

# Biodiversity and Dollo's Law: To What Extent can the Phenotypic Differences between *Misopates orontium* and *Antirrhinum majus* be Bridged by Mutagenesis?

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## ABSTRACT

According to Dollo's law, evolution is irreversible. Yet, of the eight derived features essentially distinguishing *Misopates orontium* from its closely related *Antirrhinum majus*, five differences have phenotypically been clearly diminished or fully overcome by mutant genes, so that *Misopates orontium* outwardly approaches, meets or even overlaps the features of *Antirrhinum majus* or *vice versa* (aspects of the life cycle, leaf form, flower size, flower colour and mode of fertilization). However, to date the morphological key distinguishing feature between the two genera, the strongly elongated sepals in *Misopates* (itself a feature being at odds with Dollo's law), could not be reduced to that of the length of *Antirrhinum* nor could the development of the short *Antirrhinum* sepals be extended to that of the length of *Misopates*, in spite of extensive mutagenesis programmes with both species (agreeing with Dollo's law as to the stasis of this difference). Also, the long sepal character strongly dominated almost all homeotic *Misopates* mutants. After a general discussion of Dollo's law, its relevance for our mutants (and *vice versa*) is examined according to different evolutionary viewpoints. Furthermore, two concerns are raised: (1) To what extent can the hypothesis be substantiated such that the long and short sepals could really constitute genuinely persistent ("immutable") characters? (2) To what magnitude can the unexpected constancy of a feature distinguishing genera like the sepal difference be generalized for systematics and paleontology? Moreover, four basic genetic explanations (losses of gene functions, redundancy, the origin of new genes and chromosome rearrangements) are examined in this connection, and their relevance for some pivotal questions on the origin of species is investigated. As far as the authors are aware, this is the first thorough paper on Dollo's law in botany.

**Keywords:** apomorphic, mutations, plesiomorphic, reversibility and irreversibility in plants: phenotypic, genetic, functional

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[A]n organism cannot return, even partially, to a former state already realized in the series of its ancestors.

**Louis Dollo** 1893

Evolution is discontinuous, irreversible and limited.

**Louis Dollo** 1893

Functional or physiological reversal occurs; structural or morphological reversal does not occur.

**Louis Dollo** 1903

This principle of irreversibility, properly formulated (in Dollo's own manner) as a statement about historicity, remains central in evolutionary biology.

**Stephen Jay Gould and Betsey A. Robinson** 1994

## INTRODUCTION

The celebrated Human Genome Project has been the basis for the Chimpanzee Genome Project, which was inaugurated to approach, *inter alia*, the question of the genetic basis for the fundamental anatomical and other differences between *Homo sapiens* and *Pan troglodytes*, especially in the face of the strong sequence similarities found so far at the DNA and protein levels (for a review and unexpected new results, see Britten 2002; Holmes 2004; Orwant 2004; Watanabe *et al.* 2004; Weissenbach 2004).

A similar, but more modest, project focusing on the main genetic differences between closely related genera in the plant world was started with our *Antirrhinum-Misopates* project at the Max Planck Institute for Plant Breeding Research – the genetically well-studied genus *Antirrhinum* being the counterpart to humans of the aforementioned programme. In comparison to the human-chimp-project (as well as many others studying the genetics of conscious and/or sensitive creatures with a complex nervous system and the capability of feeling pain), one of the great advantages of approaching basic questions of biodiversity by studying closely related plant genera is, among other points, the possibility to directly apply mutagenesis to address and unravel essential problems of the genetic basis of their differences as well as the question of the phenotypic reversibility (Dollo's law) of the features distinguishing the two genera.

As quoted above, according to Dollo "an organism cannot return, even partially, to a former state already realized in the series of its ancestors". This statement may directly be confronted with the morphologically most

distinguishing feature of *Misopates*, the leaflike sepals. From an evolutionary perspective leaflike sepals are assumed to be a plesiomorphic (original) character whereas clearly defined and well developed sepals standing out against the leaves are assumed to be an apomorphic (derived) trait (see viewpoints A, C<sub>1</sub>, and C<sub>2a</sub> in the discussion). Since even from the most inclusive evolutionary presuppositions *Misopates* cannot be directly derived from an original ancestral angiosperm stock with leaf-like sepals, this trait would have returned – at least phenotypically – "to a former state already realized in the series of its ancestors" (see the further discussion of the pros and cons below). Moreover, comparable cases have been found in several different angiosperm families, so that this character would even include a high number of convergent reversals. Now, if it is assumed that such reversals have happened many times independently by point (and other) mutations and selection – the question may also be raised whether such a reversal could itself be reversed by further mutations and/or other factors back again into the direction of the derived state, i.e. the differentiated sepal. This would constitute another "return to a former state already realized in the series of its ancestors".

Mutations are viewed to be the ultimate basis of any biodiversity by all biologists adhering to the synthetic theory: "...pure chance, absolutely free but blind, at the very root of the stupendous edifice of evolution: this central concept of modern biology...is the only one that squares with observed and tested fact" (Monod 1971, similarly Dawkins 1997, 2003; for an extensive documentation, see Lönning 2002). Yet, this may not be the last word and the possibilities and limits of the origin of biodiversity due to

mutations have to be further investigated (Lönnig and Saedler 2002b). So to a certain extent our project provides a test for both the potential of mutations to produce the phenomena observed as well as the validity of Dollo's law (for possible objections against the method, see the discussion). Concerning reversions, we must, of course, clearly distinguish between the different levels of biodiversity: reversions challenging Dollo's law on the phenotypic level may have nothing to do with highly improbable reversions at the DNA-level restoring perhaps the original sequences coding for, or being involved in, the generation of the original features (for possible exceptions due to transposable elements and methylation, see again the discussion below). Modifying effects of non-allelic gene mutations shifting the manifestation of a mutant gene toward the original phenotype are probably most often involved in phenotypic reversions. Several important exceptions from Dollo's law have been reported (Marshall *et al.* 1994; Teotonio and Rose 2000, 2001; Collin and Cipriani 2003). So far, the most spectacular departure from the law appears to be the "loss and recovery of wings in stick insects" (Whiting *et al.* 2003).

As for the systematic position of *Misopates*, it is so closely related to *Antirrhinum* that for centuries it was treated as one of the species of the genus *Antirrhinum* (Besler 1613; Tournefort 1700; Linné 1753; Miller 1768; Chavannes 1833) until it was raised to the level of a genus in 1840 by Rafinesque. Yet even after that revision many authors went on speaking of *Antirrhinum orontium* (Linné's original species name) instead of *Misopates orontium* well into the 20<sup>th</sup> century (see, for instance, Hegi 1928; Bonnier and Douin 1935; Harrison 1960, also *Antirrhinum orontium* is usually cited in present synonym lists). Besides, in modern gene trees the two species usually cluster closely together (Gübitz *et al.* 2003; Hileman and Baum 2003). As for the etymology of the names, the following points may be worth mentioning: *Antirrhinum*, derived from Greek, *anti*, here meaning 'like', and *rhinos*, 'nose', probably referring to the nose-like capsule in its mature state and *majus*, Latin, comparative of *magnus*, the former meaning 'larger' pertaining to the large size of the flowers (see Hartl 1974; Schubert and Wagner 2000). *Misopates*: according to Corneliuson (1997) is derived from Greek *misos*, 'to hate', and *patein*, 'to step on' (the author of the name seems to hate to step on that beautiful little plant), and the meaning of *orontium* seems to be derived from Latin *oro*, 'to speak', 'to plea', 'to beg', which may have something to do with the mouth-like form of the flower.

Surveying the differences between the two genera, it is to be noted that *M. orontium* is distinguished from the *Antirrhinum majus* essentially by the following features:

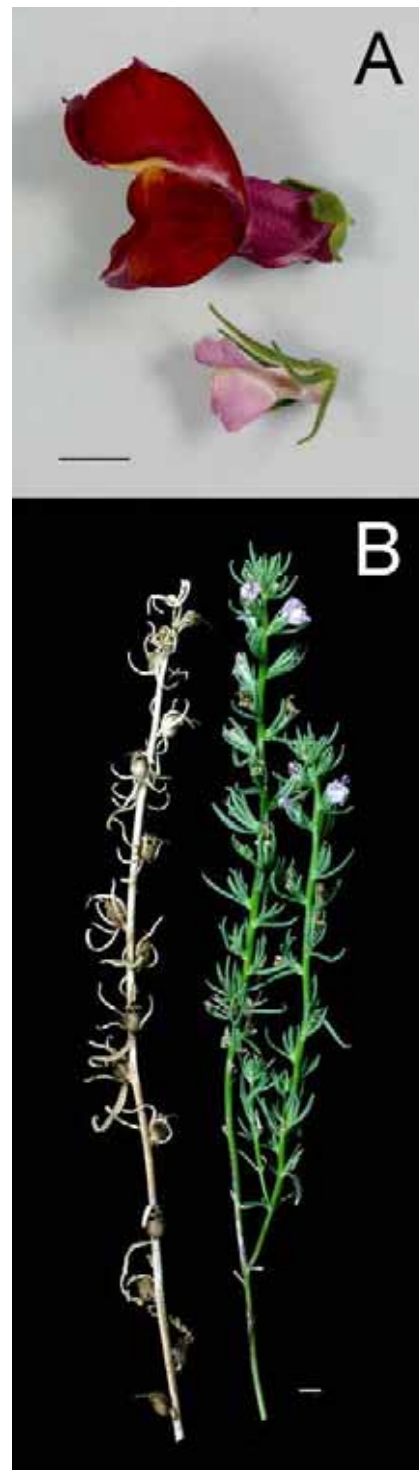
**Table 1** Major differences between *Misopates orontium* and *Antirrhinum majus*.

| Feature                  | <i>Misopates orontium</i>                         | <i>Antirrhinum majus</i>                   |
|--------------------------|---|--|
| 1) Life cycle            | Annual  | Perennial                                  |
| 2) Leaves                | 30-50 mm x 1.6-6 mm                               | 30-70 mm x 10-26 mm                        |
| 3) Sepals                | As long as or up to twice as long as the corolla  | 1/5 <sup>th</sup> of the length of corolla |
| 4) Flower (corolla) size | 11-15 mm  | 33-46 mm                                   |
| 5) Flower colour         | Light violet or almost white with violet venation | Red ("purplish pink")                      |
| 6) Fertilization         | Tendency to autogamy                              | Allogamous                                 |
| 7) Seed form             | Bowl/cuplike                                      | Elliptical/roundish                        |
| 8) Seed formation        | Also due to apomixis                              | Apomictic seed formation unknown           |

Presumably, a further significant difference between the two species appears to consist of the absence (*Misopates*) and presence (*Antirrhinum*) of larger numbers of active transposable elements (see the details under flower variegation below).

When Linné coined the name "*Antirrhinum orontium*"

in 1753 for our *Misopates orontium*, he positively characterized the species by its morphological key feature: "*calycibus corolla longioribus*" and "*calycibus flores superantibus*" ('with sepals longer than corolla' and 'with sepals towering above the flowers'). Later, the extremely elongated sepals were the reason for Lamarck even to re-name the species as "*Antirrhinum calycinum*" (Linné 1753; Lamarck quoted according to Hartl 1974) (see Fig. 1A).



**Fig. 1** Juxtaposing *Antirrhinum* and *Misopates* and *Misopates* wildtype with a mutant. (A) Comparison between *Antirrhinum majus* (above) and *Misopates orontium* (below). Note that even in absolute terms the sepals of *Misopates* are more than twice as long as those of *Antirrhinum*. (B) Wild-type (left) and mutant sister plant (*plena*, right) are of the same age. The mutant is still green and keeps on growing and flowering whilst the wild-type sister plant is already withered and dry. The similar relation was found for a femally fertile *deficiens* mutant even after seed set as well as for the *gigas* mutant L 2002/1843. Bars: The bars represent 1 cm except for 2 (C) (2 cm), 3 (B), 5 (E) 5 (F), 6 (D), 7 (B) (0.5 cm), and 3 j (1 mm).

Also, the annual life cycle and the cup-like seeds belong to the more conspicuous characters of *Misopates* distinguishing it physiologically and morphologically as a genus from *Antirrhinum*. Moreover, there is a strong fertility barrier between the two genera. Of the few tentative F<sub>1</sub> hybrids, it has been reported that they can be obtained only with *Misopates* as the mother, that the *Misopates* features proved to be dominant over those of *Antirrhinum* and that the progeny of the hybrids did not display Mendelian segregation and recombination of the many different character pairs of the two genera as normally expected (Harrison and Darby 1955; Harrison 1960). Since *Misopates* displays a strong tendency to autogamy, it is not clear how many of the tentative hybrids constituted actual F<sub>1</sub>-plants, or, as we suspect, whether perhaps almost all were just contaminations with the exception of perhaps a few cases of seed production due to apomixis (agamospermy) (see also Ernst 1918), which we have detected in *Misopates*. However, the apomictically produced seeds proved to be sterile so far (see the details below).

In relation to *Antirrhinum* in particular and the tribus of the Antirrhineae in general all the eight features specifying *Misopates* are classified as apomorphic (derived) characters, which should hardly be reversible according to Dollo's law (details in the discussion). With special emphasis on this issue as well as a possible potential of *Antirrhinum* itself to generate features bridging the phenotypic gap to *Misopates*, the following questions will be addressed and discussed in the present paper:

1) To what extent can the differences between the two genera be bridged by phenotypes due to mutant genes in either direction? In detail:

a) To what extent can the distinguishing features of the leaves, flower size, flower colour and mode of fertilization of the two genera phenotypically be bridged by mutant genes?

b) Can the (annual) *Misopates* life cycle be extended to that of *Antirrhinum* (perennial) or *vice versa*: have annual *Antirrhinum* mutants been isolated?

c) Regarding the most prominent morphological difference we focussed our attention on the following points: can the long *Misopates* sepals be reduced in perhaps one mutant step (or several small ones) to the length of *Antirrhinum* sepals or – *vice versa* – have *Antirrhinum* mutants with comparatively long sepals ever been obtained in our own experiments or those of any other researchers.

Also, for reasons given below, the subsequent questions have been investigated in the mutant phenotypes:

2) To what extent are the mutant features of leaves, bracts and sepals correlated in *Misopates*?

3) What do the corresponding homeotic flower- and inflorescence mutants of *Misopates* and *Antirrhinum* reveal about the genetic differences between the two closely allied genera?

4) Does *Misopates* display features of regressive evolution (genetical and morphological losses of functions) especially in comparison to *Antirrhinum* or the tribus Antirrhineae in general?

Concerning the origin of the essential differences between the two genera, Erwin Baur stated after pointing out that selection of "small factor-mutations" and recombination were responsible for adaptations *within* the genus *Antirrhinum*: "However, the origin of *A. majus* and *A. orontium* [now *Misopates orontium*], which belongs to another genus section [now another genus] from a common ancestral form, can hardly be envisioned by these factors. For the time being we can only confess our "ignoramus" (Baur 1930; see also Lönnig and Saedler 2002a).

In addition to a test of Dollo's law and the question on the power of mutagenesis, the following report also provides an investigation for Baur's statement.

## MATERIALS AND METHODS

Two lines of *Misopates orontium* have been used for the present studies: the nearly white flowering *M. orontium* from the Botanical Garden of Coimbra, Portugal (obtained via IPK of Gatersleben, FRG) and a typically violet flowering line derived from one wild *Misopates* plant spontaneously growing in Köln Vogelsang (the present geographical distribution of *M. orontium* includes not only the Mediterranean, but also large parts of Middle and Northern Europe, and it has become a recent invader of many further parts of the world; for the details, see Hartl 1974; Sutton 1988; Haeupler *et al.* 2003). Since there are no cultivated lines known in *Misopates* (Hartl 1974), both these lines appear to represent different varieties of the same species in the wild. Also, there was no fertility barrier between the two lines (crosses were made in both directions). Nevertheless, it should perhaps be mentioned that the *Misopates* line from Coimbra is viewed to be a species of its own by some systematists, classifying it as *Misopates calycinum* (Franco 1971). For the genetical reasons just mentioned and further points (Lönnig 2002), we do not follow this practice here. Moreover, in contrast to the description of *M. calycinum* presented by Sutton (1988), the sepals of the Coimbra line are slightly longer than those of the Vogelsang line of *M. orontium*. Although according to Stace (1997) *M. orontium* can have 2n=14 or 16 chromosomes, all *Misopates* 'species' so far investigated appear to have 2n=16 chromosomes (Sutton 1988) like the species of *Antirrhinum* (see, however, *Pseudomisopates* below).

Mutations in dry seeds were induced by fast neutrons and gamma rays in Seibersdorf (IAEA), Austria, and Forschungszentrum Jülich, FRG, respectively. Seeds of *M. orontium* from the Coimbra line were treated with fast neutrons (three packages with 3.5 Gy, 5.5 Gy, and 7 Gy respectively) in 1999 in Seibersdorf, and a second group in 2001 with gamma rays (200 Gy, 240 Gy) in Jülich. In 2001 seeds of the Vogelsang line were treated with gamma rays (200 Gy, 240 Gy), also in Jülich. Apart from the fact that the mutation frequency increases with the concentration of a mutagenic agent applied and that there can be differences concerning the various kinds of lesions on the DNA-level (especially in chemical mutagenesis), no correlation of the types and strengths of the treatments with the *types* of mutants could be detected so far. In general this seems to be in agreement with the laws of probability for a non-directed process and the results of most other mutagenesis investigations. For instance, the almost infinite variation of the different kinds and doses of applied mutagens has not resulted in more of the better mutants in mutation breeding in spite of many earlier hopes and expectations (for further points and reviews on mutation research see Auerbach 1976; Lönnig 1993, 2006).

Altogether 335,000 plants of *Misopates* have been investigated including ca. 10,800 M<sub>2</sub>-families. Moreover, during the last 22 years 1.5 million *Antirrhinum* plants including some 30,000 M<sub>2</sub>-families have been investigated by W-E L (mutagenesis by transposons, EMS, fast neutrons, gamma rays, and X rays as well as combinations of the mutagenic agencies). As for the logistic details, the same principles were followed as described in detail by Lönnig and Huijser (1994), and Kunze *et al.* (1997). To solve the question, as to what extent the main phenotypic differences between the two species can be overcome by mutagenesis encompassing a test of Dollo's law, to the best of our knowledge the present paper also reports the first (and at the same time extraordinarily large) mutagenesis programme with the wild species *M. orontium*.

### Identification of mutants

Since locus identity test crosses between *Misopates* and *Antirrhinum* are not possible, the genes so far identified were recognized by sequence analyses of the homologous *Antirrhinum/Misopates* wild-type genes and their mutant deviations. Of the many mutants only those relevant for the questions of our topic have been considered in the present paper (a general overview of all the mutants isolated – being a topic of its own – is beyond the scope of the present paper). The numbers below (like L 2002/1283) refer first to the year in which an M<sub>2</sub> was evaluated, and second to the number of the segregating family; the letter L stands for the first author's surname).

For measuring the genetic distance between the two taxa, a series of additional genes have been sequenced (also) by PCR.

If primers from one organism are also homologous to the sequence of another organism they can be used to sequence DNA from related species as has been done here for *A. majus* and *M. orontium* (the extensive tables concerning the oligos used and the positions of the oligo primers can be directly obtained from the authors). For further points on method, see Saiki *et al.* (1988).

The new *Misopates* sequences have been submitted to the EMBL Nucleotide Sequence Database and can be retrieved by the accession numbers AM162204 to AM162213 and AM396483 to AM396489.

## RESULTS

In the ensuing paragraphs, the results of the investigations are presented in accordance with the enumeration of the characteristics distinguishing the two species and genera as given in **Table 1**, followed by the topics of flower variegation and transposon activities, homeotic mutants, and genetic distance between the two species.

### Life cycle

Annuality in plants is usually viewed to be an apomorphic (derived) feature. Three mutants of the normally annual *Misopates* have displayed a clearly elongated life cycle: *A. deficiens*, a *plena*, and a *floricaula*-like (L 2002/1283) mutant. Normally the average life span found in our field trials with the wild-type *Misopates* lasted three to four months for the Vogelsang line, from which all three mutants have been derived (and up to four months in the Coimbra line). These mutants, however, now habitually live some 12 months and can be further propagated (probably indefinitely) by cuttings (until now for four years). Interestingly all three cases belong to the group of homeotic mutants (**Figs. 1B, 4A, 4B, 4D-K**). Moreover the female organs of the *deficiens*-mutant are fully fertile (instead of the stamens, compound styles and stigmas are produced). Yet even after seed-set the longevity does not seem to be reduced (extended propagation by cuttings has also been tried, but not achieved, for the wild-type).

Apart from these homeotic mutants, plants of the third generation of the leaf mutant L 2002/1843 (**Figs. 2A-C**), also derived from the Vogelsang line and further described below, reveal *gigas* growth at 15-17°C – they grow more than 230 cm long (the initial line grows up to only about 120 cm under the same conditions) and display an elongated life cycle (up to 10 months) (as for details on the term *gigas*, see Schubert and Wagner 2000). They can also be further propagated by cuttings, yet their life span appears to be limited to a few more months. Additionally, its seed production is strongly raised under these special environmental conditions. In contrast to these results at 15°C, the mutant can hardly be distinguished from the wild-type at 25°C (aside from its broad leaves).

As for *Antirrhinum*, in several of its mutants the life cycle is abbreviated, including all lethal and sublethal mutants, and this appears to be true also for some mutants with less pronounced flower and leaf aberrations (for a description of many of such mutants, see, for example, Stubbe 1966). Moreover, many culture varieties are classified as annuals in relevant horticultural volumes.

### Leaves

*Antirrhinum majus* ssp. *majus* normally displays oblong elliptic, elliptic or, more seldom, lanceolate leaves (ca. 10-26 mm in width), but in *M. orontium* linear leaves predominate (1.6-6 mm width on average) (as for their length, see **Table 1**).

In several *Antirrhinum* mutants the leaves are filiform, linear, or linear-lanceolate (for details, see **Fig. 2H**; Stubbe 1966). Moreover, some subspecies of *Antirrhinum* as *A. majus* ssp. *tortuosum*, also display long, slender leaves (4-

10 mm in width), so that many *Antirrhinum* mutants and even some wild subspecies phenotypically approach the leaf form of *M. orontium*.

Concerning leaf mutants of *M. orontium*, most of them tended to be even more slender (linear) than those of the wild-type phenotype (at least 23 candidates). However, there were two clear-cut exceptions from this rule. From the Vogelsang line of *M. orontium* a mutant (L 2002/1843) was obtained whose leaves proved to be even broader (up to 28 mm) than those of several average *A. majus* culture varieties (about 20-26 mm), yet *Antirrhinum* cv “snowman” still surpassed its width (see **Figs. 2A-C**, Snowman left above white string in c). Also, mutant L 2003/1191 approached the typical leaf form of *A. majus* ssp. *majus*. The overall ontogenetic development of the aforementioned mutant L 2002/1843 appears to be as slow as that of *A. majus* (usually anthesis of the *M. orontium* is several weeks earlier than that of *Antirrhinum*), but no organ abnormalities have been detected so far, and fertility is excellent. Yet the second mutant (L 2003/1191) displays strong flower abnormalities and its fertility is strongly reduced (further points see below).

Hence, in this character, which *prima facie* appears to be among those especially distinguishing the two species, the mutant phenotypes of the two species clearly overlap each other.

### Sepals

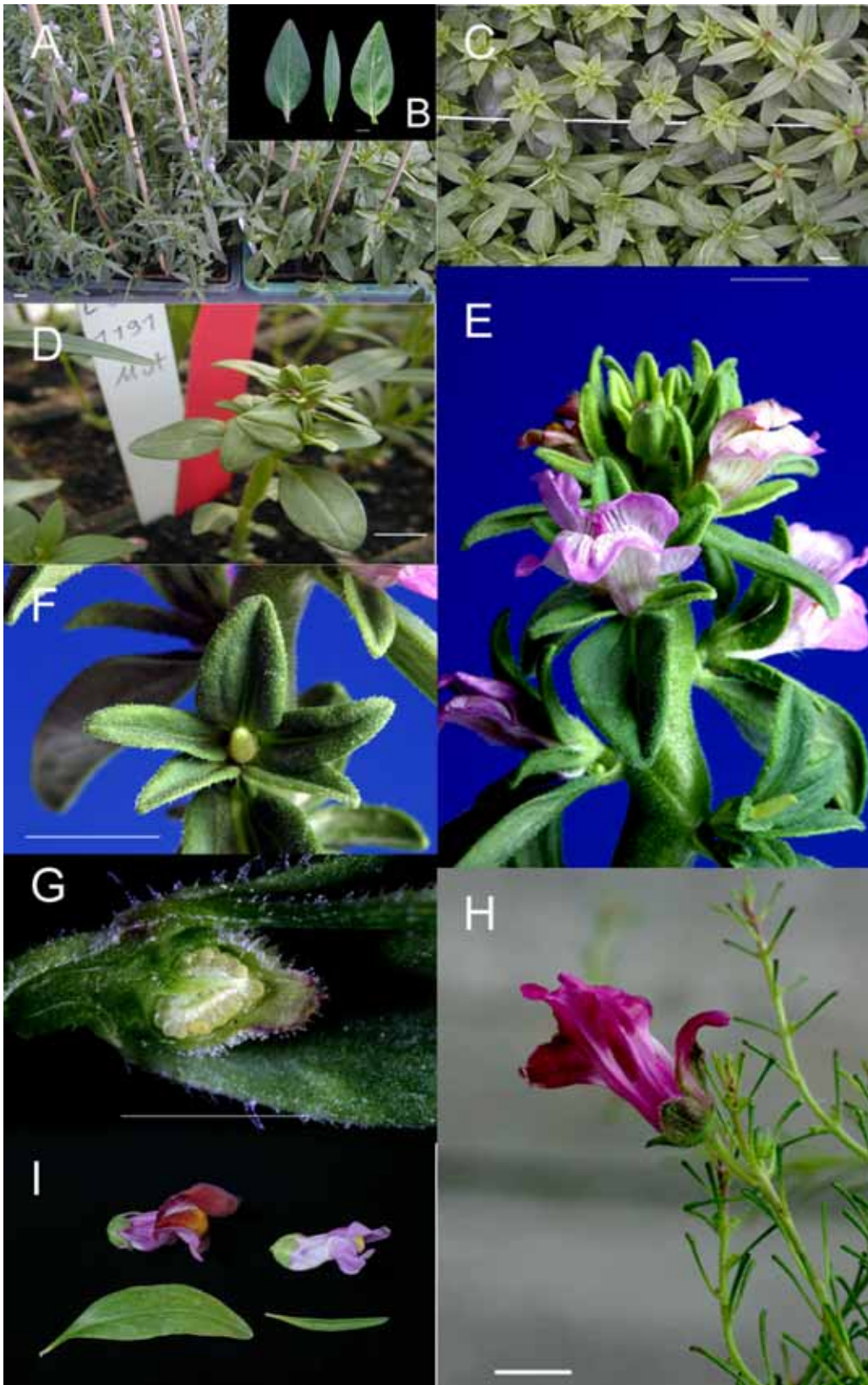
As mentioned above, the unusual sepals of *Misopates* constitute the most prominent apomorphic feature morphologically distinguishing the two genera and species from each other, even to the point of inspiring Lamarck to coin the species name ‘*calycinum*’. The sepals are as long as or up to twice as long as the corolla in *M. orontium* (developmental stages and modifications playing an important role in their variation), but the sepals of *Antirrhinum* are only ca. 1/5 the length of its corolla. Also, in absolute measurements, the *Misopates* sepals are still twice to three times as long as those of *Antirrhinum* (**Fig. 1**).

### Sepal- and leaf form correlations

Coming back to the second question of the introduction, mutant deviations in leaf form appeared to be strongly correlated in *M. orontium* for all leaf organs, i.e. leaves, bracts and sepals. So when the mutant leaves were more slender, those of the bracts and sepals displayed the same phenomenon. When the leaves proved to be broader, this was also true for the rest of the leaf organs (until now we have detected only one clear exception from this rule: in comparison to the wild-type, mutant L 2004/495 displays shorter and broader leaves, yet the sepals are not correspondingly changed). Conversely, in *A. majus* the widths of the sepals seem to be relatively independent of the other leaf organs: the sepals proved to be as broad as usual in several slender leaf mutants (for an extreme example, see the mutant *phantastica* in **Fig. 2H**) (as for *phantastica*, see Baur 1926; Waites and Hudson 1995, 2001; Waites *et al.* 1998) as well as in the subspecies *tortuosum*, the latter case displaying very slender (linear) leaves, but broad sepals (**Fig. 2I**). However, there are also *Antirrhinum* mutants where all three leaf organ types are affected by one and the same mutant gene (for example *abbreviata*, *acuminata*, *buxifolia*, *cincinnata*, *compacta*, to name but a few, for further mutants, details and references, see Stubbe 1966).

The usually strong correlation-results of leaf-mutagenesis in *Misopates* appear to be in agreement with the unanimous verdict of all morphologists commenting on the nature of the *Misopates* sepals as being essentially leaf-like (except, perhaps, the venation pattern with more parallel main veins, which corresponds to that of the sepals of *Antirrhinum*). In genetical terms this could mean that regulatory and target genes, which are expressed in *Antirrhinum* only in the leaves, but not in the bracts and/or





**Fig. 2 Leaf mutants of *Misopates* and *Antirrhinum*.** (A) (larger photograph). On the left: wild-type *Misopates orontium* with linear leaves; right (second tray): *Misopates* mutant L 2002/1843 with broad leaves. (B) (small figure within figure). From left to right: leaf of *A. majus*, normal leaf of *M. orontium*, *M. orontium* mutant L 2002/1843. (C) below the white string, except upper right: *Misopates* mutant L 2002/1843, above string, different culture varieties of *A. majus* some with moderately broader and others with more slender leaves than those of the *Misopates* mutant. (D) Broad-leafed *Misopates* mutant L 2003/1191 segregating in M2 population. (E), (F), (G): Mutant L 2003/1191 (enlarged). (E) compressed flowers of the mutants; (F) calyx consisting of broad sepals; (G) longitudinal section through ovary displaying normal looking ovules. For further details see the text. (H) *phantastica* mutant of *A. majus*. (I) Left, above and below: flower and leaf of *A. majus*, ssp. *majus*; right above and below: flower and leaf of *A. majus*, ssp. *tortuosum*: note that the width of the sepals is not correlated with the width of the leaves. (D-F) reveal that in *Misopates* there appears to be a stricter correlation for these features than in *Antirrhinum* (see also Fig. 4).

sepals, are now ectopically expressed in the *Misopates* sepals as well.

Even so, the exception of mutant 2004/495 from the correlation rule as mentioned above could be interpreted as a first hint at additional autonomous gene functions, which might be involved in the unusual calyx formation of *Misopates*.

#### **Mutagenesis of sepal length and numbers**

Two *Misopates* mutants displayed shorter sepals compared

to those of the wild-type species (Figs. 2D-G, 3A, 3B). However, in the mutant L 2000/1554, which was derived from the nearly white flowering Coimbra line from Portugal, all the leaf organs proved to be shorter than normal in harmony with the rule just mentioned. Additionally, flower form was also slightly affected, yet fertility was almost normal. In the second mutant (L 2003/1191), derived from the Vogelsang line of *Misopates*, not only all the leaf organs were reduced in length, yet increased in width, but also the petals and male and female organs proved to be all extremely shortened, and so were the internodes, but the

enlarged stem diameter (up to three times) was reminiscent of that of succulent plants (**Fig. 2D-G**). The stem was twisted. Moreover, fertility was strongly reduced in the latter mutant and the life cycle appeared to be abbreviated so that the mutants obtained so far, (1) dried up significantly earlier than the wild-types and (2) died without seed set, i.e. although ovule development appears to be normal, to date neither selfings nor pollination with wild-type pollen led to any seed set at all. Although stigma-like tissue appears at the style in early phases of development, the style appears to be closed at later stages. At present we propagate the mutant by using up the M<sub>2</sub>-seeds and by heterozygote sister plants: In 2005 the segregating mutants derived from M<sub>2</sub> sister plants again displayed their typical characters (as expected), yet grew larger and lived longer than those of the M<sub>2</sub> family.

Besides, sepal length can vary considerably in the totally sterile *plena* mutant of *Misopates*: flowers near the top of the inflorescences of older plants can show relatively short sepals (**Fig. 3C**): Yet this phenomenon hardly presents a clue to the questions raised.

The overall results concerning the problem formulated at the beginning – whether the enormously elongated *Misopates* sepals could be reduced to the length of the *Antirrhinum* sepals in perhaps one large mutant step or several smaller ones – has to be answered in the negative so far. The two mutant exceptions described above (not to mention the homeotic *plena* mutant) are hardly more than freaks, the pleiotropic effects of the mutant genes being recessive and thus due to losses of functions disturbing normal development and affecting the length and width of all leaf organs indiscriminately.

Hence, the long sepal feature has proved to be extraordinarily stable until now.

As for *Antirrhinum*, no mutants with comparably long sepals have ever been detected in any of the mutagenesis experiments over the last 100 years.

### Sepal number

In contrast to the length of the sepals of *Misopates*, their number can decidedly vary: (1) In mutant L 2002/5 the first flowers displayed only 3 to 4 more or less asymmetrically arranged sepals but the further upstream the flowers were positioned in the inflorescence, the more normal were their sepal numbers and symmetries; (2) one plant of L 2002/1323 also revealed special individual and ontogenetic asymmetries: the first flower had 7 sepals, but the following ones only 5.

No corresponding phenotypes have been described in *Antirrhinum*.

Homeotic sepal mutants with up to 7 sepals will be treated below under the section of homeotic mutants.

### Flower (corolla) size

As hinted at in the introduction, at first sight one of the more impressive differences between the two species appears to lie in the size of the flowers (see **Fig. 1**). In relative terms the size of the flowers of *A. majus* is about twice the length and width of that of the *M. orontium* flowers (for absolute measurements see **Table 1**).

Concerning the basic questions addressed in the title of the present paper, it is to be noted that in 22 cases independently arisen *Misopates* mutant candidates (seven tested, all heritable) display enlarged flowers (**Fig. 3D**). On the other hand, some *Antirrhinum* mutants show reduced flower size. Thus flower mutants of both species appear to approach each other in this feature. Interestingly, the sepals in the enlarged *Misopates* flowers were correspondingly longer (also to be seen in **Fig. 3D**). The flower size mutants of both species include fertile lines showing no or only weak pleiotropic defects in other plant organs. However, it should also be noted that mutants in both species have been detected, which swing in the opposite direction: *Misopates*

mutants with even smaller flowers than those of the wild-type and *Antirrhinum* mutants with larger flowers have been detected as well. An open question is whether the enlarged flower mutants of *Misopates* have already reached their selection limit for that character (for details on selection limits, see Lönnig 2001, 2002, 2004, 2006), or whether – by continued mutagenesis – further enlargements would be possible.

### Flower colour

Flower colour differences of isolated but otherwise similar populations of a species have often been deemed to be sufficient to suggest new species names in morphological systematics (for documentation, see Lönnig 2002). The typical *M. orontium* flower colour as given in almost all field guides is light violet with a clearly darker violet venation pattern in the upper lip. Flower colour of *A. majus* ssp. *majus* is several degrees darker (purple). So far, none of the many flower mutants of the Vogelsang line of *Misopates* has really approached this deep red of *Antirrhinum* (anthocyanin and cell shape are significant in the latter, see Noda *et al.* 1993; Schwinn *et al.* 2006), but virtually all mutant colour deviations moved in the opposite direction: a series of 19 *Misopates* mutants revealed nearly all shades of brightening until the pure white of the *nivea* lines of *Antirrhinum* was reached. So the mutants of the two species find a common level in loss of function mutations diminishing or abolishing anthocyanin synthesis.

In addition to the lack of potential to produce steps in the direction of the deep red flower colours so far in *Misopates*, another difference has been noted: the *Antirrhinum* colour mutants and recombinants displaying a whole series of yellow and bronze colour shades seem to be absent in *Misopates* (the *Sulphurea* gene?), pointing, if corroborated, to a poorer equipment in the anthocyanin pathway in *Misopates* as compared to that of *Antirrhinum*.

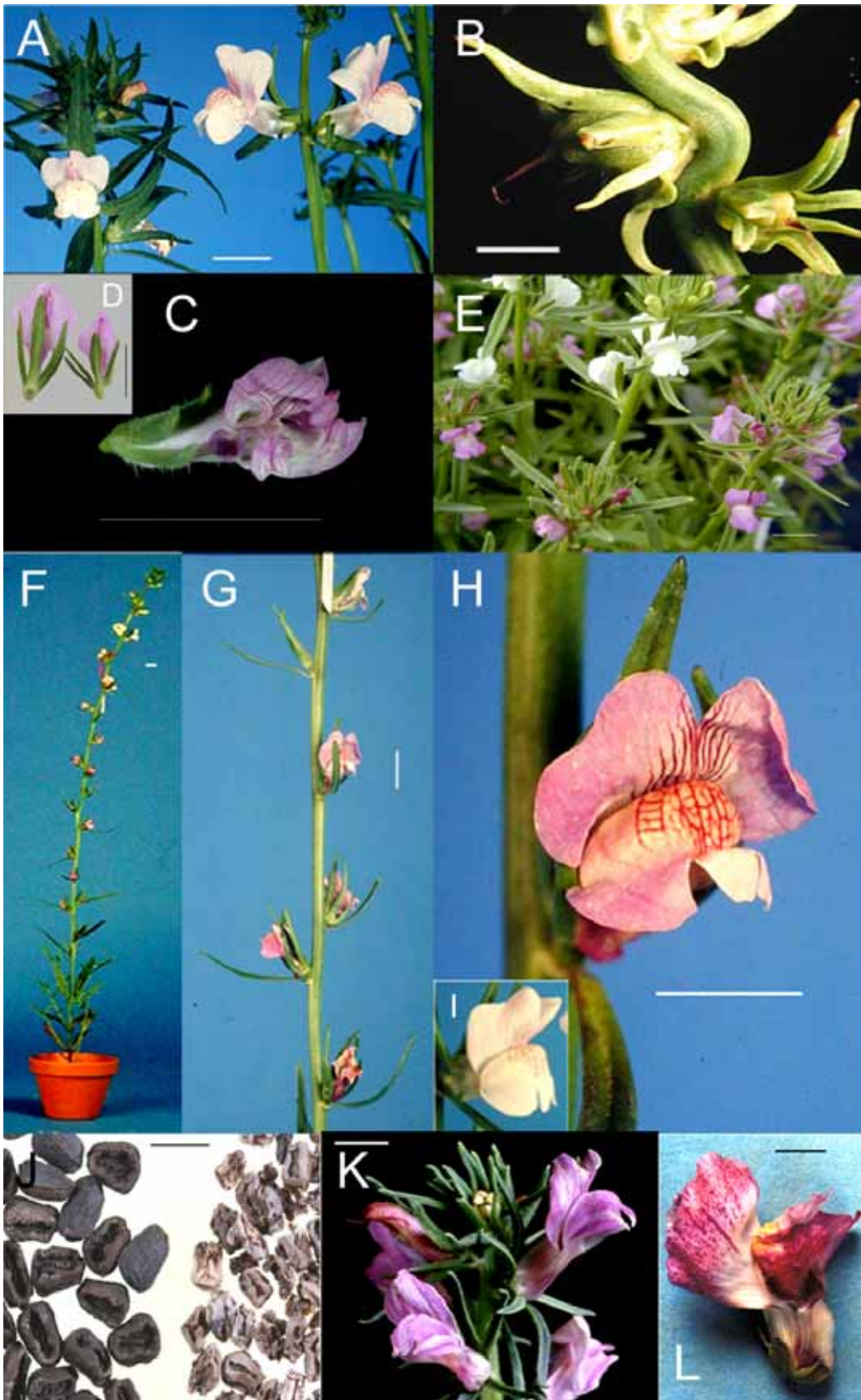
In the nearly white flowering Coimbra line of *Misopates* there appeared one mutant which proved to be violet-flowering in the field (**Fig. 3H**), so much so that at first sight we were not sure whether perhaps a contamination of the seed materials with the Vogelsang line had occurred. However, that could definitely be excluded by further investigations: upon closer inspection anthocyanin synthesis appeared to be UV light dependent in this mutant, i.e. when the UV component was missing, the flowers of the mutant remained nearly white – in contrast to the violet *M. orontium* wild-type lines (**Fig. 3F-I**). So in the field, the colour of the almost white flowering wild Coimbra line had phenotypically reverted to the more common violet of its putative ancestors.

### Fertilization

In contrast to the wild *A. majus*, there is a clear tendency to autogamy in *M. orontium*. Although the flowers of wild *Misopates* plants are diligently visited by different species of bees in the experimental field as well as in the wild, if cross-fertilization is prevented, they invariably set seeds by autogamy, self-fertilization beginning rather early in anthesis, roughly one to two days before normal petal development is finished and when the flower is still closed but the pollen sacs are already opening.

Hence, in *Misopates* the series of multiple alleles for ensuring self-sterility so characteristic of almost all wild *Antirrhinum* species appears to be absent or non-functional in the former (see also Kusaba *et al.* 2001, and Nasrallah *et al.* 2002 on *Arabidopsis thaliana* as a case of loss of self-incompatibility in the Cruciferae). However, most *A. majus* culture varieties and the (further) mutants derived from them, are self-fertile as well. Yet in *Antirrhinum* seed set due to autogamy is decidedly weaker than in *Misopates* and to guarantee full seed production in the former, *A. majus* culture varieties and mutants are usually selfed. Nevertheless, on the loss of function level the two species





**Fig. 3** Some pleiotropic mutants of and apomixis in *Misopates*. (A) Wild-type phenotype of *M. orontium* (left), and mutant L 2000/1554 (right). In the *Misopates* mutant the leaves, bracts, and sepals are altogether somewhat distorted and reduced. The leaf right below the flowers of the mutant is fused with the stem. (B) The main axes of the mutant plants regularly display this twisted/coiled phenotype whereas the branches tend to be more straight (segregation of the descendants of M<sub>3</sub> sister plants: 2593 : 807 (3.21 : 1) M<sub>2</sub> 43 : 10 (4.3 : 1)). (C) Short sepals in older inflorescences of the *plena* mutant of *Misopates*. (D) (left): Mutants with larger flowers appeared regularly in the M<sub>2</sub> populations of the *M. orontium* Vogelsang; wild-type line (right). (E) Disturbance of the anthocyanin pathway leading to one of the white flowering lines segregating in an M<sub>2</sub> population. (F-H) Putative UV sensitive mutant of *M. orontium*: the originally nearly white flowering *M. orontium* line from Coimbra turned red again (without UV light it remains white). (I) (picture within picture): wildtype phenotype. (J) Wild-type seeds compared to deranged seeds produced by apomixis. (K) Variegated mutant of *M. orontium* Vogelsang, (L) variegated flower of a descendent of Tam3 line of *nivea recurrens* 98 of *A. majus* (line kindly provided by R. Carpenter, John Innes Center, Norwich). See details in the text.

appear to approach each other again. (Loss of functional self-sterility alleles appears to have also occurred in the wild species *Antirrhinum siculum*). As for seed development without fertilization, see below the points under “seed formation due to apomixis”.

### Seed form

The bowl- or cuplike seed form of *M. orontium* shown in Fig. 3J (in contrast to the elliptical-roundish seeds of *A.*

*majus*) belongs to the more important and rather constant features distinguishing the two species. So the seeds of several *Misopates* mutants have been studied, yet to date no clear-cut deviations in mutant seed morphology have been detected. Several mutants, such as L 2000/1554 (leaves including the sepals reduced and somewhat twisted), L 2000/2064 (strong *cycloidea* allele), L 2002/ 691 (pure white flowers), L 2002/1843 (broad leaves; see above), L 2003/1191 (also broader leaves, but probably sterile, see above, M<sub>2</sub>-seeds investigated) and L 2202/2661 (larger



flowers) all manifested the seed form of the wild-type. In sterile mutants there is the difficulty that seeds of heterozygote sister plants segregating the homozygous mutant seeds or – if available –  $M_2$  seeds have to be examined. In both cases distinct, sharp and well-defined differences should be found among the seeds segregating 3 wild-type seeds to 1 mutant seed or – in cases with a deficit of recessives – correspondingly lower percentages of homozygous seed mutants would have to be discovered. In spite of the general stability of differences in the seed form between the two species, seed development is not always uniform, and it appears that reliable conclusions can be drawn only after repeated and extended investigations of larger seed materials, which remains to be done.

### Seed formation due to apomixis

As far as we are aware, apomixis has never been reported for *Antirrhinum* – or for *Misopates* either. Even so, one of us (HS) had expected it in *Misopates* because of some alleged  $F_1$ -hybrid plants between *Misopates* and *Antirrhinum* displaying almost exclusively the *Misopates* phenotype (Harrison and Darby 1955; Harrison 1960). However, in a potentially autogamous plant species self-fertilizing itself early in anthesis, the production or transfer of at least some pollen grains can hardly be absolutely excluded (even in cases of early castrations). The case is, of course, different in species with massive or even regular apomictic seed production as found, for instance, in many Asteraceae. Yet, in crosses between *Misopates* (as mother plants) and *Antirrhinum*, normally there was not any seed set at all (some 100 pollinated flowers).

Nevertheless, the problem has – at least in principle – been solved by work with our *deficiens* mutant L 2002/2851 of *Misopates* (see text below and Fig 3J). This mutant displays a strong *deficiens* phenotype and is unable to produce any pollen at all. Yet surprisingly it can produce high numbers of seed capsules filled with rather cranky looking seeds (Fig. 3J). Although none of these seeds has germinated as yet (59 capsules sown, ca. 40 seeds each), these cases of seed production clearly reveal at least a certain potential for apomixis in *Misopates* so far not detected in *Antirrhinum*. However, until some of such apomictically produced seeds really germinate, the case mentioned above for the alleged  $F_1$  plants of earlier authors cannot be taken for granted.

### Flower variegation and transposon activities

As shown in Fig. 3K mutant L 2002/2262 displayed variegated petals and concomitantly some flower abnormalities (sometimes *cycloidea*-like, often a bit twisted, and further minor petal deformations). Seed set due to self-fertilization is about a quarter of that of the wild-type. First results pointing to a high reversion rate of the mutant to the wild-type could not be corroborated in a large experiment involving 113 families (2,100 plants). Only one of these plants proved to be wild-type, yet contamination cannot be excluded in this case. As to the variegation pattern: comparing the *Misopates* phenotype closely to the ones known from *Antirrhinum*, there is one obvious difference to be noted: the pattern in *M. orontium* consists mainly of stripes longitudinal to the flower axis in contrast to *Antirrhinum* where it is generally more patchy and often distributed in the form of smaller dots (for a comparison see Fig. 3L). Other possibilities to explain petal variegation in the *Misopates* mutant could be DNA methylation. A molecular investigation of the phenomenon in *Misopates* might clarify the situation. Worth mentioning in this connection may be the fact that a conspicuously tricotyledonous phenotype derived from that mutant L 2002/2262 unexpectedly did not exhibit that feature in the following generation (as for tricotyledonous mutants with low penetrance in *Antirrhinum*, see Stubbe 1966).

In contrast to the findings in *Antirrhinum*, where most

mutants have been due to insertions of transposable elements (Sommer 1990; Kunze *et al.* 1997; Schwarz-Sommer 2003; Efremova *et al.* 2006), even after mutagenic treatments with EMS, fast neutrons or X-rays – which mutagenic agencies appear to have activated transposable elements (Schwarz-Sommer, personal communication) – no transposons have been detected in the mutant genes of *Misopates* sequenced so far (see Table 2 below). The results obtained to date appear to point to reduced transposon activities in today's *M. orontium* as compared to *A. majus*.

### Homeotic mutants

Because homeotic mutants can disclose similarities of and differences between different taxa usually hidden in their normal development, it was assumed that they might help elucidate these questions also for *Misopates* and *Antirrhinum* as well as be relevant for Dollo's law – the extent of reversibility of certain derived features of *Misopates* (see examples and discussion below).

Most of the homeotic mutant phenotypes described for *Antirrhinum majus* have also appeared in *Misopates orontium*: *cycloidea*, *hemiradialis*, *floricaula*, *squamosa*, *plena*, *deficiens*, *fimbriata* and others. Wherever possible so far, the identity of the mutants was clarified by DNA sequence analyses (Table 2).

**Table 2** The homeotic *Misopates* mutants.

| Gene        | Mutant    | Mutation        |                    |
|-------------|-----------|-----------------|--------------------|
| PLENA       | 2002-745  | small deletion* |                    |
| SQUAMOSA    | 2003-765  | large deletion* |                    |
| FLORICAULA  | 2003-369  | nu**            | 1 sub 12 del       |
|             |           | aa***           | 1 change 4 del     |
| FIMBRIATA   | 2002-1574 | nu              | 1 sub 2 del        |
|             |           | aa              | frame shift & stop |
| DEFICIENS A | 2002-823  | nu              | 21 del             |
|             |           | aa              | 7 del              |
|             |           | na              | 7 del              |
| CYCLOIDEA   | 2002-1249 | aa              | frame shift & stop |
|             |           | na              | 7 del              |
| CYCLOIDEA   | 2000-2064 | nu              | 6 del              |
|             |           | aa              | 2 del              |

\* the exact boundaries of the deletions were not identified

\*\* nucleotide

\*\*\* amino acid

Carefully comparing the homeotic *Misopates* mutants with the corresponding *Antirrhinum* phenotypes, the most dominating morphological feature distinguishing *Misopates* from the former, the long sepal character, also nearly invariably dominated the phenotypes of the homeotic mutants found to date (see Figs. 4-6). Yet, reversal of some homeotic features approaching the homeotic characters of the corresponding *Antirrhinum* mutants, as well as some (further) basic differences, were also noticed.

### Floricaula-like mutants

(Fig. 4A-C) (as for the detailed work on the corresponding *Antirrhinum* mutants, see Coen *et al.* 1990, Huijser *et al.* 1992): Because the morphological differentiation into leaves, bracts and sepals is absent in *Misopates* as compared to *Antirrhinum* and to most other members of the tribus Antirrhineae, in all our *floricaula*-like phenotypes (4 independently arisen cases, two of the mutant genes, *squa* and *flo*, have been identified so far – see Table 2) a repetition of leaf-like organs occurred in the inflorescence regions (instead of bract repetition as in *Antirrhinum*). This may seem trivial (a lost differentiation feature cannot, of course, be repeated), yet in cuttings made from three non-flowering lines (L 2000/1089, L 2000/3582, both Coimbra-derived, and L 2003/369, Vogelsang-derived), the leaves (repetitive bracts) appearing anew in the regenerated inflorescence were much smaller than those of the original mutant (Fig. 4B), somewhat reminiscent of the small *Antirrhinum* bracts (in extreme cases they were even shorter

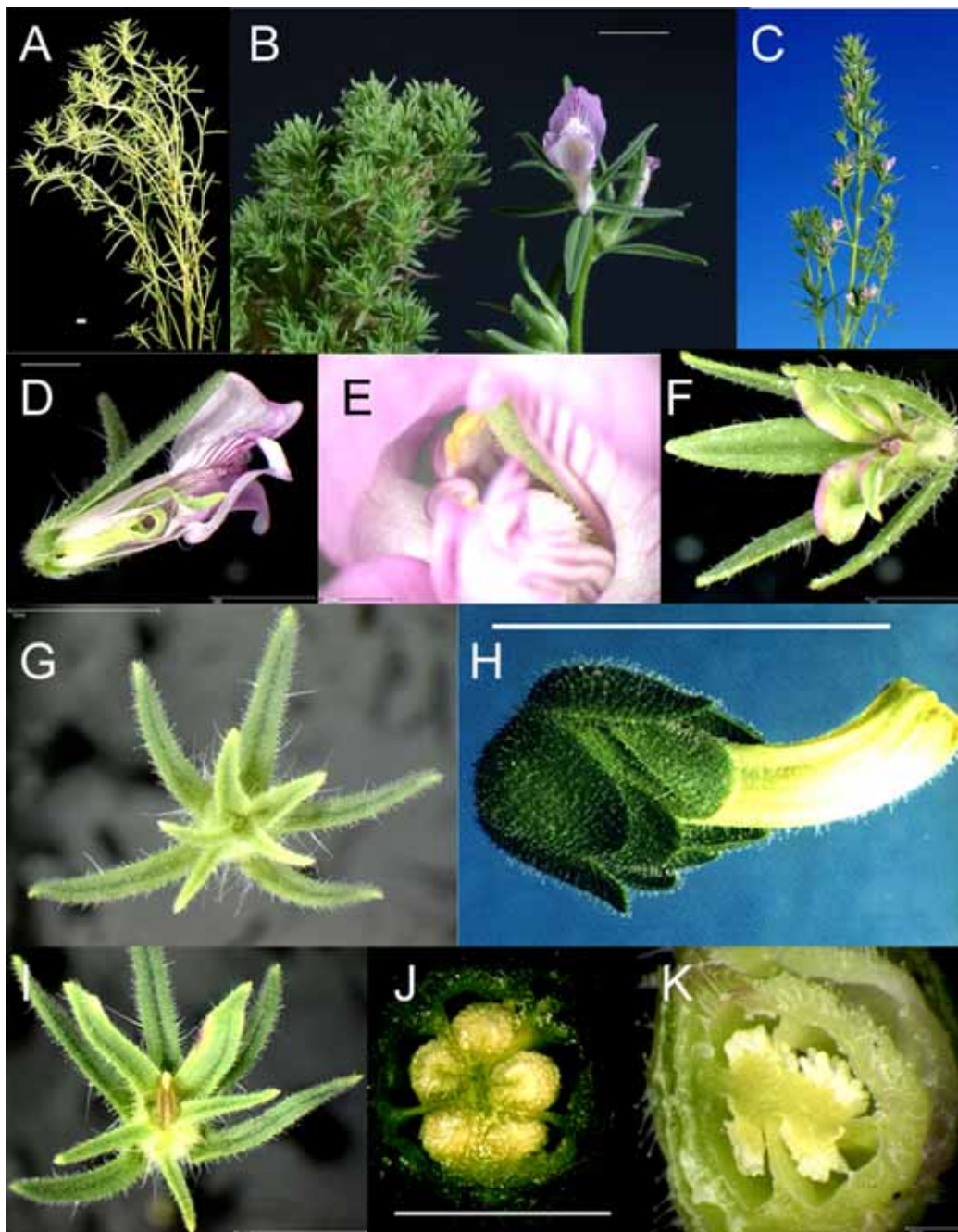
than in *Antirrhinum*). However, line 2003/765 (Vogelsang-derived; **Fig. 4C**), which regularly produces some malformed flowers (unexpectedly developing normal seeds regularly *without* artificial pollination) the repetitive leaves appear to be larger in the cuttings made so far as compared to the non-flowering phenotypes, nevertheless smaller than in the wild-type control.

### Plena

(as for *Antirrhinum*, see Bradley *et al.* 1993; Lönnig and Saedler 1994): In agreement with the most distinguishing bauplan feature of the species, the *plena* mutant of *M. orontium* (L 2002/745, derived from the Vogelsang line) is clearly different from the *plena* mutant of *Antirrhinum* by the modified reiteration of the longer sepal feature within the flower from the otherwise corresponding *Antirrhinum plena* mutants. The character reappears in the fourth whorl of the mutant flowers (**Fig. 4D, 4E**). However, the inner sepals do not constitute a simple repeat of the outer wild-type *Misopates* sepal whorl, but are modified: they are clearly shorter (on average nearly intermediate between the sepal lengths of *Antirrhinum* and *Misopates* thus approaching the situation in the *Antirrhinum* mutant), and

more tender and lighter green than those of the outer whorl, the pale green being possibly due to strong light protection in the innermost part of the flower. Moreover, the upper sepal appears to be generally longer than the lower one (some 2-3 mm). In contrast, in the *plena* mutants of *Antirrhinum* the inner sepals are about the size of the outer ones, sometimes even slightly longer (1-2 mm), but also lighter green. Besides, the *plena* mutant of *Misopates* can produce an additional shoot with 2 sepal-like leaves between the main axis and the pedicel so that at first sight it looks as if the flower had 7 sepals - a phenomenon not reported for *Antirrhinum*.

Moreover, the otherwise phenotypically closely corresponding *plena* mutant of *A. majus* obtained from the IPK (Gatersleben) develops - like our *Misopates* mutant - anthers at the upper parts of the second (inner) petal whorl. In *Antirrhinum* these anthers definitely produce some fertile pollen grains, which have successfully been used for the generation of hybrids, but in *Misopates* the anthers seem to be sterile. Yet at present it is not known whether similar sequences of the corresponding genes of the two species are mutated in comparable ways. As already mentioned above, in older inflorescences the length of the sepals of the upper flowers appears to be reduced (**Fig. 3C**).



**Fig. 4** Homeotic mutants of *Misopates orontium*. (A) *floricaula*-like mutant from segregating population displaying long repetitive bracts. (B) left: *floricaula* phenotype without any flower formation, derived from cuttings, in comparison with the wild-type: note that the repetitive bracts of the mutant are dramatically shorter than the bracts and sepals of the wild-type. (C) *squamosa* with rudimentary flower formation. (D) and (E) The *plena* mutant of *M. orontium*: note the elongated inner sepals in D. In E the anthers of the mutant are shown. However, in contrast to a well-known *Antirrhinum plena* mutant also developing anthers on petaloid organs, the anthers of the *Misopates* mutant do not produce fertile pollen. (F) and (G): weak and (G) strong *deficiens* allele of *M. orontium* in comparison to (H) strong *deficiens* allele of *Antirrhinum* ((I) strong *M. orontium* allele with style). (K) both *orontium def* alleles display only 4 locules in contrast to *Antirrhinum* displaying 5 ((J), reprinted from **Fig. 1A** in Tröbner *et al.* (1992) *The EMBO Journal* **11**, 4693-4704, ©1992 with kind permission from Nature Publishing Group).

## Deficiens

The *Deficiens* gene of *A. majus* was the first homeotic plant gene to be cloned and characterized (Sommer *et al.* 1990). As for *Misopates*, two *deficiens* (*def*) mutants have been derived from the Vogelsang line of *M. orontium* (L 2002/823 and L 2002/2851; **Fig. 4F, 4G, 4I**). In L 2002/823 the style is short and strong, the petals are not completely transformed into sepals but display residual petaloid tissue (usually the sepals of the second whorl are about half as long as the normal ones but broader and often show a light violet rim) (**Fig. 4F**). Yet the mutant is slightly temperature sensitive: Under 25°C the petaloid features are strongly reduced, under 15°C they appear as shown in **Fig. 4F** (see also the double mutants in **Fig. 7**). At the DNA level this *def* mutant is characterized by a deletion of 21 bp starting in the K-box after downstream position 255 (in the corresponding protein 7 amino acids are missing: downstream residues nos. 86 to 92 inclusively; see also **Table 2**). Interestingly, the strongly temperature sensitive mutant *def*-101 of *Antirrhinum* is due to a deletion of three base pairs of the K-box only one step downstream: amino acid in position no. 93 is missing, a deletion of a lysine residue (Schwarz-Sommer *et al.* 1992).

The sequences of the wild-type (wt) and temperature sensitive (ts) *def* mutants of *A. majus* and *M. orontium* starting at position 253 of the coding sequences of the gene ending at position 300 (the entire coding sequences is 681 bp long for a putative protein of 227 aa in *A. majus* and 684 bp/228 aa in *M. orontium*).

*A.m.* wt:  
GAGAAAATGCAAGAGCACTTGAAGAAGCTGAATGAGGT  
CAACAGGAAT

*A.m. def* 101:  
GAGAAAATGCAAGAGCACTTGAAG --- CTGAATGAGGT  
CAACAGGAAT

*M.o.* wt:  
GAGAAAATGCAAGAGCACTTGAAGAAGCTGAATGAGGT  
CAACAGGAAC

*M.o. def* (ts.):  
GAG ----- AAGCTGAATGAGGT  
CAACAGGAAC

The 3 dashes above mark the 3 deleted base pairs in *Antirrhinum* (*A.m. def* 101 standing for *Antirrhinum majus*, mutant *deficiens* 101), and the 21 dashes denote the deleted bp in the corresponding *Misopates* mutant.

The second mutant – most probably a null mutant due to deletion of seven bp, frameshift and stop – displays a strongly pronounced *deficiens* phenotype (**Table 2; Fig. 4G, 4I**): its second whorl is entirely sepal-like. Yet again – as was the case in the fourth whorl of the *plena* mutant – it is now the second whorl, which does not simply reiterate the sepals of the outer whorl. Instead, the inner sepals are shorter and more tender than the outer ones. Although in *Antirrhinum* the sepals of the second whorl are hardly distinguishable from the normal outer ones, in absolute terms the second whorl of the *Misopates* mutant again approaches the length of the corresponding whorl of the *Antirrhinum* mutant.

In contrast to the phenotype of the strong alleles of *deficiens* in *Antirrhinum*, the compound style appears to be decidedly more slender in *Misopates* (**Fig. 4I**).

Moreover, cross sections of the pistils of *Antirrhinum* and *Misopates* appear to point to a further basic difference between the two species: the *Antirrhinum* mutants manifest 5 loculi (Schwarz-Sommer *et al.* 1992), but the two *Misopates* mutants only 4 (**Fig. 4J, 4K**). Although a tiny staminoid is present in the normal *Misopates* flower, pointing to normal *Cycloidea* gene function, the involvement of that gene in the differences between the two species cannot be excluded to date inasmuch as the *Cyc* gene of *M. orontium* is 15 bp shorter than that of *A. majus* (nor can the presence of perhaps a very rudimentary fifth

locus in the pistil's early development of those *Misopates* mutants).

## Cycloidea

The two *cycloidea* phenotypes derived from the Coimbra line of *Misopates* revealed a weak (*hemiradialis*) and a strong phenotype respectively (**Fig. 5A, 5B**) (as for a detailed description and molecular characterization of similar mutants in *Antirrhinum*, see Luo *et al.* 1996, 1999; Galego and Almeida 2005; concerning *Linaria*, see some comments by Lönnig and Saedler 1997, but especially Cubas *et al.* 1999; Theißen 2000). The strong phenotype often displays 6 sepals, 6 (fused) petals, and 6 stamens, but varies and the number 5 for all three organs can also be found (often the radial phenotype appears to be very regular – much more so than in the corresponding *Antirrhinum* mutants). Moreover, the seed capsule commonly exhibits 4 pores instead of the normal number 3 in the wild-type. In contrast, the weak *hemiradialis* allele manifests the wild-type number of 5 sepals, 5 petals, 4 stamens, and 3 pores (**Fig. 5D-F**). On the other hand, the strong *cycloidea* phenotypes of *Antirrhinum* disclose 5 sepals, 5 petals and 5 stamens, the weak ones 5, 5 and 4 or 5, respectively.

However, the *Antirrhinum* double mutant *cycloidea/dichotoma* also shows 6 sepals, 6 petals and 6 stamens, the style being significantly shorter than the stamens and self-fertilisation rate is considerably lower than normal. Pore number appears to be 3 as in the wild-type. Although the weak *hemiradialis*-like phenotype of *Misopates* manifests also only 3 pores, at first sight they look as if there were 4, yet a thin slit connects the seemingly two upper ones. The mutant produces an average seed set solely due to autogamy. However, the strong *cycloidea* allele, in which the style is also somewhat shorter than the stamens, should better be selfed to obtain sufficient seed numbers. To date, it is known that one of the two independently arisen *Misopates cycloidea* phenotypes (the strong one) is, indeed, due to a mutant *cycloidea* gene (see **Table 2**), and test crosses have been carried out to identify the basis of the second mutant phenotype: the F1 is wild-type and the F2 segregates both phenotypes – so the weak allele is assumed to be due to a mutant *radialis* gene.

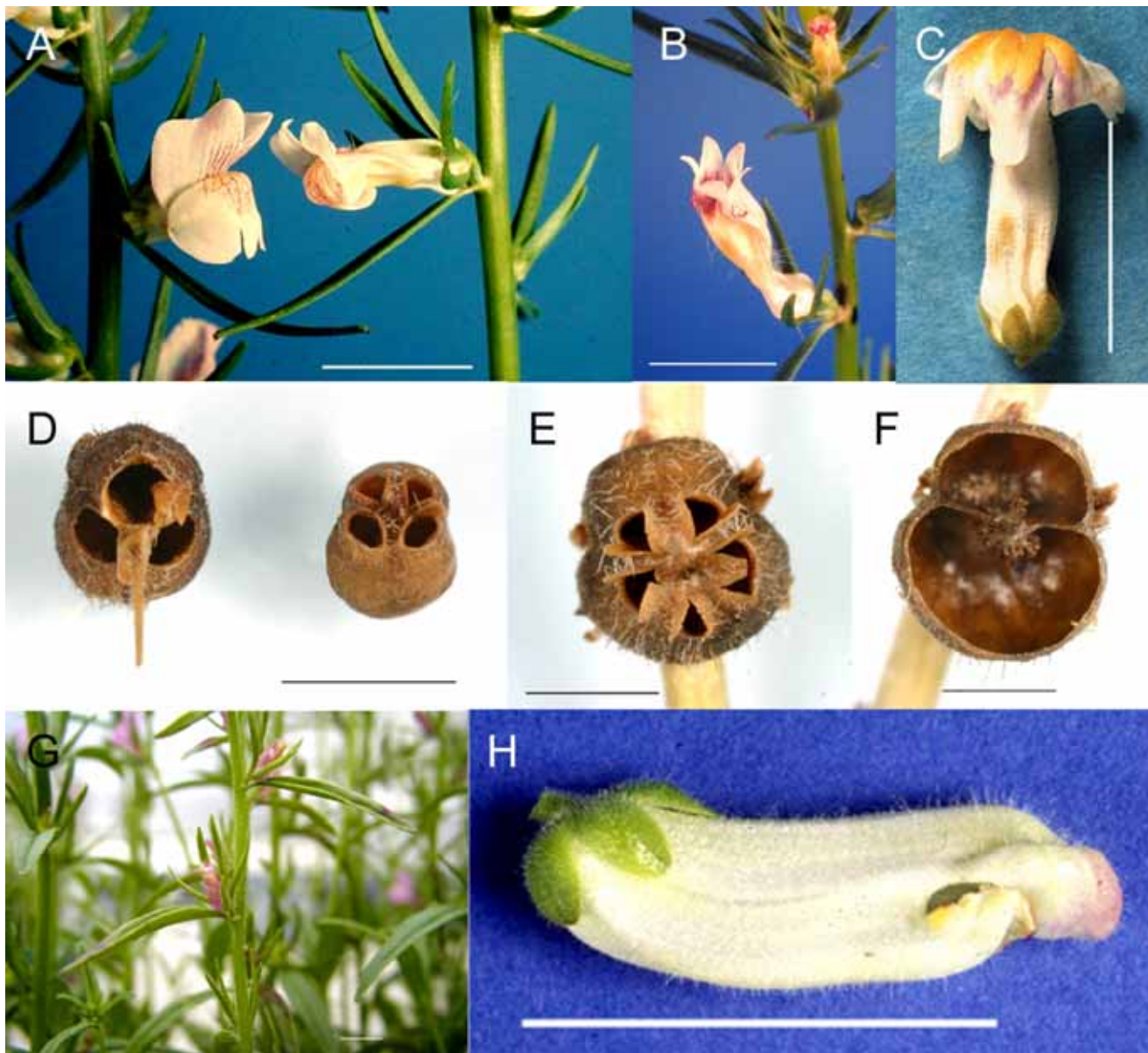
## Fistulata-like phenotypes

Also, three phenotypes were registered revealing *fistulata*-like flower aberrations (**Fig. 5G**; as for data on the *fistulata* mutant in *Antirrhinum*, see McSteen *et al.* 1998; Motte *et al.* 1998). In contrast to *Antirrhinum*, the corolla is often closed in *Misopates*, but seed set is guaranteed by autogamy. Yet, nothing is known at present of the molecular basis of the *Misopates* mutants.

## Fimbriata

(As for the investigations on the corresponding *Antirrhinum* mutants, see Simon *et al.* 1994; Ingram *et al.* 1997; Schultz *et al.* 2001, and for a comparison between *Antirrhinum* and *Arabidopsis*, Ingram *et al.* 1995): The Vogelsang-line derived *Misopates fimbriata* mutant L 2002/1574 represents one of the most striking phenotypes detected so far in *Misopates* (see **Fig. 6A-G**). A substitution and the deletion of two base pairs in an open reading frame of the gene resulting in a frame shift and a stop codon have most probably generated a null-allele (see **Table 2**). The *fimbriata* mutant displays the ensuing characteristics: a normal looking flower pedicel is followed first by 5 sepals and further by a bunch of sepal-like leaves. The sepal-like leaves are arranged around a series of nodes with strongly compressed internodes in between (see **Fig. 6A**). Whilst the plant keeps on growing and maturing, the internodes also elongate so that short branches develop. Additionally the “flowers” produce several fairly small styles and locule-like organs generating conventionally looking ovules. Although





**Fig. 5** *Cycloidea* and putative *fistulata* alleles in *Misopates*. (A) *M. orontium* wild-type of the Coimbra line in comparison with its weak *cycloidea* allele. (B) Strong *cycloidea* allele of the same line. (C) The *cycloidea* phenotype of *Antirrhinum*. (D) (left): normal *orontium* capsule with 3 pores, right: *cycloidea* normally displaying 4 pores, but as shown in (E), can also develop 5 pores, yet in any case the capsule displays only 2 loculi as in the normal initial line (F). (G) *fistulata*-like phenotype of *M. orontium* in comparison to the *fistulata* mutant of *A. majus* (H). In contrast to the latter, the *M. orontium* mutant's petals are closed.

the overall impression is that the development of these organs is strongly abnormal, rather unexpectedly these tiny organs are fertile and produce some seeds upon pollen transfer to the stigmas of the styles. The *Antirrhinum fimbriata*-null mutant can produce a basically similar phenotype, and this is even true for the mutants of the corresponding *UFO*-gene in the distantly related *A. thaliana* (Ingram *et al.* 1995).

In contrast to *Antirrhinum* the *fimbriata* null mutant of *Misopates* has never produced petaloid tissue sectors as is regularly the case in *Antirrhinum* (Fig. 6E, 6G) or the weak *deficiens* allele of *Misopates* described above.

Segregation: *fimbriata* segregation in the  $M_2$  strongly deviated from the normal ratio: wild-type plants 511:63 *fimbriata* (8.1:1). This was probably due to sectorial mutant tissue in the  $M_1$  plant (Gottschalk 1994). Segregation in the sister plants was normal: 394:124 (3.17:1).

#### **Mutants with petaloid sepals**

Mutant L 2003/971, derived from the Vogelsang line of *M. orontium*, displayed varying numbers of sepals (5-7) partially transformed into petaloid tissue sometimes fused with the lower lateral sepals (Fig. 6I, 6J). Phenotypically it is

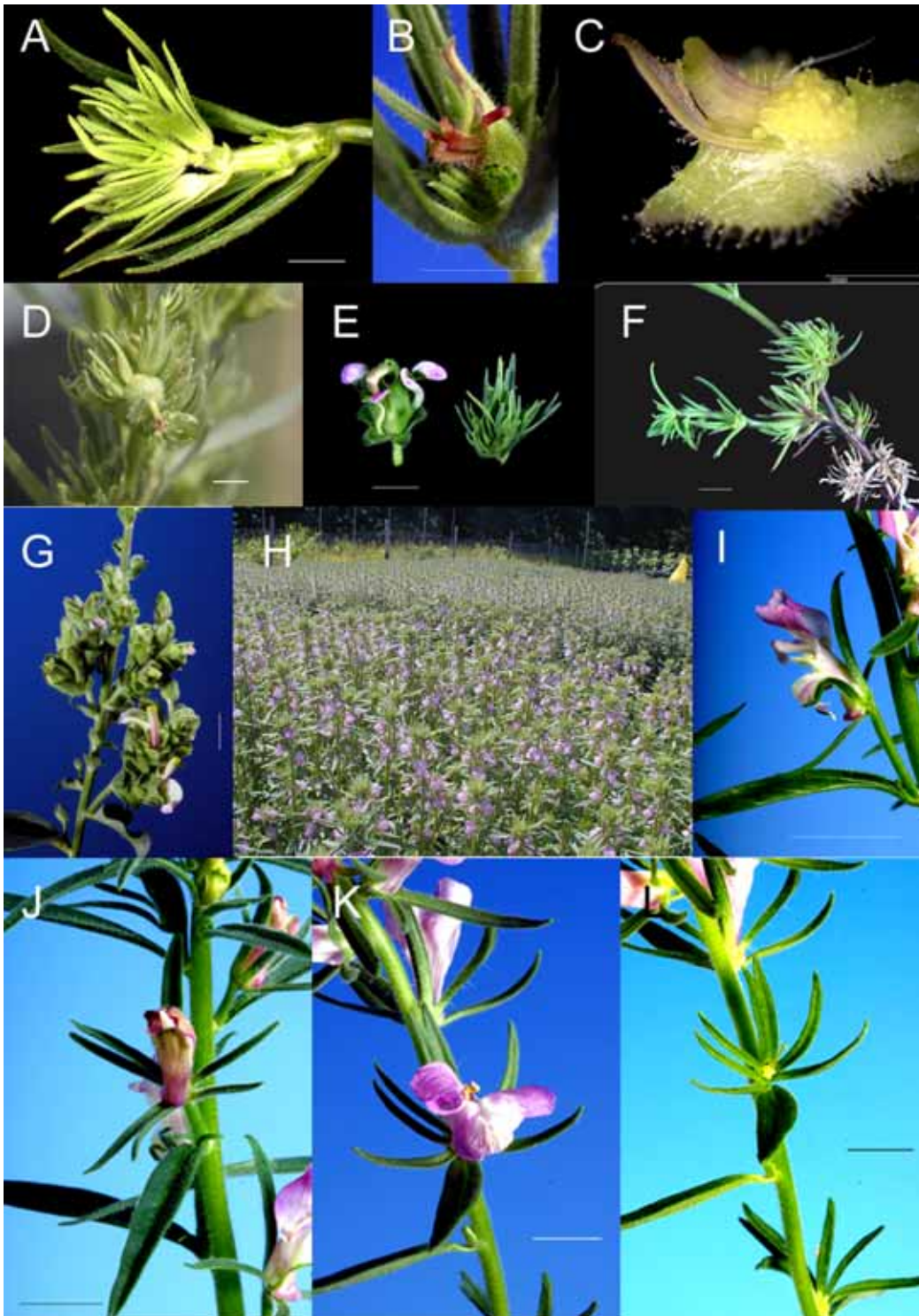
somehow intermediate between the *fimbriata*- and *cycloidea* mutants of *Antirrhinum*. Furthermore, mutant 2003/977 (petaloidy to be confirmed) manifests up to 7 sepals, of which the 2 adaxial ones can be fused with the lower lateral petals. Interestingly, the additional adaxial (median) sepal is often forked (Fig. 6K, 6L), thus representing another feature not reported for *Antirrhinum*. Both mutants display lowered fertility.

Further investigations appear to point to strong environmental influences on the expression and penetrance of these mutant features:  $M_3$  sepal number deviations were severely reduced under mild greenhouse conditions in contrast to the  $M_2$ -phenotypes raised under strongly varying field parameters.

#### **Unidentified *Misopates* mutants**

In *Misopates* there appeared several mutants for which no corresponding phenotypes have (yet?) been detected in *Antirrhinum* and there seem to be potent reasons for the hypothesis that – as in several cases described above – further homologous mutant genes and perhaps even some non-homologous ones of *Misopates* and *Antirrhinum* might be involved in the development of phenotypes differing in





**Fig. 6 (A-F)** The *fimbriata* mutant of *Misopates orontium*. (A) Mutant phenotype of a flower with elongating internodes. (B) Flower with several styles; (C) Style of younger flower enlarged displaying ovules at its base of a style. (D) Capsule with seed set upon artificial pollination in that mutant (seed set upon pollination of such styles was regular). (E) comparison of *fimbriata* null mutants of *A. majus* and *Misopates*; (F) *M. orontium fimbriata* flower internodes elongate to produce further “flowers”; (G) Similar situation in the *fimbriata* null mutant of *A. majus* (yet the *A. majus* mutant regularly displays some petaloid tissue whereas this has not been detected in *M. orontium*). (H) *Misopates* M<sub>2</sub> field. (I) *M. orontium* phenotype of L 2003/971 with the two middle sepals fused to petals. (J) The same plant displaying flower with 7 “free” sepals. (K) Plant of L 2003/977 with varying numbers of sepals (again 7 in the flower shown, the upper 2 are largely fused) and often show *cycloidea*-like petal deformations. (L) Same as (K), but flower removed. As far as the authors know, there are no *Antirrhinum* mutants, whose flowers produce 7 sepals.

some basic features of these closely related species.

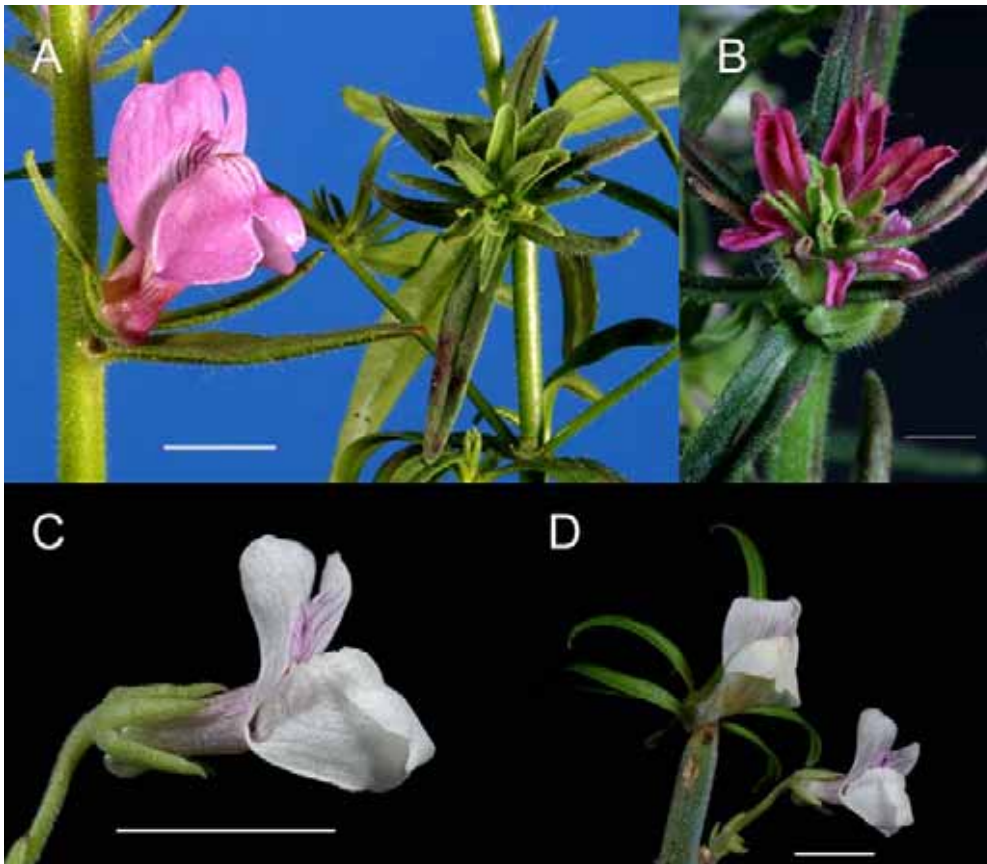
The description and discussion of such further phenotypes, as the *bizarre* mutant (reduced petals, sterile) and several others of this category of unidentified mutants, will be given in another paper.

#### Double mutants

The double mutant between the temperature-sensitive weak allele of *deficiens* and the *plena* mutant consisted either of weakly petaloid organs of partly violet colour (15°C) or solely sepaloid structure (25-30°C) – see **Fig. 7**. Further double mutants are currently being generated.

#### Dollo's law and the ABC(DE) flower developmental models

Several authors have argued with the German poet Johann Wolfgang von Goethe (1790) for a fundamental equivalence of the flower and the stem in the angiosperms (Coen and Carpenter 1993, there further references), implying that the different parts of the flower – sepals, petals, stamens, carpels – are essentially equivalent to the leaves of a shoot. This equivalence theory has had a long tradition especially in the German speaking world and has been known under the slogan “alles ist Blatt” (everything is leaf). Although determinacy, internode length, organ identity and phyllo-taxy have all been changed in the flowering plants, these novelties have often been assumed to be “simply different modifications of a common growth plan”, produced by “different permutations of a few key features of plant



**Fig. 7 A putatively temperature-sensitive double mutant of *Misopates* and phenotype of the genus *Pseudomisopates rivas*.** (A) *Misopates orontium* wild-type flower (left), right: the double mutant *deficiens/plena* on the right at 25° to 30°C, developing sepaloid petals. (B) also double mutant *deficiens/plena*, but at 15°C: the outer whorls are larger and the anthocyanin pathway of the petals is switched on. (C) *Pseudomisopates rivas martinezii*. (D) Left above: *M. orontium* in comparison to right: *P. rivas martinezii*: note the contrast in sepal length between the two genera.

growth" (Coen and Carpenter 1993, p. 1175).

In the well-known but simplified ABC model of the master regulatory genes specifying flower development, the A-function is thought to designate sepal identity, the A- and B-functions cooperate for petal formation and the solitary C-function determines carpel identity, – the A- and C-functions being antagonistic (for reviews see for example Coen and Meyerowitz 1991; Theißen and Saedler 1999; Theißen *et al.* 2000; Keck *et al.* 2003; Efremova *et al.* 2006).

Although recent research has refined the model (see for instance Theißen and Saedler 2001; Zahn *et al.* 2006) – for nature is often much more complicated than our paradigms predict or even can predict – let us assume for a moment that the homeotic mutants *deficiens* (B-mutant) and *plena* (C-mutant) discussed above are steps to the assumed ground state of angiosperm evolution, i.e. the leafy shoot. In that case the *deficiens* phenotype would represent “a former state already realized in the series of its ancestors” and, in contrast to Dollo’s law, a morphological reversal would, indeed, have occurred: the petals constitute an apomorphic feature, but a double whorl of sepals a more plesiomorphic state – as is known sepals are usually more leaf-like than petals. A similar statement could be made for the *plena* mutants: the petals, being much less differentiated than the stamens and carpels, could perhaps be viewed to constitute a step in the approach to the ground state just mentioned. Finally, the double mutant *plena/deficiens* consisting of sepaloid leaves only, could be interpreted to almost have reached that ground state in defiance of Dollo’s law, in which a shoot only displays leaves.

However, such an interpretation can be criticized for several basic reasons (Lönnig 1994):

- 1) In this scenario the assumed ground state of the angiosperms would have no sexual reproduction, which is in contradiction to any theory on the origin of species known to the authors.
- 2) In the homeotic mutants the flower organs are *not transformed* into simpler or more original ones, but the complex genetic programmes necessary for the petals, stamens and

carpels are switched off by transposons or loss-of-function-mutations and are often substituted by other less differentiated ones. To illustrate the point in the words of Sattler (1988): “If a botanist in a biology department is replaced by a zoologist (as too often happens), the latter is not a transformed botanist simply because his predecessor was a botanist; he only occupies the same position. From the sameness of position, it does not follow that members occupying it are also essentially the same; they may be similar or very different.”

3) There is no equivalence of the flower and shoot concerning sexual reproductive functions. Already at the beginnings of the 1980s, Kamalay and Goldberg (1980, 1984) had detected that “both the anther and the ovary contain approximately 10,000 diverse mRNAs that are not detectable in heterologous organ system mRNA or nuclear RNA populations” (Drews and Goldberg 1989). Since that time many further genes have been cloned and sequenced that are expressed only in the flower and hardly anywhere else (for an exhaustive literature survey see the annual bibliography of Georges Bernier, for example of the year 2001, or the following editions up to now).

The independent results of many research groups that large numbers of genes are expressed almost exclusively in the flower distinctly disproves the simple equivalence of flowers and shoots required by Goethe and his adherents.

4) As is now generally known to biologists, the MADS-box sequences are conserved from yeast to humans. Losses of functions in MADS-box genes perturb the various organisms quite differently, and – most important for the question of the ABC(DE) flower developmental models with regard to Dollo’s law – there is no trend to categorize the deviations as atavisms (for a further review on the developmental models, see Davies and Schwarz-Sommer 1994 and the more recent literature just mentioned above). To take again the first molecularly investigated homeotic flower mutant: hardly anyone would claim that the different (promoter-, MADS-box and K-box) alleles of the *DEFICIENS* gene of *A. majus* effectuating various degrees



of greenish petals (not to speak of the temperature-sensitive allele *defA-101*) would be the atavistic gene sequences of the angiosperms and thus be relevant for Dollo's law. The same can doubtlessly be maintained for the alleles of the corresponding *APETALA3* gene in *Arabidopsis* (Jack *et al.* 1992). The substitution of a complex developmental programme necessary for the formation of special flower organs by an earlier and simpler one does not indispensably confirm the equivalence of both (for further points see Lönnig 1994). Thus, clear evidence for the relevance of the ABC(DE) models of flower development for Dollo's law appears to be missing at present.

### Genetic distance between *Misopates* and *Antirrhinum*

The genetic distance (base substitutions) for protein coding sequences between humans and chimpanzees has been calculated to be about 1.23%, including the indels in noncoding sequences now roughly 5%, but up to about 80% of the proteins are different (Britten 2002; Watanabe *et al.* 2004; Weissenbach 2004; Glazko *et al.* 2005). In **Table 3** we have presented the divergence data for 18 nuclear genes for *M. orontium* and *A. majus* (comparison of altogether 14,140 bp). Although the distances vary strongly from gene to gene (see the details in **Table 3**), a putative medium overall divergence of 5.64 bp substitutions per 100 can be projected for the genomes of the two taxa, that is slightly more than 4.5 times the distance between the coding sequences of humans and chimpanzees.

**Table 3** Genetic distances between *Antirrhinum majus* (Am) and *Misopates orontium* (Mo).

| Gene                           | Sd/100 | Sn/100 | Sum   | Sd                 |      |     | AS dif-ferent | Reading frame |
|--------------------------------|--------|--------|-------|--------------------|------|-----|---------------|---------------|
|                                |        |        |       | +Sn                | Sd   | Sn  |               |               |
| <i>AmMADS11</i>                | 3.10   | 1.55   | 4.65  | 30                 | 20   | 10  | 15            | 645           |
| <i>CENtroradialis</i>          | 2.38   | 4.76   | 7.14  | 39                 | 13   | 26  | 9             | 546           |
| <i>Chalcone synthase (CHS)</i> | 0.94   | 5.88   | 6.82  | 80                 | 11   | 69  | 9             | 1173          |
| <i>CYCloidea</i>               | 3.23   | 5.54   | 8.77  | 76                 | 28   | 48  | 19            | 867           |
| <i>DEFiciens</i>               | 0.73   | 6.11   | 6.84  | 47                 | 5    | 42  | 3             | 687           |
| <i>DEFH7*</i>                  | 2.42   | 3.06   | 5.48  | 34                 | 15   | 19  | 11            | 621           |
| <i>DEFH21</i>                  | 0.67   | 4.15   | 4.82  | 36                 | 5    | 31  | 3             | 747           |
| <i>DEFH24</i>                  | 6.11   | 3.49   | 9.61  | 66                 | 42   | 24  | 19            | 687           |
| <i>DEFH49</i>                  | 0.13   | 3.63   | 3.76  | 28                 | 1    | 27  | 1             | 744           |
| <i>DEFH68</i>                  | 1.38   | 2.14   | 3.52  | 23                 | 9    | 14  | 6             | 654           |
| <i>DEFH200</i>                 | 0.96   | 2.33   | 3.29  | 24                 | 7    | 17  | 5             | 729           |
| <i>FARinelli</i>               | 1.34   | 2.82   | 4.17  | 31                 | 10   | 21  | 8             | 744           |
| <i>FIMbriata</i>               | 2.70   | 4.78   | 7.63  | 99                 | 35   | 62  | 27            | 1298          |
| <i>FLORicaula</i>              | 1.34   | 7.04   | 8.38  | 100                | 16   | 84  | 9             | 1194          |
| <i>GLOBosa</i>                 | 0.15   | 2.47   | 2.62  | 17                 | 1    | 16  | 1             | 648           |
| <i>INCOmposita</i>             | 1.17   | 2.19   | 3.35  | 23                 | 8    | 15  | 6             | 686           |
| <i>PLEna</i>                   | 1.94   | 1.38   | 3.32  | 24                 | 14   | 10  | 7             | 723           |
| <i>SQUAmosa</i>                | 0.80   | 1.87   | 2.68  | 20                 | 6    | 14  | 6             | 747           |
| Sum:                           | 31.49  | 65.20  | 96.84 | 797                | 246  | 549 | 164           | 14,140        |
|                                |        |        |       | Average difference |      |     |               |               |
|                                |        |        |       | Bases/100          | 5.64 |     |               |               |

\*In the scientific literature only the abbreviations for the *Deficiens* homologues are given: *DEFH7*, *DEFH21* etc. The comparisons include only the reading frame regions including the stop codon, with the exception of *INCOmposita*, where the stop codon has not been sequenced. The introns and intergenic regions show high divergence and where not aligned. The column "reading frame" displays the total number of nucleotides.

On several phylogenetic presuppositions (and after several revisions during the last decades) not be discussed in this paper, a divergence time of 4.6 to 6.2 million years has been estimated for the two latter taxa (Chen and Li 2001), and according to a more recent review about 8 million years (Junker and Scherer 2006). A tentative extrapolation from these hypotheses to the pair *Misopates/Antirrhinum* would mean a divergence time between 21 to 36 million years.

## DISCUSSION

### General discussion of Dollo's law

Before we discuss the relation of our empirical results to biodiversity and to Dollo's law we should make some introductory remarks on Dollo's law of irreversibility.

As far as the authors are aware, perhaps the most thorough discussion of Dollo's law (including the translation of original French papers into the English language) has been presented by Gould (1970) and the basic points later reinforced by Gould and Robinson (1994). In the first paper the author contrasts Dollo's law against several other largely discredited evolutionary laws, among them orthogenesis, recapitulation, Cope's and Williston's laws arguing that irreversibility on the other hand "is a notion quite different from the standard set of such "laws"", because, in Dollo's sense, "this would require that the organism retrace, exactly and in the same order, an extremely large number of steps" (Gould pp. 189, 198). Muller explained the point by "the sheer statistical improbability, amounting to an impossibility, of evolution ever arriving at the same complex genetic end-result twice" (Muller 1939). Reasons pointing in the same direction but from a thermodynamic vantage point have been presented by Blum 1962, see also Gould 1970).

Dollo himself explicated his law as follows (1913): "The irreversibility of evolution is not simply an empirical law resting on facts of observation, as many have believed. It has deeper causes which lead it, in the last analysis, to a question of probabilities as with other natural laws...In order for it [evolution] to be reversible, we would have to admit the intervention of causes exactly inverse to those which gave rise to the individual variations which were the source of the first transformation and also to their fixation in an exactly inverse order – a circumstance so complex that we cannot imagine that it has ever occurred."

From the geneticist's point of view, however, there are now several themes, which should be briefly considered to replenish the discussion (the first point could have been contemplated by Dollo himself to a certain extent, at least after the so-called rediscovery of Mendel's laws in 1900).

**1. The basic difference between genotype and phenotype in classical (Mendelian) genetics.** In the text above we have repeatedly referred to differences between *Antirrhinum* and *Misopates*, which have *phenotypically* been clearly diminished or fully overcome by mutant genes, so that *Misopates outwardly* approaches, meets or even overlaps features of *Antirrhinum majus*. In polygenic traits many individual mutant genes can result in similar phenotypic aberrations: so totally different sequence deviations can lead to identical phenotypes. Double mutants (mutations in combination with suppressor mutations) can result in the reversal to near wild-type phenotypes although at the DNA level the original sequences may be entirely lost.

**2. Transposable elements (TEs).** Could a Dachshund or a Chihuahua ever revert to the phenotype of his ancestor, the wolf? Certainly not – if several losses of gene functions due to deletions or other complex sequences deviations are involved. However, if the key regulators were simply blocked by non-autonomous TEs which could be reactivated by some transposases *in trans* of active transposons (due to recombination or tranformation), a reversion to the original wild-type gene function and corresponding phenotypes should be possible – astounding as it may appear especially in this case. Yet at least partial reversions to the wild-type phenotypes have been reported for several crop plants both under human care and in the wild. Strongly deviating phenotypes have, indeed, reverted to wild-type in the controlled experiments of many TE investigations: the *deficiens* mutant of *A. majus* regularly reverts to the wild-type phenotype when active TEs are involved (see **Figs. 1, 4H**; Sommer *et al.* 1990). The same is true for active TEs blocking genes of the anthocyanin pathway or other

pathways or functions (for further examples, see Nevers *et al.* 1986; Lönnig and Saedler 1994; Kunze *et al.* 1997).

**3. Epigenetics.** The original DNA sequences of genes can even be entirely conserved, yet due to methylation the gene functions remain unexpressed. Reversal to the original function due to demethylation has also been repeatedly reported (for recent reviews, see Kalisz and Purugganan 2004; Tariq and Paszkowski 2004). So, even in the absence of any mutant DNA sequence variations strong phenotypic aberrations can be produced, be ‘inherited’ and revert to the wild-type after many generations (applying this to our Dachshund or Chihuahua examples, the ‘inhibited wolf’ could reappear in almost one step due to demethylation of the key regulatory genes).

**4. Reversibility by recombination within species and genera.** When an original species splits up into a series of subspecies by differential losses of gene functions, the original phenotype and even genotype may be retrieved in one or several steps of recombination as far as free recombination is possible (for details, see Lönnig 2002).

**5. The law of the homologous series in hereditary variation** (Vavilov 1922). This law describes, emphasizes, and predicts recurrent morphological and physiological phenotypes in related and non-related taxa. Von Sengbusch (2003) has summed up the law as follows:

(a) “Genetically closely related species and genera are characterized by similar series of hereditary variation with such a regularity that, knowing the series of forms within one species, one can predict the existence of parallel forms in other species and genera. The genetically closer the genera and Linnaeons within the general system are, the greater is the similarity in the series of their variation.”

(b) “Whole plant families are generally characterized by a definite pattern of variation penetrating all the genera and species comprising a family.” The author provides the ensuing example: “Brittle ears’ of gramineae is regarded as a primitive trait that occurs only exceptionally in cultivated varieties. The cross of certain varieties with stable ears results sometimes in offspring with brittle ears. Such setbacks are called atavisms...” (for further examples, see Vavilov 1922, 1951; Scherer 1993).

Thus such ‘atavisms’ would (phenotypically) “constitute a former state already realized in the series of its ancestors”.

The famous reversal cases of melanism in *Biston betularia* and in approximately 100 additional butterfly species may be subsumed under the rule of recurrent variation, which is closely related to Vavilov’s law of the homologous series in hereditary variation, yet in contrast to the latter accentuates the cyclic and finite nature of functional reversals and variation *per se* within microevolution (Lönnig 1995, 2002, 2003b, 2005, 2006).

**6. Gene mutations.** Concerning point mutations (partly overlapping with points 5 and 7), reversals have been described for several plant and animal species (for reviews, see Gottschalk 1971; Lönnig 2002; Lewin 2003). Among many other topics, Gottschalk (1971) has reviewed all the cases of alterations in the sepal whorl known until then and believed to be due to gene mutations. Strongly elongated sepals were found in *Lycopersicon* (mutants *macrocalyx* and *torosa*) and *Papaver* (*leafy calyx*). Later Vrebalov *et al.* (2002) sequenced the *Rin* locus of tomato (in the *rin* mutant fruits fail to ripen and the calyx is enlarged). It was detected that two mutant genes were involved in this phenotype, *LeMADS-RIN* in ripening and *LeMADS-MC* in sepal development and inflorescence determinacy, *LeMADS-MC* being the homologous gene of *Squamosa* of *Antirrhinum* and *Ap1* of *Arabidopsis*, respectively.

The phenomenon of calycanthemy, the transformation of sepals into petals so that the flower displays two petal

whorls in extreme cases, has been detected in many angiosperm species and it has been classified as “back differentiation” (“Rückdifferenzierung”, i. e. reversals to a less differentiated state – Gottschalk 1971). As to selection, which is thought to be a key factor for determining selection values, so far only the mutant *calyciflora* of *Nicotiana tabacum* (“subject to splitting of corolla tube and other morphological irregularities...three or four of the sepals are always roseate colored in whole or in part” – White 1916) was deemed to be “fully fertile”, yet – even apart from its morphological irregularities – one may doubt whether it would have any chance of survival in the wild, inasmuch as even *N. tabacum* has never been found there. Most of the calycanthemy mutants display further hefty anomalies; many disclose reduced fertility or are sterile.

Many additional examples of calycanthemy are found in less recent works on plant teratology (Masters 1869; Penzig 1922). Although hardly any of these cases were genetically analyzed so that a part of the instances might be simply due to modifications, the potential wealth of the information intrinsic in that older material becomes immediately apparent when one applies Stubbe’s rule to it, derived from his *Antirrhinum* studies (1966): “All alterations due to different environmental factors (modifications) have also been detected as mutants, but not all phenotypes due to mutations can be replicated by environmentally elicited modifications”. Concerning calycanthemy He *et al.* (2004) have raised the question whether nature has taken advantage of the possibility to ectopically express B-function genes, like *Def* and *Glo* in the sepal whorl thus producing two whorls of petals as found in the Liliaceae (further points on calycanthemy, see below).

**7. Constancy of developmental pathways.** Several authors have argued that the developmental programmes and pathways underlying (even) complex morphological features lost in the history of a genus or family can nevertheless be maintained on the genetic level for millions of years and have, indeed, been reactivated in several cases (Marshall *et al.* 1994; Collin and Cipriani 2003; Whiting *et al.* 2003).

On an experimental basis (probably in part due to point mutations, too) phenotypic reversal has been reported for *Drosophila* (Hall 1995; Teotónio and Rose 2000; and reviewed by the latter authors in 2001). Reversals from antibiotic resistance to sensitivity – predominantly due to losses of plasmids carrying resistance factors – has been detected in many lines of different species of microorganisms (see, for example, Madigan *et al.* 2000). Bull (2000) also mentions reversibility in attenuated virus of Sabin poliovirus vaccine, in domesticated organisms when returned to the wild, and of traits when artificial selection in lab experiments is relaxed. Last, but not least the famous reversals in the Axolotl (*Ambystoma mexicanum*), a larval salamander of mountain lakes of Mexico that usually lives without metamorphosing, should be mentioned in this paragraph (for details see, for example, Armstrong and Malacinski 1989).

**8. Reversals from polyploidy to diploidy.** A range of examples has been presented and discussed by Gottschalk (1976). Although Grant (1981) assumes that reversals from polyploidy to diploidy will not overturn polyploid trends, he specifies: “The haploid or polyploid progeny of a tetraploid parent is, of course, diploid. Such polyploids do arise spontaneously in polyploid plant populations.” For recent literature on polyploidy, see Bennett (2004), as well as 25 further papers on the topic in the same (special) issue of the Biological Journal of the Linnean Society, edited by Leitch *et al.* (2004).

**9. Homeomorphy.** Additionally, a series of phenotypic reversals have been implied and described in paleontology under the term “homeomorphy” for more than 50 years now (homeomorphy being “the result of a convergence which

has gone so far as to produce a similarity affecting the whole outer appearance of the homeomorphs to such a degree that the one may be mistaken for the other unless certain internal or other characters requiring close examination are studied" – Haas and Simpson 1946). Since this phenomenon has repeatedly been described *within* certain species and genera, it may be relevant for Dollo's law. Such reversibility has been reported for foraminifera, brachiopods, trilobites, conodonts, tabulate corals, rudist bivalves, and others.

### Relevance of mutagenesis with *Misopates* and *Antirrhinum* for biodiversity and Dollo's law

The assumption of the significance of mutants in general and homeotic mutants in particular mentioned for our topic in the introduction and later, *inter alia* that the homeotic mutants may disclose further similarities of and differences between various taxa usually hidden in the wild-type and non-homeotic mutant developments as well as be relevant for Dollo's law – appears to be fully vindicated by the results obtained:

1) The (expected) similarities found at the level of the mutants, especially the homeotic deviations, reinforce the inference drawn from the ontogenies of the wild-type phenotypes of the two taxa to their common basic developmental patterns. Nevertheless, the temperature sensitivity in the weak *deficiens* allele due to a deletion in 21 bp as described above was, in fact, more than we had anticipated.

2) Conversely, most of the following differences and reversions in the corresponding mutants and phenotypes of the two species could hardly have been predicted for *Misopates* from the mere inspection of the wild-type on the basis of contemporary phylogenetic hypotheses:

- (a) reversion into the direction of the putative original perennial life cycle (as mentioned above, normal life cycle of 3 to 4 months of *Misopates* extended to about 10 months in a non-homeotic mutant and to more than 4 years now in some (repeated) cuttings of homeotic mutants; this process can probably be extended indefinitely),
- (b) supposed reversion to broad leaves,
- (c) reversion of the almost white flowering Coimbra line to a violet flower colour,
- (d) putative reversion to larger flowers,
- (e) the strong reduction of sepal length in the cuttings of the *floricaula*-like mutants,
- (f) the intermediate length of the inner 'sepals' as well as the reduction of the outer sepals at the top of older inflorescences in the *plena* mutant (perhaps also its sterile anthers),
- (g) the intermediate length of the second sepal whorl in the *deficiens* mutants and the short and broad style in the weak allele and the extraordinary slender and long style in the strong one,
- (h) the absence of the fifth loculus in the *deficiens* mutants,
- (i) the closed corolla in *fistulata*-like phenotypes,
- (j) the absence of petaloid sectors in the *fimbriata* mutant comparable to those in the corresponding *Antirrhinum* phenotype,
- (k) sepal number variation in one and the same mutant (up to 7 sepals),
- (l) (at present) some hardly or not identifiable mutants.

Points (a) to (g) could be relevant for Dollo's law (see discussion below). Moreover, referring to further mutants mentioned in the first part of the present paper, the ensuing differences pertinent for the biodiversity question in general could hardly have been predicted from the mere inspection from the wild-type phenotypes either:

- (m) the absence of yellow flowering mutants (so far?),
- (n) seed formation due to apomixis,
- (o) four pores in the seed capsule of the *cycloidea* mutant of *Misopates*,
- (p) until now, no hints for a reactivation of TEs by mutagenic agencies.

3) The differences between *Misopates* and *Antirrhinum* in combination with the fact that the most conspicuous morphological distinction between the two taxa, the enormously elongated sepals of *Misopates*, could not be reduced as an isolated morphological feature to that of the length of those of *Antirrhinum* (or *vice versa*), appears to point to a deeply rooted genetic and developmental disparity between them. This seems to be in accord with the overall distance that can be inferred from the sequence comparisons reported above.

### Objections against the relevance of the experimental mutants for Dollo's law

We will begin the discussion of this part by several objections that could be raised against the applicability of the presumed reversals reported above to falsify Dollo's law and subsequently present the counter arguments:

First, nobody can be sure what the original features of the stem group of the family or tribus really consisted of. Second, the putative reversals reported here all belong to micro-evolutionary events, mostly due to *losses of gene functions*; however, Dollo's law is essentially describing morphological macroevolution generally thought to be the result of *gains of gene functions* (often including new promoter- and gene sequences). Third, Dollo's law was meant only for complex not for simple morphological structures.

### The complexity criterion of Dollo's law

Continuing the discussion with the third more general point (to return to the two others subsequently), one could, indeed, argue that at the genotypic level Dollo's law is correct for any complex DNA rearrangements whose reversal would demand the "intervention of causes exactly inverse to those which gave rise to the individual variations which were the source of the first transformation and also to their fixation in an exactly inverse order – a circumstance so complex that we cannot imagine that it has ever occurred." Applying this scenario to our Dachshound and Chihuahua examples (see general discussion of Dollo's law above), the original wolf DNA-sequences and corresponding phenotypes would never reappear even in millions of generations (except for phenotypes perhaps in the unlikely case that for any mutant gene a corresponding suppressor allele generating the original wild-type phenotype could be devised).

Or, to directly address some genetic differences between *Misopates* and *Antirrhinum*, – the divergence between the taxa given in **Table 3** will never revert to a common sequence by random mutations, although individual bp can.

There is, however, no question that – when formulating his law – Dollo did not (and could not) think in terms of genetics at all (inasmuch as there was no discipline of genetics in any modern sense before 1900). He rather followed the "immortal Charles Darwin" (Dollo 1893), whose basic ideas on evolution Dollo had unreservedly accepted ("Louis Dollo was the most Darwinian of paleontologists in his time" – Gould and Robinson 1994). Now Darwin had formulated his Lamarckian pangenesis hypothesis according to which the causes, "which gave rise to the individual variations" etc. (see above) were the imprints of the environment on the organism becoming hereditary by the "gemmules" produced by the entire body continuously travelling to the germ cells indelibly preparing them for the inheritance of the acquired characters for the following generations (for further details and references, see Lönnig 2003a; – "indelibly" refers to Dollo's "virtue of the



indestructibility of the past"). In conjunction with Dollo's strictly deterministic view of the natural world (he "was educated in the mechanistic tradition that dominated late nineteenth-century science" – Gould 1970), irreversibility was an unavoidable inference for any complex constellation in evolution – and complex is what the constellations have always been.

Gould and others have tried to defend Dollo's law arguing that "he applied irreversibility only to complicated morphologies", but Dollo himself had definitely stated that (to repeat) "structural or morphological reversal does not occur", or "an organism cannot return, even partially, to a former state already realized in the series of its ancestors". Moreover, Gould admitted that if the qualifying term "complex" is used to exclude any possible counter-instance, the statement becomes unfalsifiable" (Gould 1970).

To sum up the complexity objection, Dollo derived his law of irreversibility of evolution from the doubtful (some authors, especially of viewpoints C<sub>1</sub> and C<sub>2</sub>, – see below – would prefer to use the adjective "false") mechanistic and deterministic perspective dominating the end of the 19<sup>th</sup> century in combination with a basically imperfect view of heredity. Apart from the missing distinction between phenotype and genotype and the further points mentioned above (mostly simply unknown to him), he himself did not clearly define "complex" (impossible) versus "simple" (possible) reversals. Since, *sensu lato*, even both kinds of reversals have been described (see again Marshall *et al.* 1994; Bull 2000; Teotónio and Rose 2000, 2001; Collin and Cipriani 2003; Whiting *et al.* 2003), experimentally induced mutants could obviously also be relevant for Dollo's postulate of general irreversibility – including the mutants of *Misopates* and *Antirrhinum*.

### Micro- and macroevolution

The second objection mentioned above (the 'putative reversals reported here all belong to micro-evolutionary events due to losses of gene functions; however, Dollo's law is essentially describing morphological macroevolution mostly due to gains of gene functions (often including new promoter- and gene sequences)') has in part already been answered in the last paragraphs. However, if the objection were correct, also the micro-evolutionary events due to temporary losses of gene functions by TEs and methylation mentioned in the general discussion above might be classified as irrelevant for Dollo's law.

Yet, Dollo did not distinguish between micro- and macroevolution at all, nor, as mentioned above, did he delineate simple from complex reversions. Additionally, the absence of the differentiation between genotypes and phenotypes (whose distinction would have been possible for Dollo after 1900) appears to be a *proton pseudos* of Dollo's law. As is well known today, losses of gene function can be compensated by suppressor- and modifier mutations to restore the original phenotype's morphological structures (for some suppressor mutations with morphological effects see, for example, Hong and Spreitzer 1998; Hsieh 2000; Resnick *et al.* 2006), so phenotypic reversibility appears to be possible *in both directions*, losses and gains of functions – at least to a certain extent. The phenotype of *Misopates* mutant L 2002/1843, for example, displaying the broad leaves normally found in *A. majus* is certainly due to a loss of function mutation: all its features are fully recessive when crossed with the wild-type, recessiveness being a rather sure indicator of a loss of gene function. Yet there will hardly be any risk in predicting that by further mutation a mutant phenotype with slender leaves will reappear – being very similar to the original character of the *Misopates* wild-type (Vogelsang) line.

Conversely, the violet coloured mutant of the *Misopates* Coimbra line is certainly a gain of function, which in turn could be lost again in further mutagenesis experiments. The basis of this gain of function is presently unknown;

demethylation of a regulator of the anthocyanin pathway could be among the possible causes. In any case, it is taken for granted that the necessary DNA sequences are still present in the Coimbra line.

From the *neo*-Darwinian standpoint of the origin of species and higher systematic categories by a putative process called "additive typogenesis" (see, for example, Heberer 1971; Cizhak *et al.* 1996; Sauer and Rehfeld 1999) macroevolution should – even to a large extent – be reversible by what may be called "subtractive typolysis", i.e. by simple losses of the additionally acquired morphological features. However, since most new characters arise, not by simple additions but by integration of complex networks of gene functions rendering many systems to be irreducibly complex (Behe 1996, 2004; for a review, see Lönnig 2004), such systems cannot – in agreement with Dollo's law – simply revert to the original state without destroying the entire integration pattern guaranteeing the survival of a species.

Nevertheless, extensive research on "regressive evolution" (for several review points, see Kunze *et al.* 1997; Lönnig 2002) has described a range of different species, genera and families, in which morphological features have, in fact, been lost due to mutations (yet within certain limits), in some cases perhaps reverting to some earlier and simpler morphological state. In many cases of regressive evolution, the life forms were given the status of species and genera of their own – the phenomenon thus partly belongs to "transspecific evolution", the latter being the identification mark of macroevolution according to Mayr's definition (Mayr 2001).

Yet, nobody will deny that the origin of higher systematic categories and Baupläne demands more than losses of gene functions (after all, 'it is hard to think of oneself as an inactivated amoeba'; Crow 1981), and for the rise of these taxa as well as for the inception of irreducible complex systems, the debate continues whether mutations and selection alone will be sufficient to produce all the new genetic functions and innovations necessary for the cytoplasm, membranes, and cell walls (see, for instance, Behe 1996; Lönnig and Saedler 2002; Swift 2002; Campbell and Meyer 2003; Dembski 2004; Lönnig 2004, 2005).

### The status of the original features of the tribus *Antirrhineae*

According to the third objection 'nobody can be sure what the original features of the stem group of the family or tribus really consisted of'. This is certainly correct: most inferences concerning the reconstruction of the history of any higher taxa must remain chiefly hypothetical. In spite of some strong overall assertions, the uncertainty of the deductions as to the details is true for any evolutionary theory including the modern synthetic theory of evolution. Moreover, several researchers fully committed to naturalism prefer theories that question whether there has ever been something like a stem group of the tribus *Antirrhineae* at all (Nilsson 1953; Schwabe and Warr 1984; Schwabe 1986; Schwabe and Büllsbach 1998; Schwabe 2001, 2002, 2004; Schwabe, personal communication 2003, 2004).

On the other hand it should be noted that even most critics of the modern synthesis accept direct phylogenetic connections within almost all of the lower systematic taxa at least up to the tribus or family level of the plant and animal kingdoms (for further points, see Lönnig and Saedler 2002; Meyer 2004). Yet for various rational reasons they reject the extrapolation from the mode of origin of the lower taxa to that of the higher systematic categories – i.e. usually the origin of the tribus or families and/or higher taxa themselves (for discussions of the details, see Lönnig 2002; Campbell and Meyer 2003). For our purposes we will refer to these three groups of researchers as representing viewpoints A, B, and C in the following text: A: modern synthesis, B: genomic potential hypothesis, and C: tendency to postulate a discontinuous origin of higher taxa either in combination

with purely naturalistic interpretations (C<sub>1</sub>: Margulis and Sagan 1997; Schwarz 1999; Erwin 2000, 2004; Jablonski *et al.* 2000; Gould 2002; Müller and Newman 2003; Valentine and Jablonski 2003; Valentine 2004; Theißen 2005; and last but not least, Dollo himself) or, from a more or less typological vantage point, often including ID (C<sub>2</sub>), the hypothesis that, for example, irreducible complexity in certain organs and physiological processes (not to be discussed here) is real and not only apparent and is best explained by intelligent design. The latter, in turn, can be grouped into researchers postulating phylogenetic connections to putatively preceding taxa (C<sub>2a</sub>) (Behe 1996; Denton 1998; Berlinski 2003a, 2003b; Conway-Morris 2003a, 2003b), and those doubting additional evolutionary links on a macro-evolutionary scale (C<sub>2b</sub>) (Dembski 1998, 2002, 2003, 2004; Junker and Scherer 2001; Junker 2002; Swift 2002; and many further authors, in part already quoted above – see Campbell and Meyer 2003; Meyer 2004).

Apart from the fact that we do not aim to present a complete literature survey on the different positions here, but refer mainly to some relevant recent contributions, it should perhaps also be added that the following discussion is not meant to scientifically recount and evaluate the different views mentioned above. Rather, it is essentially dedicated to an interpretation of the applicability of Dollo's law within the different frameworks and hypotheses, which are (also) presented in contemporary peer-reviewed scientific journals and/or peer-reviewed books. Only where a topic demands direct comparison of different ideas (like natural selection, see below), a few points will be referred to. (As for the different viewpoints, it need hardly be mentioned that the minority view of yesterday has become the standard vantage point of today and there is no convincing reason to believe that this will be any different in the future.)

Assuming with the majority of contemporary biologists (A, C<sub>1</sub>, C<sub>2a</sub>, and C<sub>2b</sub>) that there was, indeed, a common ancestral species of all the members of the Antirrhineae, – which characteristic features could have distinguished it? In the abstract and introduction of this paper we have spoken of “the eight derived features essentially distinguishing *Misopates orontium* from its closely related *Antirrhinum majus*”: (1) annuality, (2) linear leaves, (3) long sepals, (4) small flowers, (5) light violet flower colour, (6) autogamy, (7) cuplike seed form and (8) apomictic seed production (for details, see **Table 1**). Concerning the sepals we have remarked that “since even from the most inclusive evolutionary presuppositions *Misopates* cannot be directly derived from an original ancestral angiosperm stock with leaflike sepals, according to viewpoints A, C<sub>1</sub> and C<sub>2a</sub> this trait would have returned – at least phenotypically – (quoting Dollo) “to a former state already realized in the series of its ancestors”. According to the frame of reference C<sub>2b</sub>, the realization of *Misopates* would, in the absence of putative ancestors at the root of the angiosperms, signify a return to a more generalized (original) ground-plan of the flowering plants, i.e. to one without an advanced differentiation into leaves, bracts and sepals. Consistent with viewpoint B, “Mutations are a reality of life, but a chain of reinforcing constructive mutations are not part of reality” and because initial DNA (RNA) accumulations “remain species-specific through all generations” (Schwabe 2004), the evolutionary tree would be an illusion, for life is assumed to be “polyphyletic from its inception” (Schwabe 2001). This is supposed to be a fact even within the tribus of the Antirrhineae (Schwabe, personal communication). Although for totally different genetical reasons, this position not only agrees with Dollo “that structural or morphological reversal does not occur”, but also denies that “functional or physiological reversal occurs” in the evolutionary sense quoted above (some critical remarks on the distinction between morphological and physiological reversals are given below).

However, let's return to the present majority viewpoints and continue our discussion with additional questions on

the status of the leaf-like *Misopates* sepals.

### Further questions concerning the status of leaf-like sepals

So focussing first on the sepals as the most distinguishing morphological character of *Misopates* the following question might arise: would the reverse order from leaf-like sepals to differentiated ones appear to be equally (or even more) probable from current evolutionary premises (viewpoints A, C<sub>1</sub> and C<sub>2a,b</sub>)? Assuming a common ancestor with leaf-like sepals as found in *Misopates* for the contemporary 328 species of the Antirrhineae (Sutton 1988), this would signify the highly convergent, multiple origin of clearly distinct sepals from the more original (plesiomorphic or “primitive”) state. Because multiple independent mutations regularly leading to basically the same, or at least very similar, complex morphological differentiation processes appear to be less likely than loss-of-function-based reversals to a more or less original state (“ground-state”) in a few genera than the reverse in perhaps dozens of taxa, the leaf-like sepals would constitute the apomorphic (derived) state for the three genera *within this group*. (There are altogether 27 genera in the Antirrhineae, three of which – *Misopates*, *Linaria*, and *Holzneria* – display strongly elongated sepals in relation to the size of the corolla, yet in *Linaria* only one species out of the exactly 150 counted at present exhibits that feature, but six species out of seven in *Misopates* and the two *Holzneria* species. The exception in *Misopates* appears to be *M. chrysothales* from Morocco developing a corolla size of 11.5-14 mm and the length of adaxial sepal lobe being 6-7.5 mm according to Sutton 1988. However, the data for the latter species should be checked.)

As noted above, several authors have concluded that the developmental programmes and pathways underlying (even) complex but lost morphological features can be maintained for millions of years at the genetic level (Marshall *et al.* 1994; Collin and Cipriani 2003; Whiting *et al.* 2003), and reversions have experimentally been determined in *Drosophila* (Teotónio and Rose 2000) and other organisms (Bull 2000; Teotónio and Rose 2001), the question might be raised whether the strongly reduced bracts of the three *floricaula*-like phenotypes point to a similar, but perhaps rudimentary genetic situation in *Misopates* for this and other characters.

However, the results so far obtained by mutagenesis that – as isolated organs – neither the long leaf-like sepals of *Misopates* could be reduced to that of the length of *Antirrhinum* sepals nor the short differentiated sepals of *Antirrhinum* be extended to the length of the former, may suggest a hypothesis that in these two taxa the sepal problem goes deeper still (see further discussion below).

Concerning Dollo's law, the few isolated cases of leaf-like sepals within the tribus Antirrhineae, including the insulated case of *Linaria chalepensis* of the overall 150 *Linaria* species (Sutton 1988), seem to point to reversions to a more basic and undifferentiated state on the premises and data so far considered and obtained (viewpoints A, C<sub>1</sub>, C<sub>2a</sub>).

Nevertheless, in the tribus Antirrhineae there are also several further delicate sepal differentiations along with some more or less slight elongations of the sepals in relation to the corolla that appear to be more than just reversions to an original state (see Sutton 1988, for the sepal distinctions in several species of *Schweinfurthia*, where the adaxial calyx lobe is exceeding and overlapping the four lateral lobes and is also exceeding the corolla-tube, *Linaria paradoxa* with three fused adaxial lobes, *Kicksia membranacea* with broad sepals at base but slim elongations at the tips (“obspathulate to linear-lanceolate, acuminate”), *Howellia ovata* with sepals similar to *Schweinfurthia*, *Holmgrenantha petrophila* with “conspicuously spinulose-dentate” sepals etc.).

Also, the unusual sepal development in several genera of the Solanaceae, the inflated-calyx-syndrome in *Physalis*, *Nicandra*, *Przewalskia* and others, is phenotypically, genetically and developmentally distinctly different from

just a reversal to a “former state already realized in the series of its ancestors” (for a recently discovered example of a distinct case of independently arisen, but astoundingly similar structures in plants, see Glover *et al.* 2004, with further instances in the discussion; see also Theißen 2004); as to *Physalis*, see He and Saedler (2005).

### Calycanthemy

As mentioned above, the phenomenon of calycanthemy has been reported in many plant families and been interpreted to be reversals into the direction of a more original morphological state of the flower by some authors. Besides, the question has been raised by He *et al.* (2004) whether nature has taken advantage of this possibility in the case of the monocot family Liliaceae, like lilies and tulips, displaying two whorls of petals (tepals) in their perianth. Thus, according to viewpoint C<sub>1</sub>, the discovery of B-function in all the tepals in *Tulipa gesneriana* (Kanno *et al.* 2003) has been interpreted to be molecular support for the hypothesis of a mutant ectopic expansion of the B-function into an assumed former normal sepal whorl. However, the present majority viewpoint A (interestingly in agreement with viewpoints B and C<sub>2b</sub>) has commonly rejected the hopeful monster theory of Goldschmidt especially for higher systematic categories because of the functional and thus selective disadvantages of virtually all the originally promising ‘macromutants’ detected and closely investigated so far. The stark morphological irregularities and the lowered fertility of our *Misopates* mutants L 2003/971 and 2003/977 (see text above and **Fig. 6I-L**) are in agreement with this latter view. Yet, viewpoint C<sub>2a</sub> could perhaps postulate macromutational steps transcending the selectively disadvantageous features of the calycanthemous mutants found so far.

### Mutants with petaloid sepals

As mentioned above, mutant L 2003/971 displayed varying numbers of sepals (5-7) partially transformed into petaloid tissue and sometimes fused with the lower lateral sepals (**Fig. 6I, 6J**). Phenotypically it is somewhat intermediate between the *fimbriata*- and *cycloidea* mutants of *Antirrhinum*. Furthermore, the candidate mutant 2003/977 manifests up to 7 sepals, of which the 2 adaxial ones can be fused with the lower lateral petals. Interestingly, the additional adaxial (median) sepal is often forked (**Fig. 6K, 6L**), thus representing another feature not reported for *Antirrhinum*.

### Annuality versus perenniality

Fourteen out of the 27 genera of the Antirrhineae are classified as perennials, 8 as annuals, 1 genus has annual and biennial species (*Nuttallanthus*), 4 genera have annuals and perennials (*Chaenorrhinum* [“rarely annual”], *Cymbalaria* [perennial but “sometimes behaving as annuals”], *Kicksia*, *Sairocarpus*), and 1 genus even has annual, biennial, and perennial herbs (*Linaria*). Almost all the close relatives of *Misopates* are perennials (*Antirrhinum*, *Asarina*, *Cymbalaria*, *Kicksia*, yet *Chaenorrhinum* is also (if only) “rarely annual” and *Linaria* can do ‘everything’) (quotations from Sutton 1988).

In line with several authors working on other plant genera (Lesins and Lesins 1979; Quirós and Bauchan 1988; Barrett *et al.* 1996), the simplest way to explain the status of annuality in *Misopates* appears to be a perennial ancestral population of the tribus Antirrhineae with the potential to either maintain that state or to differentiate into biennial and annual descendents. These specializations of the life cycle could have been mainly due to losses of gene functions, for in several cases investigated, perennials were dominant over annuals (this was always the case, for example, in the many interspecific hybrids between annual and perennial species of *Hordeum* – von Bothmer *et al.* 1983, for further examples, see Thomas *et al.* 2000). Now, recessiveness usually means losses of gene functions (for the details, see Lönnig 2002). Otherwise any of these

features would independently have arisen time and again by more or less improbable specific point mutations (i.e. Remane’s ‘Differenzierungsmutationen’ in contrast to losses of functions). The *Misopates* mutants with extended life cycles (see the details under point 1) seem to hint at a certain capacity to revert into the direction of the original state. Another interpretation could be the involvement of TEs (Lönnig and Saedler 2002), or epigenetic regulation (see above), both in the generation of alternative life cycles, including a large potential of reversibility, i.e. “to return to a former state already realized in the series of its ancestors”.

Referring to a not unproblematic molecular interpretation of the history of the genus *Medicago* – based on the most parsimonious reconstruction, the ancestral population is hypothesized to have consisted of autogamous annual individuals succeeded by recurrent evolution into the direction of perenniality and outcrossing. Nevertheless, “assuming an outcrossing, perennial ancestral state (partly supported by morphological features) does not fundamentally change the reconstruction” (Bena *et al.* 1998) – Thomas *et al.* (2000) comment: “However, a general conclusion that can be drawn from this and other studies of life-history is that annuality and perenniality are traits that recur time and again across the taxonomic range and that, with the right selection pressure, the propensity to generate either form of phenotype can be realized without the need for large-scale genetic innovation.”

So whatever happened in the cases of *Misopates* and *Antirrhinum*, Dollo’s law would have been violated either totally or in part by any of these scenarios.

This appears to be equally true for the viewpoints A, C<sub>1</sub>, and C<sub>2a</sub> and C<sub>2b</sub> mentioned and briefly defined above. However, since the further vantage point B does not postulate any different ancestral species, all these annual and perennial forms would have been generated independently of each other and a “return to a former state already realized in the series of its ancestors” would be impossible.

### Leaf form

Variation of leaf form from linear to almost orbicular occurs regularly within many genera of the Antirrhineae, including *Antirrhinum* itself (see Hartl 1974; Sutton 1988). Phenotypic reversibility into both directions appears to be possible. However, (almost) orbicular leaves have not been determined for any of the 7 species of the genus *Misopates*. Yet, the width of leaves of *Misopates* mutant L 2002/1843 (**Fig. 2A-C**) surpasses those of all the wild species of the genus and may be viewed as a reversal to an originally more inclusive spectrum of phenotypes in its polyvalent ancestor. It has to be investigated whether a loss of function mutation of perhaps a suppressor in mutant 2002/1843 may have released a more basic developmental state for a larger photosynthetic area (interestingly, the mutant is also as late in flowering as *Antirrhinum majus* and displays slightly enlarged flowers).

Also, the regular-repetitive range of leaf form variation within so many angiosperm genera, tribus, and families (see any work on plant taxonomy comparing several taxa) suggests polyvalent ancestors with a stark genetic potential to differentiate into lines with distinct leaf types (simple vs. compound leaves, different venation patterns, margins etc.), including an established capability to revert to earlier phenotypes.

As for the Antirrhineae, leaves vary from filiform, linear and lanceolate to cordate, sagittate, and reniform to ovate-cordate (*Asarina*).

In accord with viewpoints A, C<sub>1</sub>, and C<sub>2a</sub>, and C<sub>2b</sub> the case of the *Misopates* leaf mutant 2002/1843 suggests that Dollo’s verdict on the irreversibility to a preceding morphological state might be revised as follows regarding leaf form at the phenotypic level: “an organism can return, at least partially, to a former state already realized in the series of its ancestors”. As for viewpoint B, see especially the last paragraph on annuality vs. perenniality above.



### Flower form

Historically the *cycloidea* and/or *radialis* mutants of *Antirrhinum* and many other plants species with zygomorphic flowers have frequently been interpreted to be perfect textbook examples of reversals “to a former state...” – the assumed original radial flower form (viewpoints A, C<sub>1</sub>, and C<sub>2a</sub>, see, for example, Gustafsson 1979; Strasburger 2002). And since in *Misopates* the ‘reversal’ of the strong *cycloidea* allele (Fig. 5B) seems to be even more perfect than in *Antirrhinum*, this example could now be added to the list of putative atavisms so far known in the plant kingdom. However, several authors have emphatically rejected the interpretation of an atavism here for the following reasons: (1) Zygomorphic plant species can give rise to distinct kinds of radial mutants differing strongly from each another in their morphology. A species cannot have had differently looking ancestors as the basic starting point. (2) The radial flower form is often due to the 5 to 6 times repetition of a flower part already highly differentiated and synorganized for a special function within the zygomorphic flower form (i.e. usually reiteration of the middle part of the lower lip with lobe, gibba or spur and further unique structures of the ‘palate’, and the abaxial part of the flower tube like the hairs lining the abaxial folds, which ‘may serve to guide the tongue of the insect to the nectar at the base of the tube’; for references, see Sutton 1988) as well as total or almost total suppression of the upper lip. (3) Normal flower function is often strongly hampered by severe constriction of the entrance and other malformations and malfunctions (pollinators excluded, autogamy reduced, lower seed production and seed sterility, also, owing to nearly upright position, early fouling of all flower parts due to rain water in the flower under field conditions in *Antirrhinum*). For further information, see Linnaeus 1749; Penzig 1922; Goebel 1928; Arber 1970; Hartl 1974; Endress 1992; Luo *et al.* 1996, 1999; Lönnig and Saedler 1997; Cubas *et al.* 1999; Theißen 2000.

The *cycloidea* phenotypes of *Misopates*, *Antirrhinum* and others could, perhaps, rather be quoted as examples for Dollo's and Gould's conviction that certain forms can – more or less superficially – approach a previous phyletic state but never fully reattain it. However, due to the many special structures and functional aberrations just enumerated, the *cycloidea* and *radialis* cases might not even qualify to be “so near but not quite” (Gould and Robinson 1994) to the assumed original state according to viewpoints A, C<sub>1</sub>, and C<sub>2a</sub>. So B and C<sub>2b</sub> could perhaps classify the *cycloidea* and similar mutants solely as teratological phenomena. Yet interestingly, these aberrant radial forms themselves can phenotypically perfectly revert to the original zygomorphic symmetry – either with no change on the DNA-level at all (in cases of demethylation) or, if transposons are involved, with complete restoration of the original DNA sequence in the 1% to 6% of transposon excisions without footprints and almost complete restoration in the functional rest with footprints (for the details on transposon excision in plants, see, for example, Scott *et al.* 1996).

For several further difficulties concerning the *cycloidea* gene family, including DNA sequence diversity for species of *Antirrhinum* and *Misopates*, see Vieira *et al.* (1999), and the reviews by Gübitz *et al.* (2003), and Hileman and Baum (2003); for facts and interpretations on some legume *cyc* genes according to different evolutionary models, see Ree *et al.* (2004).

As to flower form and Dollo's law, the case of *Pseudomisopates rivas martinezii*, originally described as *Misopates rivas martinezii* (Sánchez-Mata 1988; Güemes 1997) may be mentioned here. In contrast to *Misopates*, *Pseudomisopates* is a self-sterile, spinous perennial displaying sepals shorter than corolla-tube, having only two pores in an ovoid capsule, generating seeds different in surface structure and overall form as compared to those of *Misopates*. Additionally, this species has a horizontal rhizome, and  $n = 7$  chromosomes (*Misopates*  $n = 8$ ).

*Pseudomisopates* may perhaps be interesting for Dollo's law concerning corolla form and function: although being relatively removed from *Misopates* morphologically and physiologically, corolla morphology of *Pseudomisopates* is very similar to that of *Misopates* (Fig. 7C, 7D). Yet, whether this similarity is it due to common ancestry, convergence, or a reversal in a history of several more or less slightly different corolla forms and colours cannot be answered at present.

### Flower size

The supposition that the 22 independently arisen *Misopates* mutant candidates with larger flowers (Fig. 3D) be phenotypic revertants into the direction of an earlier ancestral state rests mainly on the fact that most of the 20 European species of *Antirrhinum* – being the closest relatives of *Misopates* – have larger flowers than the latter (for *A. majus*, see Table 1), and that the one case of really small flowers within *Antirrhinum* (*Antirrhinum valentinum*: corolla 12-15 mm), being “a narrow endemic of south-eastern Spain” (Sutton 1988), appears to be the apomorphic state within this genus. Besides, the regular appearance of *Misopates* mutants with larger flowers suggests a relatively easy genetic (or epigenetic?) step in this direction. Also, flower size in *Misopates* may be evaluated in connection with its annual life cycle and its tendency to autogamy. Charnov and Schaffer (1973) had come to the conclusion that annuality would be preferred in hostile environments (for a further development of the argument, see Thompson *et al.* 2000). Interestingly, all annuals are selfers in the above mentioned genus *Medicago* guaranteeing seed set in the absence of any pollinators, but there are selfers and outcrossers in the perennials (for further points, see Bena *et al.* 1998). An apomorphic (derived) short life cycle with relatively small autogamous flowers could help coping with adverse conditions.

Moreover, rather intense variation of flower size has been reported for nearly any genus with several species in the angiosperms. Like annuality and perenniality as well as several distinct leaf forms, differences in flower size “recur time and again across the taxonomic range”. Thus, for viewpoints A, C<sub>1</sub>, C<sub>2a</sub>, and C<sub>2b</sub> the assumption that phenotypes with larger flowers had also appeared in the series of polyvalent ancestors of *Misopates* does not appear to be far-fetched.

### Flower colour

Because from viewpoints A, C<sub>1</sub>, and C<sub>2a</sub>, the absence of the anthocyanin pathway describes a more basal or “primitive” state in the plant kingdom, any mutations subtracting or destroying this pathway may be viewed to be a phenotypic (in case of complete deletions even a genotypic) reversal “to a former state already realized in the series of its ancestors”, which would apply to virtually all the 19 colour mutants of the Vogelsang line of *M. orontium* (see the details under “flower colour” of the results and Fig. 3E). For viewpoint B and C<sub>2b</sub> these mutants would perhaps represent only more basic physiological states in the genomic configurations (B) or design (C<sub>2b</sub>) of flower colours.

In agreement with the inferences of Marshall *et al.* (1994), Collin and Cipriani (2003), and Whiting *et al.* (2003) referred to above, the reversion to the violet flower colour in the almost white flowering Coimbra line of *Misopates* requires that the developmental pathway underlying this complex physiological feature phenotypically lost in the history of this subspecies has nevertheless been maintained at the genetic level for all the time this subspecies has existed and has now, if only to a certain degree, been reactivated in the mutant representing another candidate for the thesis that “an organism can return, at least partially, to a former state already realized in the series of its ancestors”. One could, however, perhaps object that flower colour is not a morphological feature and thus lies outside the boundaries of Dollo's law of irreversibility (see further points below).

### Fertilization

In a tribus where cross fertilization is the rule, a tendency to autogamy and the absence of functional self-sterility alleles usually describes a derived (apomorphic) state of fertilization. We predict that – as in *Arabidopsis* (Kusaba *et al.* 2001; Nasralla *et al.* 2002) – the inactivated self-sterility alleles will be found by further molecular investigations. Yet from the evolutionary vantage points of A, C<sub>1</sub>, C<sub>2a</sub>, and C<sub>2b</sub> the absence of these functions may again be viewed to be a return to “a former state already realized in the series of its ancestors”, for the apomorphic autogamy can be interpreted to be the original state before the invention of the self-sterility alleles. Whether this putative pathway itself can be reactivated depends on the complexity of its deactivation mechanism: in case of several deletions and other complex mutations the probability will almost be zero and a reversal to functional self-sterility alleles would be due to ‘a circumstance so complex that we cannot imagine that it will ever occur’, to reformulate another passage of Dollo quoted above. If, however, the deactivation is due to reversible cut and paste mechanisms of TEs or epigenetic factors, its reactivation should be possible.

From viewpoint C<sub>2b</sub> the absence of functioning self-sterility alleles could simply be interpreted as a reversal to a more basic physiological state in the design of the fertilization mechanisms in angiosperms. However, viewpoint B (“Life is polyphyletic from its inception, there is no major branching” – Schwabe 2001) may perhaps have some basic problems with a rudimentary system of self-sterility alleles or would have to include this phenomenon in minor branching.

Again, as in the case of flower colour, it could be objected that differences and reversions in fertilization systems do not represent morphological features and hence have nothing to do with Dollo’s law of irreversibility. Rather it seems to be in agreement with his assertion that “functional or physiological reversals occur”. However, a differentiation of reversibility for structural and morphological characters *vs.* functional and physiological features is itself problematic and cannot be taken for granted without a further genetic and developmental foundation. For the underlying genetic and developmental basis of both, reversions in flower colour and self-sterility appear to be no less complex than the basis for many purely morphological features. If correct, this argument would constitute another valid point questioning Dollo’s law.

### Seed form

The cuplike seed form in *Misopates* (Fig. 3J) is clearly a complex apomorphic feature characterizing all 7 species of this genus. To the authors’ knowledge, this character does not occur anywhere else in the tribus Antirrhineae. To date, seed form reversions are unknown in *Misopates*.

### Apomixis

Apomixis is viewed to be an apomorphic character having been generated convergently multiple times in the history of a large number of angiosperm genera. Thus, from viewpoints A, C<sub>1</sub>, C<sub>2a</sub>, and C<sub>2b</sub>, it cannot be excluded that this feature had already been realized in the series of its ancestors, subsequently been lost and then been reactivated again. Hardly anything is known at present about the occurrence and distribution of this feature in 27 genera of the Antirrhineae.

### Photosynthetic apparatus

Dozens of mutants of the photosynthetic apparatus have been detected in our experiments – such mutants are, in fact, the most common ones in any mutagenesis trials with plants. The results indicate that there is an irreducibly complex core system as well as a variable part, the latter being especially esteemed in breeding colour varieties (including chimeras) in ornamentals. In the former any losses-of-function-mutations abolish the activity of the entire photosynthetic apparatus, and have to be all

classified as lethal mutations, at least if the entire photosynthetic area of a plant is affected (usually white seedlings are still formed which soon die after the nutrients of the seeds are used up).

Although the origin of the irreducibly complex core system will not easily be explained by selection of successive point mutations in duplicated genes, – from evolutionary standpoint A, especially that of “additive typogenesis”, many of these losses of functions in the variable part could perhaps be interpreted as steps returning to “a former state already realized in the series of its ancestors”, i.e. steps on the way to an optimally functioning photosynthetic apparatus. Phylogenetically, C<sub>1</sub> appears to be largely in accord with A (although not entirely with the causes generating the systems). From the vantage points of C<sub>2b</sub> and B, however, most of these still functioning mutants represent secondary deviations most probably never representing any of their ancestors. Yet C<sub>2b</sub> could perhaps argue that a less differentiated photosynthetic apparatus may be viewed as a reversal to a more basic or possibly to an imperfect physiological state in the design of photosynthesis.

As for the possible objection that photosynthesis does not belong to morphology proper, see comment under the topic of fertilization.

### Significance of sepal variation and sepal constancy in *Misopates orontium*

Since several phenotypic differences distinguishing *M. orontium* from its closely related species *A. majus*, like properties of the life cycle, leaf form, flower size, flower colour, and fertilization, have phenotypically been diminished or fully overcome by mutant genes in otherwise normal phenotypes, – what possibilities of variation could exist in the rest of the differences, especially the long sepal feature being the morphologically most conspicuous trait so that Lamarck even suggested to coin the name “*calycinum*” for the species?

As mentioned above, reduction of the length of sepals has been found so far only as a by-product of syndromes more or less strongly disturbing normal development producing “monster plants” in *Misopates*. Conversely, mutants with long sepals have never been detected in *A. majus* in more than a hundred years of research with mutants and recombinants of this species in several institutes nor in any culture varieties.

Before addressing the basic question whether this constancy may be simply due to limited materials or could hint at a deeper meaning in relation to systematics in general we will first discuss some basic findings of systematics and paleontology relevant for this problem.

### The question of the general constancy of taxonomically relevant features

In contrast to Dollo, Darwin had proposed a theory of continuous evolution for the origin of new species and higher taxa by selection of innumerable “small steps”, “steps not greater than those separating fine varieties”, “insensibly fine steps”, “for natural selection can act only by taking advantage of slight successive variations; she can never take a leap, but must advance by the shortest and slowest steps” (Darwin 1859, 1896).

In accordance with Darwin, the essence of the synthetic theory of evolution (modern synthesis, neo-Darwinism) is a slow, steady, and gradual origin of species by piecemeal selection of mutations, which “have only slight or even invisible effects on the phenotype” (Mayr 1970). Against this setting of continuous evolution it is easily comprehensible why Mayr, the doyen of the modern synthesis, has just recently called the phenomenon of morphological stasis (constancy) – so persistently characterizing the fossil record (for a review see Lönnig and Saedler 2002) – to be one of the basic unresolved problems of evolutionary biology. He specified the problem in a

recent interview as follows (Mayr 2002):

In evolutionary biology we have species like horseshoe crabs. The horseshoe crab goes back in the fossil record over two hundred million years without any major changes. So obviously they have a very invariant genome type, right? Wrong, they don't. Study the genotype of a series of horseshoe crabs and you'll find there's a great deal of genetic variation. How come, in spite of all this genetic variation, they haven't changed at all in over two hundred million years while other members of their ecosystem in which they were living two hundred million years ago are either extinct or have developed into something totally different? Why did the horseshoe crabs not change? That's the kind of question that completely stumps us at the present time.

Beginning with the founder of embryology, Karl Ernst von Baer, many biologists have raised basic objections against the idea of gradual evolution (although Huxley had already expressed some dissatisfaction with gradualism as the almost exclusive mode of evolution before). Von Baer inferred that – “if small steps would have led to essential changes, these continuous alterations could only have been caused by continuous effects and that the accumulation of small changes would have generated a chaos of indeterminable forms”, thus disorder and confusion would be specifying the objects of taxonomy (von Baer 1886, see also Lamprecht 1966, 1974). However, von Baer submitted many arguments that “most species are very clearly delimited” and “provocatively constant”. Moreover, von Baer contested the idea that the initial “insensibly fine steps” could already have clear selective advantages. Like Dollo, von Baer postulated discontinuous evolution, but from a combination of viewpoints C<sub>1</sub>, C<sub>2a</sub> and C<sub>2b</sub> (see above).

Two of the great pioneers of general and systematic botany, Augustin Pyrame De Candolle, and Christian Konrad Sprengel made the following comments on the cardinal characters distinguishing species and genera from each other (1819/1821/1978, excerpts from pp. 95-97, – note the authors' emphasis on the terms “invariable” (invariably), “invariableness” and “constant” in the ensuing paragraph):

By Species (*species*), we understand a number of plants, which agree with one another in invariable marks. In this matter every thing depends upon the idea of invariableness. When an organ, or property of it is changed neither by difference of soil, of climate, or of treatment, nor by continued breeding, this organ or property is said to be invariable. When, for instance, we have remarked for centuries, that *Centifolia* has always unarmed leaf-stalks, we say correctly, that this property of the *Centifolia* is invariable...What we know is, that from as early a time as the human race has left memorials of its existence upon the earth, the separate species of plants have maintained the same properties invariably...All properties of plants which are subject to change, form either a Subspecies (*subspecies*), or a variety (*varietas*)...By a *Genus* we understand the sum of the species which agree in certain constant properties of the essential parts. (p. 152) The generic character (*character genericus*) is the expression of the peculiar and invariable marks by which a genus of plants is distinguished from all others...every generic character must state shortly and distinctly the common marks which belong invariably to all species of the same genus. (p. 153) The generic character of the higher plants is borrowed solely from the organs of fructification. [Italics by the authors.]

Before proceeding to a discussion of the basic question, to what extent our work on *Misopates* and *Antirrhinum* is relevant for these statements on “invariableness” and *vice versa*, – expositions, which are roughly 200 years old and were first published 40 years before Darwin's Origin in 1859, let us directly turn to some comments of modern systematics on the same questions (the arguments on the following points can also be found in Lönnig 2004).

Stace comments in agreement with almost all contemporary authors (1989):

Although flowers are no longer regarded as ‘essential’ and therefore taxonomically particularly important, they still provide the bulk of information contained in the diagnosis of angiosperm taxa. This is because

in general the flowers appear to be more conservative than do most other organs.

Before he had already remarked that “This reliance on the flower is remarkable when one considers that most of the time the majority of angiosperms lack any flowers at all”. This appears to be also true for seed- and fruit-structures. Concerning the conservative key systematic characters he further points out that “endemorphic vegetative characters are more conservative than exomorphic ones” and continues:

Conservative characters are...most useful in delineating the higher taxa, where the emphasis is on the recognition of similarity between the members of a taxon.

Yet, for species and systematic categories below the species, he insists that the non-conservative characters seem to dominate.

And he is most probably correct in his analysis: considering the general shift in systematics during the last 250 years (Haeckel's verdict for zoology that “related species which had been united within a genus by Linné and within a family by Cuvier, now constitute an inclusive order with several families and many genera” – implying that many of Linné's species have been elevated to the position of genera during the last centuries – is also valid for botany: the history of the systematic status of *Antirrhinum* in relation to *Misopates*, not to mention the genera and species of the tribus Antirrhineae in general, may be used as a paradigm to illustrate this fact (see introduction above; for further details, see Lönnig 2002). Hence, one may conclude that the essentials have hardly changed in morphological systematics: The *invariable characters* delineating species and genera according to Linné, Cuvier, De Candolle, Sprengel and many others are now the *conservative characters delineating higher taxa* including the morphologically defined genera, tribus and families of today.

### Stasis of systematic categories in time: some examples

Taking the descriptions of Linné for *Antirrhinum* and *Misopates* given some 250 years ago in his *Species Plantarum*, there is no difficulty in identifying the different species of today on the basis of his characterizations (see Linné 1753) – only the names have evolved. The same is true for the drawings and descriptions of plant species by Leonhard Fuchs (1543), Tabernaemontanus (1588) on maize and many other plants, even the portrayals by Dioskurides A.D. 78 and 288. Also, George Cuvier had absolutely no problem in identifying the mummified animals of old Egypt being several thousand years old (Cuvier 1830).

Yet, usually concerning such examples it is objected that they are simply nothing on the evolutionary time table. So let us have a look at the last 2.3 million years of European life history: Intriguingly this is characterized by “comparatively slow rates of evolution” (Lang 1994), and the author continues: “At the end of the tertiary the organisms consisted of species, almost all of which can be assigned to present genera, a large section even to living species. This applies not only for the European flora but also for its fauna.” The actualistic inferences drawn from present ecological indicator values to solve elementary questions of quaternary paleontology are based on “this obviously far-reaching constancy of life forms down to the species” (Lang).

Additionally, about half of the genera of flowering plants found in geological formations dated to be 37 million years old have been assigned to present genera (Stanley 1986), and many well-known present plant families and genera have even been identified in cretaceous formations (taxa sometimes dated to be older than 100 million years before present).

Or, to take a glimpse at another well-known plant group,



the bryophytes. Agashe reports (1995):

Members of both the major groups of bryophytes, i.e. Hepaticopsida (liverworts) and Bryopsida (mosses), are well represented in the known fossils. However, a detailed comparative study with modern bryophytes indicated that the group has remained almost unchanged since the Paleozoic time. Hence the fossil bryophytes do not help us much in understanding evolution except for the fact that they formed a prominent part of the vegetation from the Paleozoic onwards.

Hence, the widespread bryophytes have survived in their present *gestalt*, i.e. "almost unchanged", some 400 million years on earth.

A comprehensive survey about the phenomenon of the constancy of morphological characters and of entire taxa in the fossil record is beyond the scope of the present paper (for further Details, see Benton 1993; and for a review, see Lönning and Saedler 2002b). The theory of punctuated equilibrium (Eldredge and Gould 1972; Gould and Eldredge 1993; Gould 2002), was developed to come to grips with the general phenomenon of abrupt appearance and stasis (constancy of the *gestalt* of organisms usually documented for millions of years) in the fossil record. The well-known "living fossils" are only a relatively small part of that general phenomenon.

For some recently discovered examples of the enormous stasis of morphological features, see Gao and Shubin (2003), Mayr (2004), and Gandolfo *et al.* (2004).

### Molecular stasis

In the following paragraphs we will briefly examine the question whether molecular biology has contributed basic facts in accord with the findings on stasis found in systematics, morphology, and paleontology just mentioned.

As noted above, "several authors have argued that the developmental programmes and pathways underlying (even) complex morphological features lost in the history of a genus or family can nevertheless be maintained on the genetic level for millions of years and have, indeed, been reactivated in several cases (Marshall *et al.* 1994; Collin and Cipriani 2003; Whiting *et al.* 2003). This inference is in agreement with Ohno's earlier hypothesis (1985) that even dispensable DNA-sequences "would not readily disappear from the genome of mammals (in particular) and vertebrates (in general). Instead they will linger for millions of years."

Perhaps the peak of molecular stasis has been described by Lazcano and Miller (1996):

After the explosive metabolic evolution that took place soon after the beginning of life, the basic genetic processes and major molecular traits have persisted essentially unchanged for more than three-and-a-half billion years, perhaps owing to the linkages of the genes involved and the complex interactions between different metabolic routes. At a macroevolutionary level, this represents a case of conservation that is even more striking than the maintenance of the major body plans that appeared at the base of the Cambrian, and which have remained basically unchanged for 600 million years.

Moreover, at the beginning of the 1990s, a series of discoveries of a totally unexpected constancy (or virtual constancy) of gene functions in comparative developmental biology had led to a corresponding chain of comments describing that amazement. Quoting a few key phrases to illustrate the point: Shapiro (1993) spoke of "a big surprise" that "was totally unexpected". De Robertis (1994) commented that "...it is safe to say that no one would have predicted the degree of conservation in the molecular mechanisms that control development". Nüsslein-Volhard (1996) called this phenomenon a "great surprise" (see also Lewin 1994; Hultmark 1994; Cohn and Tickle 1996). For a detailed discussion, see Lönning (2003a, 2004).

Thus, the "basic genetic processes and major molecular traits" are thought to have "persisted essentially unchanged

for more than three-and-a-half billion years", and the molecular mechanisms of animal ontogenesis more than a billion years. On the background of the then prevailing idea of the synthetic theory, that 'each particular gene is going to accumulate many changes over long periods of time and that this was how one organism turned into another' (Shapiro), the discovery of molecular conservation was, indeed, 'totally unexpected' and 'to a degree beyond anyone's wildest expectations' (De Robertis 1994).

Similar conservative sequences and functions dated to be at least some 300 million years old, are involved in the development of plants (Sommer *et al.* 1990; Schwarz-Sommer *et al.* 1990, 1992; Davies and Schwarz-Sommer 1994; Theißen and Saedler 1995, 1999; Theißen *et al.* 2000; Becker *et al.* 2001; Münster *et al.* 2002; Becker *et al.* 2003; De Bodt *et al.* 2003; Kirst *et al.* 2003; Kufuji *et al.* 2003; Zhang *et al.* 2004; and many others).

Now, since all these "old features", anatomically as well as molecularly, are still with us, first the basic genetical question for viewpoints A, C<sub>1</sub>, C<sub>2a</sub>, and C<sub>2b</sub> should be addressed, how it is possible to derive stable features in any given plant or animal species by mutations in their genomes and to what extent could the answers be relevant for our results obtained so far for the main differences between *A. majus* and *M. orontium*.

Could this molecular stasis be the basis for the morphological constancy as found in paleontology – as well as of reversibility? But if so, how to generate stable characters by mutagenesis or otherwise at all?

### The origin of stable characters

1) *Stable features due to losses of gene functions.* The simplest possibility to generate constant characters by gene mutations is to inactivate gene functions, which are redundant under special ecological conditions (see Lönning and Saedler 1997). To give an easily comprehensible example: In thousands of angiosperm genera, species with coloured flowers exist side by side with species displaying white flowers, - "white" usually signifying a loss of function in the anthocyanin pathway or limited expression of the respective genes within such closely related species or subspecies. Provided that a sufficient loss of genetic information has taken place (for instance by deletions of the key gene functions), so that reversions to the original state can safely be excluded, the new feature (in this case "white flowers") will be a stable character for aeons of time and over limitless geographical distribution.

Applying this inference to the origin of the features distinguishing *Misopates* from *Antirrhinum*, if the leaf-like bracts and sepals were due to the irreversible losses of functions of a repressor and/or its target genes, the feature would largely be constant. The sepal structure could then only be modified by an indirect route, i.e. the genes affecting the basic leaf programme now ectopically expressed. So in this case we should expect an essentially consistent correlation of mutant leaf deviations with corresponding aberrations in the bracts and sepals in *Misopates*. Apart from one exception, this was the rule we have found so far: Whenever there was a change in the sepals, a corresponding difference was also detected in the leaves and *vice versa* (see the leaf and sepal mutants mentioned above).

2) *Redundancy by gene duplications.* In a more or less simplistic model, generation of genetic redundancy by gene duplications could be another method to create rather constant features: the more extensive the redundancy the more stable the character (for the intricacies often found for real gene duplications, see Kunze *et al.* 1997; Lönning 2002). Although this model does not guarantee absolute stability, functional gene duplications are thought to buffer the features of any anatomical or other system against mutant deviations of many kinds. Yet in contrast to the first possibility (constancy of features generated by losses of

gene functions) redundancy could – at least in theory – gradually be reduced until only the original functional gene is left, which, upon further mutation, would then also affect the constant character. To avoid this effect, partial redundancy tightly connected with the overall functions of a species system could be a solution (see below). Mutations in partially redundant genes being disadvantageous for the survival of the plant would quickly be lost by natural selection.

Although the generation of such redundancy can easily be imagined and given genomes correspondingly be interpreted, as far as the authors are aware, as yet there are no reports for any experimental generation of a comparable case of a mutable systematic feature becoming stable by individual gene duplications in a diploid organism. The situation appears to be somewhat different in polyploid organisms: experimentally induced polyploidy can have, indeed, a certain buffer function against the effects of gene mutations (Stebbins 1950, 1971; for a review see Lönnig 2002). For it appears to be clear that a recessive mutation, which normally has some effects on the phenotype in the homozygous state in the following generation in autogamous plants, will not or hardly be detected in – for example – an octoploid plant line: the majority of dominant alleles buffers the potential effects of the recessive ones.

**3) New genes with double functions:** Another possibility to produce characters stable enough to be relevant for systematics could be the generation of genes with double functions, one for a key morphological feature and concomitantly a second function for a basic role in an important physiological process, so that, again, any loss of function mutations would be selected against whenever appearing in the wild and in extreme cases also in cultivated populations. In several plant species the gene coding for chalcon synthase (CHS) appears to have such a double function being (1) at the basis of the anthocyanin pathway and simultaneously displaying (2) basic functions for resistances of several kinds (frost, disease, UV), so that no wild populations without *Nivea* functions (the gene coding for CHS) are known in *Antirrhinum* or *Misopates* so far. Moreover, some plant species are endowed with more than one copy of the *Nivea* gene. However the basic problem remains: how to derive a new non-redundant gene with double functions from an old one by duplications and substitutions? Viewpoints B to C might find the problem especially interesting. (The problems how to derive entirely new gene functions and gene reaction chains from old ones have also been discussed by Kunze *et al.* 1997, Axe 2000, 2004, Lönnig 2002, Behe and Snoke 2004).

A network of interdependent functions – substantially irreducibly complex – is hinted at in the comment of Lazcano and Miller quoted above trying to explain the basic genetic processes and major molecular traits that have persisted essentially unchanged for more than three-and-a-half billion years “perhaps owing to the linkages of the genes involved and the *complex interactions between different metabolic routes*” (italics added).

**4) Chromosome rearrangements.** “Normal” chromosome rearrangements as well as those due to transposons could be involved in some of the possibilities mentioned above. Due to rapidly growing evidences for chromosomal hot spots of transposable element visits in combination with the data of the theory of the chromosome field (Lima de Faria 1986, 1999), we have discussed the possibility of partly predetermined chromosome rearrangements, which could also be relevant for the origin of new chromosome races and species in the plant- and animal kingdoms (Lönnig and Saedler 2002b). According to the investigations of Harrison (1956, 1960) the chromosomes of *Antirrhinum* and *Misopates* display so many differences so that pairing is strongly disturbed, leading to sterility in his putative F<sub>1</sub> plants. However, to date no chromosome map of *Misopates* is available to compare it with the data known from

*Antirrhinum* (for the latter, see Schwarz-Sommer *et al.* 2003). Granted the findings reported by Harrison are correct, gene position effects could also be involved in the generation of the differences between *A. majus* and *M. orontium*. Yet, because gene positions should in part be reversible by further chromosome rearrangements, character reversion to the original “wild-type” should be considered for this possibility (except, of course, for chromosome rearrangements implying the deletion of the genes in question).

### Data pointing to regressive evolution (losses of gene functions) in *Misopates* in comparison with *Antirrhinum*

Among several other authors, it was especially Gould who has emphasized the point that there is no general trend for improvement in evolution (Gould 1998, 2002). Moreover, regressive evolution due to losses of gene functions has been widely documented for the animal and plant world alike (for a brief review, see Lönnig 2002). As for *Misopates*, the data found so far mostly appear to point to the absence of gene functions possibly by regressive evolution in *Misopates* as compared to *Antirrhinum*, yet the following hypotheses of our discussion have to be further checked and investigated:

**1)** Loss of function of a repressor and/or its target genes otherwise involved in the differentiation of leaves, bracts and sepals present in almost all the other 328 species of the tribus Antirrhineae. In *Misopates* the main leaf programme runs directly up into the sepals. In this connection it is relevant to note that *within the tribus* the *Misopates* bracts and sepals definitely represent apomorphic, not plesiomorphic, features.

**2)** Perhaps loss of function of a gene necessary to produce functional pollen in the *plena* mutant of *Misopates* with anthers (the corresponding phenotype of *Antirrhinum* mutant is fertile). However, since none of the mutants have been sequenced so far, at present it could be argued that the situation may be different at the DNA level of the corresponding loci.

**3)** In the *deficiens* mutants of *Misopates* the fifth locule appears to be absent, probably pointing to another absence or loss or reduction of gene function.

Moreover, the phenotype of the double mutant *cyc/dich* of *A. majus* has already been realized by the monoallelic strong *cycloidea* mutant of *Misopates* also described above.

Thus, some monogenic mutants of *Misopates* appear to display the phenotypes of double mutants of *Antirrhinum*.

**4)** As already noted above, the almost white flower colour in the wild Coimbra *Misopates orontium* line is probably due to a “loss” or suppression of function and can phenotypically revert to the violet of its assumed ancestors.

### Natural selection

If *Misopates* has evolved its dramatically long sepals as a shelter for the inner flower whorls – the hypothesis most often advanced to explain the origin of this feature – the question might immediately be raised, how all the other 99% of the Antirrhineae (or the angiosperms in general) have managed to survive and successfully flourish without correspondingly elongated sepals.

As already emphasized, Darwin had suggested a theory of gradual evolution for the origin of new species and higher systematic categories by selection of – to repeat this vital point – innumerable “small steps”, “steps not greater than those separating fine varieties”, “insensibly fine steps”, “for natural selection can act only by taking advantage of slight successive variations; she can never take a leap, but must advance by the shortest and slowest steps” (Darwin

1859, 1896). And among other topics it was mentioned that von Baer contested the idea that the initial “insensibly fine steps” could already have had clear selective advantages and that both, Dollo (viewpoint C<sub>1</sub>) and von Baer (C<sub>1</sub> in combination with C<sub>2a</sub> and C<sub>2b</sub>) had postulated a discontinuous origin of species and higher taxa (followed by a long line of biologists up to the present day).

Briefly extending this controversy to the origin of the enormously elongated sepals of *Misopates orontium*, the difficulties of both positions (entirely or in part) may immediately become translucent for this case, too.

Assuming with viewpoints A, C<sub>1</sub>, C<sub>2a</sub> and C<sub>2b</sub> that the common ancestor of the tribus Antirrhineae displayed relatively short sepals in comparison to the corolla as one of its original characteristics – which could have been (1) the genetic factors focussing especially on the length of the sepals (of the many morphological characters) by rounds of repetitive mutations to “stretch” them in an extended series of insensibly fine steps to their present magnitude in cooperation with (2) an enormous selection pressure again concentrating particularly on this morphological character? Also, similar questions might be raised for the less conspicuous, but also derived morphological features, especially the cuplike seeds, alone or in combination (perhaps by the effects of pleiotropic genes)!

For some physiological characters the problems seem to be easier, although insensibly fine steps seem to reach their limits here, too. Sometimes it is argued that the morphological deviations might be just by-products of physiological changes. Moreover, Mayr (1963) claimed that “one can never assert with confidence that a given structure does not have selective significance.” And Simpson (1953) argued that “the fallibility of personal judgements as to the adaptive value of particular characters...is notorious”, – referring especially to features of animals quite unlike any now living. Dobzhansky (1975) asserted that “not even a biologist of Grassé’s experience can judge reliably which characters are useful, neutral, or harmful in a given species.” These statements may illustrate the frequency and depth of the problem. Yet the perceptive reader may wonder whether such statements can ever be falsified (Grassé 1977; Brady 1982; ReMine 1993; Wesson 1997; Müller and Newman 2003).

Also, the saltational view of C<sub>1</sub> will meet enormous problems regarding the origin of the long sepal feature if the latter is part of an irreducibly complex system. Yet, in that case B and C<sub>2</sub> (both) will see corroborating evidence of their views.

If, however, the long sepals of *M. orontium* were just due to some neutral losses of gene functions (perhaps best classified under C<sub>1</sub>), these losses should be detectable in comparison with related species displaying normal sepals.

## CONCLUSION

Dollo’s statement that “an organism cannot return, even partially, to a former state already realized in the series of its ancestors” and that “structural or morphological reversal does not occur” etc. (see above) has to be qualified for several apomorphic features of *Misopates* (longevity, leaf form, flower size and colour and mode of fertilization), at least phenotypically. However, as to the most important morphological difference between *Misopates* and *Antirrhinum*, i.e. the length of the sepals, so far no convergence due to mutations has been achieved and this agreement with Dollo’s law raises the possibility that the unexpected constancy of this key feature may be due to an irreversible genetic constitution in its present state (see the details above). Future investigations will solve this question.

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