploids because they have an inheritance intermediate between disomic and tetrasomic. Tetraploid *R. amphibia* has larger genetic diversity than diploid conspecifics, in accordance with predictions of neutral theory. Besides mutation and recurrent formation, gene exchange with diploids through unreduced gametes may provide a source of genetic variation in tetraploid *R. amphibia*. Introgression from tetraploid *R. sylvestris* may provide an alternative source of variation. *R. amphibia* and *R. sylvestris* appear to be adapted to different flooding regimes: *R. amphibia* grows best under waterlogged conditions; *R. sylvestris* grows best under well-drained conditions, but may also be better capable of underwater photosynthesis than *R. amphibia*. Hybrids resembled *R. amphibia* in their responses to waterlogging and submergence, which may promote backcrossing to *R. amphibia*. There are no postmating barriers to hybridisation. Hybrids are as fit in terms of seed production as the parental species and there are no postmating barriers to backcrossing. This confirms earlier conclusions that hybrids between *R. amphibia* and *R. sylvestris* are not a dead end, and that introgression is the most likely consequence of hybridisation in this system.

Samenvatting

Het evolutionaire belang van hybridisatie is al jaren onderwerp van discussie. Velen hangen de mening aan dat hybriden slechts bij uitzondering voorkomen, over het algemeen een lage 'fitness' hebben en daarom een evolutionair doodlopend spoor vormen. Echter, zelfs hybriden met een lage 'fitness' kunnen een evolutionaire rol spelen en bijdragen aan het soortvormingsproces van de hyrbridiserende lijnen door een proces dat 'reinforcement' genoemd wordt: het fenomeen dat reeds gedivergeerde lijnen steeds meer reproductief geisoleerd raken, omdat het vormen van hybriden voor beiden nadelig is. Anderen hangen de mening aan dat – zelfs als hybriden gemiddeld genomen een lage fitness hebben – er op individueel niveau enkele hybriden kunnen zijn met een even grote of zelfs grotere fitness dan de oudersoorten. Zulke 'fitte' hybriden zouden kunnen terugkruisen met de ouderlijke lijnen en zo leiden tot 'introgressieve hybridisatie', uitwisseling van genetisch materiaal tussen gedivergeerde lijnen (bijvoorbeeld tussen soorten). Of, als zulke hybriden niet terugkruisen met de ouderlijke lijnen, dan zouden ze uiteindelijk nieuwe onafhankelijke lijnen kunnen vormen (hybride soortvorming).

Een algemeen voorkomend mechanisme van hybride soortvorming is middels een proces dat allopolyploidisatie genoemd wordt, waarbij hybridisatie tussen soorten gekoppeld is aan een verdubbeling van het genoom. Allopolyploiden worden meestal gekenmerkt door een disoom overervingspatroon: ze produceren gameten die twee sets chromosomen bevatten, één set van elke ouderlijke soort. Als gevolg daarvan zijn hybriden altijd heterozygoot voor loci waarvoor de ouders verschilden, hetgeen gefixeerde heterozygotie genoemd wordt.

Ook kruisingen binnen een soort kunnen leiden tot polyploiden. Polyploiden die op die manier ontstaan worden autopolyploiden genoemd. Autopolyploiden worden meestal gekenmerkt door een polysoom overervingspatroon: ze produceren gameten met alle mogelijke combinaties van ouderlijke allelen. Allo- en autopolyploiden zijn de uitersten van een gradient, met zogenaamde segmentele allopolyploiden ertussen. De mate van divergentie tussen de gekruiste lijnen bepaalt de positie van een nieuw gevormde polyploid. In de klassieke definitie worden segmentele allopolyploiden gekenmerkt door disome overervingspatronen voor sommige loci, en polysome overerving voor andere loci.

Overerving kan in theorie ook intermediair zijn tussen disoom en tetrasoom, hoewel zo'n toestand instabiel kan zijn door recombinatie.

Tot 70% van alle angiospermen heeft een polyploide achtergrond en veel tegenwoordige diploiden zijn waarschijnlijk ooit polyploid geweest. Men verklaart het evolutionaire succes van polyploiden voornamelijk door de hybride oorsprong en daarmee samenhangende grotere genetische diversiteit van allopolyploiden. Hoewel autopolyploiden wellicht niet zo algemeen voorkomen als allopolyploiden, zijn ze zeker niet zeldzaam. Bovendien, hoewel autopolyploiden geen gefixeerde heterozygotie hebben, theoretisch kunnen ze een grotere genetische diversiteit herbergen dan diploiden (een tetraploid heeft bijvoorbeeld twee keer zoveel onafhankelijke allelen per locus als een diploid). De vraag dient zich aan of, en onder welke condities, autopolyploidie een wezenlijk evolutionair mechanisme kan zijn.

Rorippa amphibia komt voor in diploide en tetraploide vormen en *R. sylvestris* in tetraploide and hexaploide vormen. De soorten zijn duidelijk verschillend qua morfologie and habitatpreferentie, maar zijn volledig interfertiel op het tetraploide niveau. Onder natuurlijke omstandigheden komen 'zwermen' van hybriden voor langs diverse rivieren. De aanwezigheid van hybriden, polyploiden, en de nauwe verwantschap met de modelsoort *Arabidopsis thaliana*, maken *Rorippa* tot een uniek systeem om hybridisatie en polyploidisatie te bestuderen. In Hoofstuk 6 is de sequentie-similariteit tussen *Rorippa* en *Arabidopsis* gebruikt om een set geconserveerde primers te ontwikkelen voor microsatelliet loci, hetgeen amplificatie van dezelfde loci in beide soorten mogelijk maakt. Deze primers worden toegepast in Hoofdstuk 2 en 3.

De eerste invalshoek van dit proefschrift betreft de oorsprong van tetraploiden. Om vast te stellen of tetraploiden van *R. amphibia* en *R. sylvestris* een allo- of autopolyploide oorsprong hebben gehad, heb ik de overerving van microsatelliet allelen in de nakomelingen van tetraploide *R. amphibia* en *R. sylvestris* planten geanalyseerd (Hoofdstuk 2). Met traditionele chi-kwadraat gebaseerde methoden kan alleen getest worden of disome of tetrasome overervingsmodellen al dan niet verworpen kunnen worden. Ik heb een nieuwe 'likelihood' gebaseerde methode ontwikkeld waarmee ook intermediare overervingsmodellen geëvalueerd kunnen worden. Overerving bleek tetrasoom te zijn voor zowel *R. amphibia* als *R. Sylvestris*, wat betekent dat tetraploiden in dit systeem waarschijnlijk een autotetraploide oorsprong hebben. Verder heb ik getest of intermediaire overervingsmodellen zouden kunnen gelden

voor eerste generatie (F1) hybriden. Dit zou je kunnen verwachten aangezien *R. amphibia* en *R. sylvestris* enerzijds genoeg overeenkomst hebben om geslaagde hybridisatie mogelijk te maken, en anderzijds toch voldoende gedivergeerd zijn om de status van soort te krijgen. Het bleek inderdaad zo te zijn dat intermediaire overervingsmodellen de waargenomen overervingspatronen beter verklaarden dan volledig disome of tetrasome modellen. De mogelijkheid van overerving intermediair tussen disoom en tetrasoom heeft belangrijke consequenties voor 'linkage mapping' en populatiegenetica en dus ook voor kweekprogramma's van tetraploiden. Methoden die zijn ontwikkeld voor ofwel disome ofwel tetrasome tetraploiden zijn wellicht niet algemeen toepasbaar, vooral in systemen waar hybridisatie veel voorkomt.

De tweede invalshoek van dit proefschrift betreft het succes van polyploiden. In *R. sylvestris* zijn diploiden waarschijnlijk uitgestorven, en in *R. amphibia* hebben de tetraploiden een grotere verspreiding dan diploiden. Volgens neutrale theorie kunnen (auto)tetraploiden meer genetische variatie herbergen dan diploiden indien een evenwicht betaat tussen mutatie en drift. Echter, als autotetraploiden een zogenaamde onstaansbottleneck hebben doorgemaakt (een periode met zeer beperkte populatiegrootte), dan kan het een behoorlijke (evolutionaire) tijd duren voordat een dergelijk evenwicht bereikt wordt. De aanwezigheid van diploiden en tetraploiden met hetzelfde voortplantingssysteem (beiden zelf-incompatibel) en een vergelijkbare morfologie (en waarschijnlijk ecologie) biedt een uitgelezen mogelijkheid om te testen of een grotere genetische diversiteit de sleutel zou kunnen zijn tot het succes van polyploiden in dit systeem. Daarom hebben we neutrale (microsatelliet) variatie vergeleken tussen diploide en tetraploide *R. amphibia* (Hoofdstuk 3) en aangetoond dat tetraploiden van *R. amphibia* meer genetische diversiteit herbergen dan diploide soortgenoten, zoals neutrale theorie voorspelt. Tetraploiden kunnen deze variatie verkregen hebben door een combinatie van mutatie, herhaaldelijke vorming, voortdurende genetische uitwisseling met diploiden of introgressie van genen van verwante, tetraploide soorten. De mogelijkheid van de laatste twee processen worden onder de loep genomen in Hoofdstuk 4. Tetraploiden van *R. amphibia* zijn gekruist met diploide soortgenoten, tetraploiden van *R. sylvestris* en F1 hybriden. De resultaten lieten zien dat er een sterke barriere is tussen tetraploiden en diploiden van *R. amphibia.* Echter, genetische uitwisseling met diploiden is niet geheel onmogelijk omdat diploiden ongereduceerde gameten kunnen vormen. Dit kan met name een belangrijke rol spelen in situaties waarbij tetraploiden geen compatibele partners hebben, bijvoorbeeld in de vestigingsfase direct na hun ontstaan. De resultaten lieten verder zien dat er nagenoeg geen

kruisingsbarriere is tussen tetraploiden van *R. amphibia* en *R. sylvestris* en dat F1 hybriden eenvoudig terugkruisen met beide oudersoorten. Derhalve is introgressie van genen van tetraploide *R. sylvestris* een mogelijke alternatieve bron van variatie in tetraploide *R. amphibia*.

Dit brengt ons bij de derde invalshoek van dit proefschrift: de rol van ecologische divergentie. De onderzoekssoorten verschillen in hun habitatpreferentie. *R. amphibia* groeit vooral op plaatsen met stabiele waterstanden, vaak als emergente planten of in dichte rietvegetatie. *R. sylvestris* groeit op meer open plaatsen die voor langere perioden overstroomd kunnen zijn, maar die ook volledig kunnen uitdrogen in de zomer. Middels een experimentele aanpak heb ik geprobeerde om mogelijke verschillen te detecteren in de reacties van beide soorten in relatie tot tolerantie van overstroming (Hoofdstuk 5). Drie waterniveaus werden toegepast om natuurlijke overstromingsregimes te simuleren: normaal gedraineerd, met de wortels overstroomd en volledig overstroomd. Worteloverstroming reduceerde de groei in *R. sylvestris* in vergelijking tot de normaal gedraineerde behandeling, terwijl het nauwelijks een effect had op de groei van *R. amphibia*. Beide soorten waren in staat tot groei van de scheut over de periode van twee weken volledige overstroming, maar alleen in *R. sylvestris* ging dit niet gepaard met een afname in wortelbiomassa. Dit suggereert dat *R. sylvestris* beter in staat is om onder water te fotosynthetiseren, en daarom de koolwaterstof-reserves in de wortels niet hoeft aan te spreken. Het experiment liet tevens zien dat hybriden vooral lijken op *R. amphibia* in hun reactie op beide typen overstroming. Dit suggereert dat de habitat van hybriden vooral zal overlappen met die van *R. amphibia* op plaatsen waar het voorkomen van de oudersoorten met name bepaald wordt door de heersende overstromingsregimes. Een dergelijke overlap zou belangrijke gevolgen kunnen hebben voor de waarschijnlijkheid en de richting van introgressie.

Conclusies

Tetraploide *R. amphibia* en *R. sylvestris* hebben tetrasome overerving en dus een autotetraploide oorsprong. Eerste generatie hybriden tussen de soorten gedragen zich noch als pure auto-, noch als pure allopolyploiden, aangezien ze een overervingspatroon hebben dat intermediair is tussen disome en tetrasome overerving. Tetraploide *R. amphibia* heeft een grotere genetische diversiteit dan diploide soortgenoten, in overeenstemming met neutral

theorie. Afgezien van mutaties en herhaaldelijke vorming, kan genetische uitwisseling met diploiden middels ongereduceerde gameten een bron van variatie zijn voor tetraploide *R. amphibia*. Introgressie van tetraploide *R. sylvestris* kan een alternatieve bron van variatie vormen. *R. amphibia* and *R. sylvestris* zijn klaarblijkelijk aangepast aan verschillende overstromingsregimes. *R. amphibia* groeit het beste in situaties waarbij de wortels overstroomd zijn; *R. sylvestris* groeit het best in normal gedraineerde situaties, maar lijkt ook beter in staat te zijn om onder water te fotosynthetiseren dan *R. amphibia*. Hybriden leken vooral op *R. amphibia* in hun reacties op overstroming, hetgeen terugkruisen met *R. amphibia* in de hand zou kunnen werken. Na de bestuiving zijn er geen barrieres tot de vorming van hybriden. Hybriden zijn even 'fit' als de oudersoorten qua zaadproductie en er zijn ook geen barrieres tot terugkruisen. Dit bevestigt eerdere conclusies dat hybriden tussen *R. amphibia* and *R. sylvestris* geen evolutionair doodlopend spoor vormen en dat introgressie de meest waarschijnlijke evolutionaire consequentie zal zijn van hybridisatie in dit systeem.

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On the author (Curriculum vitae)

Ik ben geboren op 19 januari in de strenge winter van 1979. Ik groeide op in het dorpje Kudelstaart, maar verhuisde naar Amstelveen op tienjarige leeftijd. In 1997 slaagde ik voor mijn VWO examen op het Amstelveen College en ging direct door naar Anna's Hoeve om biologie te gaan studeren aan de Universiteit van Amsterdam. Mijn eerste afstudeeronderzoek (Master) bracht me naar de nevelwouden van Costa Rica, waar ik de populatiestructuur van planten in de ondergroei onderzocht, in reactie op mogelijke klimaatverandering. Mijn tweede afstudeeronderzoek bracht me naar de geheel andere kant van de wereld, naar Irkoetsk in zuidelijk Siberië. Daar onderzocht ik de morfologie en mogelijke herkomst van een slak die het unieke en geïsoleerde ecosysteem van het 'antieke' Baikalmeer had gekoloniseerd. Ik studeerde af in 2002 en ging weer direct door met het volgende project, waarvan dit proefschrift het resultaat is. Zelfs terwijl ik de laatste hand leg aan deze niet-wetenschappelijke onderdelen van mijn proefschrift, ben ik al weer begonnen aan een nieuw project. De komende jaren zal ik werken aan de evolutie van zelf-incompatibiliteit van planten aan de University of Glasgow.

*****

I was born on the 19th of January, in the harsh winter of 1979. I grew up in the village of Kudelstaart, but moved to Amstelveen at 10 years' age. In 1997, I graduated from the Amstelveen College and went straight on to Anna's Hoeve to take up the study of Biology at the Universiteit van Amsterdam. My first Master's project brought me to the cloudforests of Costa Rica, studying the population structure of understory plant species potentially responding to recent climate change. My second Master's project brought me to the other end of the world, to Irkutsk in southern Siberia. There I studied the morphology and potential origin of a snail that had invaded the unique and isolated system of the ancient Lake Baikal. I graduated in 2002 and again, went straight on to the next project, of which the thesis at hand is the product. Even as I write these very last non-scientific bits of my thesis, I have already started a new project. The coming years I will be working on the evolution of the self-incompatibility system of plants at the University of Glasgow.

Marc Stift, Glasgow, 20-05-2007.