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AFLP analysis of genetic similarity between the species of the genus *Lolium*

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Abstract: The genus *Lolium* is represented by selfpollinated *L. loliaceum*, *L. persicum*, *L. remotum* and *L. temulentum* and outpollinated *L. multiflorum, L. perenne* and *L. rigidum*. The aim of the present study was the analysis of phylogenetic relationships between all *Lolium* species on the base of AFLP markers. All *Lolium* species were analysed with 8 pairs of AFLP primers. Each pair of primers resulted in the amplification of large number of AFLP fragments. Most fragments were polymorphic and many of them were restricted to a single species. Only 10 fragments were monomorphic across all species tested. The UPGMA clustering grouped all species into two groups. The first group was represented by three selfpollinated species, *L. persicum*, *L. remotum* and *L. temulentum* while the second one is formed by three outpollinated species, i.e. *L. multiflorum*, *L. perenne* and *L. rigidum*. Out of these species, *L. multiflorum* and *L. perenne* are joined with the highest genetic similarity providing further credence to our earlier conclusion that both taxa can not be regarded as biological species. The grouping of *L. loliaceum* within outpollinated species may suggest that this taxon evolved within a group of outpollinated taxa, followed by several point mutations responsible for self-fertility.

Key words: *Lolium*, AFLP markers, genetic diversity, evolution

1. Introduction

The species of the genus *Lolium* are common in the temperate regions of Europe, Asia and North America. They have also been introduced to Australia and New Zealand. Traditionally, the genus is divided into selfpollinated and outpollinated species. The former is represented by 4 species, i.e. *L. loliaceum*, *L. persicum*, *L. remotum* and *L. temulentum* and the later by 3 species, *L. multiflorum*, *L. perenne* and *L. rigidum*. All of them are recognised on the basis of their manner of reproduction in addition to morphological characters such as spike and leaf morphology and growth habit as well. However, the occurrence of partial reproductive barriers between some of the *Lolium* representatives suggests that at least some of them can not be regarded as biological species. In general, there is no barrier at all between outpollinated species, which are fully interfertile and extensive gene flow is observed. In contrast, selfpollinated species are characterised by partial reproductive barriers but hybridisation is observed within the group of inbreeders (i.e. between *L. remotum* and *L. temulentum*) and also selfpollinated species can cross with *L. perenne* and *L. multiflorum*.

The analysis of the genus *Lolium* using isozymes has shown that all species belong to a single gene pool with the same allozymes in addition to a lack of species specific markers (Polok 2005). The slight differences were only found in allele frequencies. The DNA data, including RAPD, ISJ and bacterial *katG* gene supported the very high genetic similarity of *L. multiflorum* and *L. perenne* (Zieliński & Polok 2005). These two outpollinated species were clearly distinct from selfpollinated *L. temulentum*. Thus, the molecular data proved to be very effective in the analysis of evolutionary relationships within the genus *Lolium*.

The aim of the present study is the analysis of phylogenetic relationships between all *Lolium* species on the basis of AFLP markers (Amplified Fragment Length Polymorphism). The application of high-throughput technologies that generate a huge number of markers, should focus a light on the evolution of the genus and in the end should lead to the classification of the genus based on biological species.

2. Material and methods

Seeds of 7 *Lolium* species were obtained from the Institute of Grassland and Environmental Research Gene Bank in Aberystwyth, UK (*L. loliaceum*, *L. persicum*, *L. remotum*, *L. rigidum*), Nordic Gene Bank (*L. temulentum*) and Poznań Breeding Station, Poland (*L. multiflorum*, *L. perenne*). The analysis of bulked samples was based on the pooling genomic DNA from 10-20 individuals per species. A modified version of CTAB method (Murray & Thompson 1980) was used for the DNA isolation. AFLP templates were prepared as described by Vos *et al*. (1995), except that 125 ng of genomic DNA and 1.25 U of restriction enzyme (*EcoRI* and *MseI*) were used. Adaptors and primers were synthesised by IDI DNA Technology. Preamplification and selective amplification were performed as described by Vos *et al*. (1995). Amplified fragments were separated on 6% polyacrylamide gel and visualized by silver staining. Gels were dried on the glass plate and scanned at 300 dpi using Mustek flatbad scanner and saved as .jpg image files for analysis. AFLP bands were scored for presence (1) and absence (0). Fragments of the same size in different genotypes were considered to be the same alleles. Genetic similarity among bulked samples was based on Nei & Li (1979).

3. Results and discussion

Each pair of primers resulted in the amplification of a large number of AFLP fragments from the 7 species of the genus *Lolium* (Fig. 1). A total of 879 bands were identified using 8 pair of primers. The number of scored loci per species varied from 160 to 318 with an average of 109 per reaction. Most fragments were polymorphic (98.8%) and many of them were restricted to a single species. Only 10 fragments were monomorphic across all species tested.

Cluster analysis, presented in the form of a dendrogram (Fig. 2) grouped all species into two groups and was consistent with the traditional division of the genus and our earlier results based on DNA markers (Polok 2005). The first group is represented by three selfpollinated species, *L. persicum*, *L. remotum* and *L. temulentum*. The relatively high similarity coefficient between them (I=0.73-0.75) may reflect a relatively recent split between these species. This observation correlates well with results based upon analysis of selected STS (Sequenced Specific Tags) from cereals and *Lolium* ITS (Haluskova & Polok, in preparation). Moreover, the three species were unique and clearly distinct in comparison with outpollinated species amplification profiles generated by primers complementary to the bacterial *katG* gene and *IS6110* (Polok, unpublished data). These bacterial specific sequence-based markers proved to be a very effective species specific marker system (Zieliński & Polok 2005).

The second major cluster is formed by three outpollinated species, i.e. *L. multiflorum*, *L. perenne* and *L. rigidum*. Out of these species, *L. multiflorum* and *L. perenne* are joined as a distinct minor cluster with the highest genetic similarity (0.83). These results provide further credence to our earlier conclusion that both taxa can not be regarded as biological species

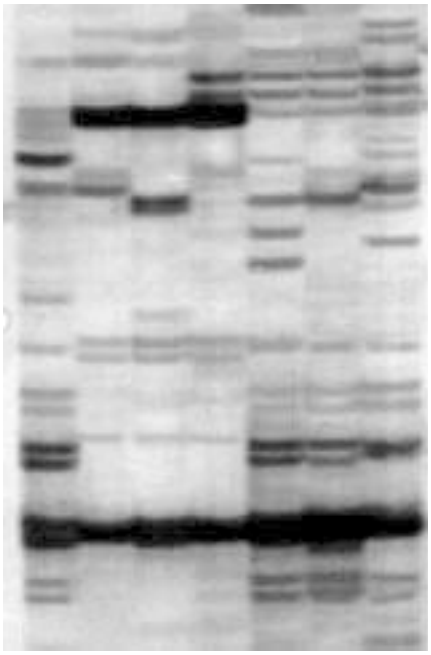

Fig. 1. AFLP profiles of *Lolium* species
Explanations: from left – *L. loliaceum, L. persicum, L. remotum, L. temulentum, L. multiflorum, L. perenne, L. rigidum*

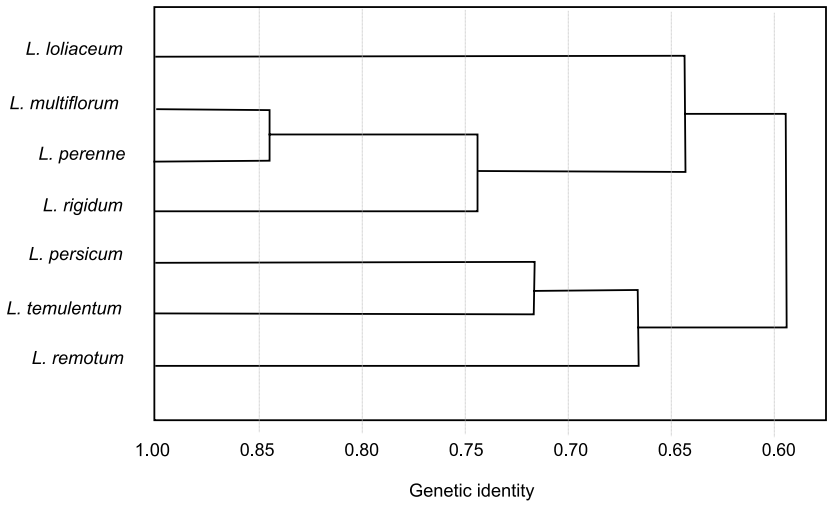

Fig. 2. UPGMA grouping of the seven *Lolium* species on the base of genetic distance

(Zieliński *et al*. 1997; Zieliński & Polok 2005). The lack of a reproductive barrier between both taxa was confirmed by Polok (2005) who observed the Mendelian segregation of about 90% of DNA, enzymatic and

morphological markers in F_2 derived from a cross between *L. multiflorum* and *L. perenne*. Interestingly, this analysis showed the 1-gene or 2-gene mode of inheritance of significant taxonomic characters such as the presence of awns, growth type and fluorescence of seminal roots. It is likely, therefore, that both botanical taxa differ only in a few genes justifying their classification as subspecies. The first suggestion that *L. multiflorum* and *L. perenne* may be classified as a single species (or subspecies) was made by Bulińska-Radomska (1985). Similarly, Loos (1993a, 1993b) on the basis of isoenzyme and morphological analysis pointed out that the differences between both species correspond rather with inter-population than inter-specific variation.

On the AFLP based dendrogram, the third outpollinated species, *L. rigidum* joined with *L. multiflorum* and *L. perenne* with 0.71 similarity and then joined with a cluster of selfpollinated species. Its placement may suggest that it may be a progenitor of all *Lolium* species as it has been postulated by Charmet & Balfourier (1994).

Out of seven *Lolium* species, the most surprising is the placement of selfpollinated *L. loliaceum*, which formed one major cluster with outpollinated taxa. However, the selected *Gramineae* STS markers generated the identical amplification products in *L. loliaceum*, *L. multiflorum, L. perenne* and *L. rigidum* and were different from the other inbreeders, *L. persicum, L. remotum* and *L. temulentum* (J. Haluskova, personal communication). Comparison of *Lolium* species based on bacterial specific sequences (*katG* and *IS6110*) as well as transpozons also grouped *L. loliaceum* together with outpollinated species instead of selfpollinated ones (Polok, unpublished data). Thus, the AFLP data seems to confirm that this taxon evolved within a group of outpollinated species, followed by several point mutations responsible for self-fertility.

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